

Special Issue Reprint

Advances in Phenolic Based Complexes

Edited by
Kiril B. Gavazov

mdpi.com/journal/molecules

Advances in Phenolic Based Complexes

Advances in Phenolic Based Complexes

Guest Editor

Kiril B. Gavazov



Basel • Beijing • Wuhan • Barcelona • Belgrade • Novi Sad • Cluj • Manchester

Guest Editor

Kiril B. Gavazov

Department of Chemical

Sciences

Medical University of Plovdiv

Plovdiv

Bulgaria

Editorial Office

MDPI AG

Grosspeteranlage 5

4052 Basel, Switzerland

This is a reprint of the Special Issue, published open access by the journal *Molecules* (ISSN 1420-3049), freely accessible at: https://www.mdpi.com/journal/molecules/special_issues/S7S59BBQJ9.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. <i>Journal Name</i> Year , Volume Number, Page Range.
--

ISBN 978-3-7258-8719-4 (Hbk)

ISBN 978-3-7258-8720-0 (PDF)

<https://doi.org/10.3390/books978-3-7258-8720-0>

© 2026 by the authors. Articles in this reprint are Open Access and distributed under the Creative Commons Attribution (CC BY) license. The reprint as a whole is distributed by MDPI under the terms and conditions of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) license (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

Contents

About the Editor	vii
Kiril Gavazov	
Editorial for the Special Issue “Advances in Phenolic Based Complexes”	
Reprinted from: <i>Molecules</i> 2026 , <i>31</i> , 2593, https://doi.org/10.3390/molecules31152593	1
Jan Hočevár, Jernej Iskra and Estelle Leonard	
Phenolic Azobenzene as Ligand for Cation Complexation—Syntheses and Applications	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 2499, https://doi.org/10.3390/molecules30122499	10
Shun Zhang, Kewei Zuo, Lijun Zhang, Chenlu Zhang and Juan Shi	
Preparation and Properties of Chitosan Complexes Consisting of <i>Artemisia argyi</i> Volatile Oil Nanoemulsion	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 585, https://doi.org/10.3390/molecules30030585	45
Petya V. Racheva, Antoaneta D. Saravanska, Galya K. Toncheva, Denitsa D. Kiradzhiyska, Nikolina P. Milcheva, Vidka V. Divarova, et al.	
A Semi-Micro Extraction Spectrophotometric Determination of Iron Using 4-Nitrocatechol and Xylometazoline Hydrochloride	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 899, https://doi.org/10.3390/molecules30040899	64
Daniel Hinojosa-Nogueira, Cristina María Díaz-Perdigones, María José García-López, Ascensión Marcos, María P. Portillo, Rosa María Lamuela-Raventós, et al.	
Beer-Derived (Poly)phenol Metabolism in Individuals With and Without Metabolic Syndrome: A Comparative Dietary Intervention	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 2932, https://doi.org/10.3390/molecules30142932	80
Almanelly Agabo-Martínez, Erika Gomez-Chang, Erick Hernández-Hipólito, Elizabet Estrada-Muñiz, Carolina Escobedo-Martínez, Marco A. Obregón-Mendoza, et al.	
Anti- <i>Helicobacter pylori</i> Activity and Gastroprotective Effects of Diacetylcurcumin and Four Metal Derivatives	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 3849, https://doi.org/10.3390/molecules30193849	96
Rosario Tavera-Hernández, Rubén Sánchez-Obregón, Marco A. Obregón-Mendoza, Antonio Nieto-Camacho, María Teresa Ramírez-Apan, Leidys L. Pérez-González and Raúl G. Enríquez	
Synthesis of First Copper Metal Complex of C=C Extended Curcuminoid Analogue: Structure, β -Cyclodextrin Association, and Biological Properties	
Reprinted from: <i>Molecules</i> 2025 , <i>30</i> , 3943, https://doi.org/10.3390/molecules30193943	119
Elif Ildız and Elif Yürümez Canpolat	
Phenolic-Rich Wild Edible Macrofungi: Antimicrobial Activity and Antioxidant Potential	
Reprinted from: <i>Molecules</i> 2026 , <i>31</i> , 978, https://doi.org/10.3390/molecules31060978	142
Nikola Stamenković, Filip Nikolić, Aleksandar Matić, Dragana AntoniĆ Reljin, Marija Milovančević, Danijela Paunović and Olga Radulović	
The Role of Phenolic Profile of Salt-Stressed Duckweed (<i>Lemna minor</i>) in Synthesis and Biological Activity of Green ZnO Nanoparticles	
Reprinted from: <i>Molecules</i> 2026 , <i>31</i> , 2326, https://doi.org/10.3390/molecules31132326	165

About the Editor

Kiril B. Gavazov

Kiril B. Gavazov is a Full Professor in the Department of Chemical Sciences at the Medical University of Plovdiv, Bulgaria. He teaches General and Inorganic Chemistry in the Master of Pharmacy program. His research focuses on the synthesis and characterization of novel coordination compounds and ion-association complexes, with particular emphasis on their analytical applications, including the development of preconcentration techniques based on the principles of green analytical chemistry.

Editorial

Editorial for the Special Issue “Advances in Phenolic Based Complexes”

Kiril Gavazov

Department of Chemical Sciences, Faculty of Pharmacy, Medical University of Plovdiv, 120 Buxton Bros Str., 4004 Plovdiv, Bulgaria; kiril.gavazov@mu-plovdiv.bg

Abstract

Research on phenolic compounds and their complexes continues to advance rapidly, driven by interdisciplinary efforts that expand both their fundamental scientific significance and practical applications. This Special Issue of *Molecules* presents recent developments in the chemistry and applications of these complexes through contributions from researchers across three continents. The published papers can be organized into five thematic sections: (i) synthesis and structural characterization; (ii) biological activity and therapeutic potential; (iii) analytical applications and sensors; (iv) nanotechnology and advanced delivery systems; and (v) metabolism and environmental factors. Among the contributions is a review article on the application of phenolic azobenzene ligands for cation complexation. Collectively, the eight contributions provide an up-to-date overview of the field, highlighting recent advances, emerging trends, innovative approaches, and future research directions in the chemistry and applications of phenolic-based complexes.

Keywords: phenolic compounds; metal complexes; coordination chemistry; photoisomerization; nanoparticles; green chemistry; biological activity; therapeutic applications; analysis; (poly)phenols

1. Introduction

Phenolic compounds constitute a diverse class of aromatic molecules characterized by one or more hydroxyl groups directly attached to an aromatic ring. As weak acids, they readily undergo deprotonation to form phenoxide ions under alkaline conditions, while their susceptibility to oxidation leads to the formation of quinones—highly reactive intermediates involved in numerous chemical and biological processes. The electron-donating effect of the hydroxyl group increases the electron density of the aromatic ring, thereby enhancing its reactivity and enabling a wide range of chemical transformations.

Owing to their structural diversity and versatile coordination behavior, phenolic compounds have attracted considerable interest in coordination chemistry, analytical chemistry, supramolecular chemistry, and materials science [1–7]. Depending on the number and relative positions of their hydroxyl groups, they can be broadly classified into monohydric phenols, phenols with isolated hydroxyl groups, ortho-hydroxylated phenols (catechol derivatives), peri-hydroxylated phenols, multifunctional phenolic derivatives, and polyphenols. Their ability to coordinate metal ions through different binding modes has led to the development of a wide variety of metal complexes, with applications ranging from chemical sensing to catalysis and biomedicine [6–12].

A key aspect of progress in these materials is the development of multifunctional ligands, such as phenolic azobenzene derivatives. These molecules have the ability to

form complexes with metal cations and possess the unique property of the azobenzene core for reversible photoisomerization. This combination makes them perfect for creating “smart” materials and colorimetric sensors that can detect heavy metals [13–16]. Similarly, catechol-based ligands are of significant interest due to their affinity for transition metal ions. This feature allows for their use in metal ion determination following environmentally friendly preconcentration procedures [17–21].

The incorporation of phenolic compounds and their metal complexes into nanotechnology has further broadened their range of applications. Plant- and macrofungi-derived phenolic extracts have been successfully employed in the “green synthesis” of metal nanoparticles [22–26], where natural phenolic metabolites function simultaneously as reducing and stabilizing agents. In parallel, the encapsulation of phenolic compounds in chitosan-based nanocomposites or nanoemulsion systems has been shown to improve their solubility, stability, bioavailability, and controlled release, thereby enhancing their antioxidant, antimicrobial, and other biological activities [27–32].

The application potential of phenolic-based complexes continues to expand across numerous scientific disciplines. In materials science, they contribute to the development of functional materials and the optimization of dyes for dye-sensitized solar cells, whereas in the biomedical field, metal complexes of phenolic compounds have demonstrated considerable promise as antimicrobial, gastroprotective, antidiabetic, antioxidant, and anticancer agents [1–3,11]. At the same time, advances in analytical chemistry have enabled the development of sensitive, simple, robust, and environmentally sustainable methods for metal ion determination [33,34], while studies on the metabolism and bioavailability of dietary phenolics are improving our understanding of their health-promoting effects [35–40].

The aim of this Special Issue (SI) was to provide an international forum for presenting recent advances in the synthesis, structural characterization, physicochemical properties, biological activities, analytical applications, and emerging technological uses of phenolic-based complexes. The eight contributions included in this Special Issue illustrate the broad scope of current research in this rapidly evolving field, highlighting innovative developments in coordination chemistry, nanotechnology, analytical science, and biomedicine while emphasizing the growing importance of phenolic compounds in addressing contemporary scientific and technological challenges.

2. Thematic Sections

The contributions to this SI can be grouped into five major thematic sections that highlight recent advances in the synthesis, analytical utility, biomedical potential, and environmental aspects of these complexes.

2.1. Synthesis and Structural Features

This section encompasses studies focused on the design, synthesis, and structural characterization of phenolic ligands and their metal complexes.

- Phenolic azobenzenes as ligands: Novel phenolic azobenzene derivatives capable of reversible photoisomerization and coordination with metal cations have been synthesized and investigated, demonstrating their potential as photoresponsive ligands [Contribution 1].
- Metal complexes of curcuminoids: Copper complexes of curcumin analogues containing an extended unsaturated chain have been synthesized and structurally characterized using X-ray crystallography and EPR spectroscopy [Contribution 2].
- Iron coordination with 4-nitrocatechol: A stable low-spin Fe(III) complex with 4-nitrocatechol has been obtained, and its molecular structure has been elucidated

through quantum-chemical calculations, supported by spectrophotometric data [Contribution 3].

2.2. Biological Activity and Therapeutic Potential

A substantial proportion of the contributions investigate the biological properties of phenolic-based complexes and their potential biomedical applications.

- Antibacterial and anti-urease activity: Metal complexes of diacetylcurcumin with Cu(II), Zn(II), Mn(II), and Mg(II) exhibit significant inhibitory activity against *Helicobacter pylori* and its urease enzyme, highlighting their promise as antimicrobial agents [Contribution 4].
- Antidiabetic and antiproliferative properties: Copper complexes of curcuminoids, including β -cyclodextrin-associated systems, demonstrate considerable inhibitory activity toward β -glucosidase, indicating potential applications in diabetes management and oncology [Contribution 2].
- Broad-spectrum biological activity: Phenolic compounds derived from natural products (in particular macrofungi) display diverse biological effects, including antioxidant, anti-inflammatory, and antitumor activities. Additionally, their ability to chelate transition metals contributes to mitigating the formation of reactive oxygen species [Contribution 5].

2.3. Analytical Applications and Chemical Sensors

This section showcases the utility of phenolic complexes in analytical chemistry and sensing technologies.

- Colorimetric sensors: Hydroxyazobenzene derivatives function as selective colorimetric chemosensors by undergoing distinct color changes upon binding to metal ions such as Cu(II), Cr(III), and Hg(II) [Contribution 1].
- Spectrophotometric analysis: A semi-micro extraction–spectrophotometric method has been developed for the sensitive determination of Fe(III). The method has been successfully implemented in the analysis of pharmaceutical formulations and industrial samples [Contribution 3].

2.4. Nanotechnology and Advanced Delivery Systems

This section highlights recent advances in improving the stability, bioavailability, and functional performance of phenolic compounds through nanotechnology-based approaches.

- Chitosan composites and nanoemulsions: Chitosan-based composites incorporating nanoemulsions of essential oils, such as *Artemisia argyi*, have been developed to enhance physicochemical stability, antioxidant capacity, and the controlled release of bioactive compounds [Contribution 6].
- Green synthesis of nanoparticles: Plant-derived phenolic compounds, including those from duckweed (*Lemna* spp.), have been utilized as reducing and stabilizing agents for the environmentally friendly synthesis of zinc oxide nanoparticles (ZnO NPs), thereby improving their biological performance [Contribution 7].

2.5. Metabolism and Environmental Influences

This section explores the factors affecting the metabolism of phenolic compounds and the influence of environmental conditions on their biosynthesis.

- Phenolic metabolism in metabolic syndrome: Studies investigating the metabolism of beer-derived phenolic compounds demonstrate that metabolic syndrome significantly influences the formation of bioactive conjugates and microbial catabolites,

- emphasizing the role of individual physiological status in phenolic bioavailability [Contribution 8].
- Effects of salt stress: Salt stress has been shown to alter the phenolic composition of plants by increasing flavonoid accumulation, which subsequently modulates the physicochemical characteristics and biological properties of the nanoparticles synthesized from these plant extracts [Contribution 7].

3. An Overview of the Individual Contributions

Leonard et al. [Contribution 1] presented a comprehensive review highlighting phenolic azobenzenes as multifunctional compounds with considerable potential in coordination chemistry. The authors summarize the principal synthetic strategies for these molecules, including azo coupling reactions and the oxidative coupling of anilines, while discussing the influence of substituent effects on reaction efficiency and product yields.

A major focus of the review is the ability of phenolic azobenzenes to function as ligands for metal cations, with particular emphasis on the crucial role of the phenolic hydroxyl group in metal coordination and complex stabilization. The review further examines the distinctive photochemical properties of these compounds, especially their reversible trans-cis photoisomerization upon light irradiation.

The combination of efficient metal-binding properties and reversible photoswitching makes phenolic azobenzenes attractive building blocks for a broad range of functional applications, including colorimetric and optical sensors, molecular switches, catalysts, and sensitizers for dye-sensitized solar cells. Overall, the review demonstrates that rational structural design of phenolic azobenzenes enables fine tuning of their coordination behavior, photophysical properties, and chemical reactivity, thereby broadening their applicability in advanced functional materials and molecular devices.

Tavera-Hernández et al. [Contribution 2] reported the synthesis and comprehensive characterization of novel curcuminoid analogues featuring an extended 11-carbon π -conjugated chain, together with their boron and copper complexes. The study is particularly noteworthy for describing the first Cu(II) complex of a C=C-extended curcuminoid analogue and investigating its structural, supramolecular, and biological properties.

The synthesized compounds were thoroughly characterized using a combination of spectroscopic methods, including NMR spectroscopy, and single-crystal X-ray diffraction analysis. These techniques confirmed their molecular structures and provided detailed insight into their structural features and optical properties.

Biological evaluation revealed that the synthesized compounds exhibit significant inhibitory activity against α -glucosidase, highlighting their potential as lead candidates for the development of novel therapeutics for type 2 diabetes mellitus. Overall, the study demonstrates that the combination of metal complexation and supramolecular encapsulation represents an effective strategy for enhancing the physicochemical and biological properties of curcuminoid derivatives. In particular, the newly synthesized copper complex emerges as a promising bioactive compound with favorable biological activity and a potentially advantageous safety profile.

Racheva et al. [Contribution 3] developed an innovative semi-micro extraction-spectrophotometric procedure for the determination of Fe(III) based on the formation of a ternary ion-association complex with 4-nitrocatechol and xylometazoline hydrochloride. The authors systematically optimized the experimental conditions to achieve high sensitivity, selectivity, and precision. The composition and structure of the extracted complex were elucidated through a combination of experimental investigations and quantum-chemical calculations performed at the B3LYP/6-311G level of theory.

The proposed procedure provides a simple, sensitive, and cost-effective approach for iron determination. In addition, it requires only small volumes of organic solvent, thereby reducing solvent consumption and making the procedure more environmentally friendly and consistent with the principles of green analytical chemistry.

Agabo-Martínez et al. [Contribution 4] investigated the therapeutic potential of diacetylcurcumin (DAC) and its metal complexes as alternative agents for the treatment of *Helicobacter pylori* infections, a major cause of gastritis, peptic ulcers, and other gastric disorders. The study evaluated the antibacterial activity, urease inhibitory effects, and gastroprotective properties of DAC complexes containing different metal ions.

Among the investigated compounds, the Cu(II) and Zn(II) complexes exhibited the strongest antibacterial activity against *H. pylori* and effectively inhibited urease, a key virulence factor that enables the bacterium to survive in the highly acidic gastric environment. Furthermore, in vivo studies in a mouse model demonstrated significant gastroprotective effects, with both complexes markedly reducing gastric mucosal damage.

The results indicate that metal coordination enhances the stability and biological activity of DAC while preserving a favorable safety profile. Overall, the study identifies DAC-based metal complexes, particularly the Cu(II) and Zn(II) derivatives, as promising candidates for the development of novel therapeutic strategies for the treatment of *H. pylori*-associated infections and gastric diseases.

Ildiz and Canpolat [Contribution 5] investigated the biological properties of four species of wild edible macrofungi collected in Türkiye—*Tuber aestivum*, *Terfezia clavariyi*, *Agaricus arvensis*, and *Bovistella utriformis*—with particular emphasis on their phenolic composition, antioxidant capacity, and antimicrobial activity. The study compared extracts obtained using solvents of different polarity and examined the relationship between phenolic content and biological activity.

The results demonstrated that methanolic extracts exhibited significantly higher antioxidant and antimicrobial activities than hexane extracts, reflecting the greater extraction efficiency of polar phenolic compounds. These extracts showed pronounced inhibitory effects, particularly against Gram-positive bacteria and multidrug-resistant bacterial strains. Among the investigated species, *B. utriformis* and *A. arvensis* contained the highest total phenolic contents and exhibited the strongest free radical scavenging activities.

Phytochemical analyses identified several bioactive phenolic compounds, including catechin and cinnamic acid, which are likely to contribute to the observed antioxidant and antimicrobial effects. Overall, the study highlights wild edible macrofungi as valuable natural sources of phenolic compounds with considerable potential for the development of functional foods, nutraceuticals, and novel antioxidant and antimicrobial agents.

Shi et al. [Contribution 6] presented an innovative strategy to enhance the therapeutic potential of *Artemisia argyi* essential oil, a natural product with well-established antibacterial and antioxidant properties but limited practical application due to its poor aqueous solubility, volatility, and chemical instability. To overcome these limitations, the authors developed a chitosan-based nanoemulsion system that serves as a stable carrier for the controlled release of the essential oil's bioactive constituents.

Comprehensive physicochemical characterization confirmed the successful preparation of the nanocomposite and demonstrated its improved stability and encapsulation efficiency. Biological evaluation further revealed that the chitosan-based nanoemulsion significantly enhanced the antibacterial activity of the essential oil against resistant bacterial strains while preserving its antioxidant capacity. Compared with the free essential oil, the nanoformulation exhibited nearly a twofold increase in antibacterial efficacy, demonstrating the synergistic benefits of chitosan encapsulation and nanoemulsion technology.

In addition to its potential biomedical applications, the developed nanocomposite shows considerable promise for use in food preservation and cosmetic formulations, where improved stability and controlled release of bioactive compounds are highly desirable. Overall, the study demonstrates that chitosan-based nanoemulsion technology provides an environmentally friendly and effective platform for enhancing the stability, bioavailability, and biological performance of natural products, thereby expanding their potential applications in healthcare, food technology, and cosmetics.

Radulović et al. [Contribution 7] investigated the influence of salt stress on the phenolic profile of the aquatic plant *Lemna minor* (duckweed) and its subsequent effect on the green synthesis of zinc(II) oxide nanoparticles (ZnO NPs). The study demonstrated that increasing salinity during cultivation promoted the accumulation of phenolic compounds and flavonoids, which served as natural reducing and stabilizing agents during nanoparticle synthesis.

The synthesized ZnO NPs were comprehensively characterized to assess their structural and physicochemical properties. The results showed that the nanoparticles possessed a well-defined crystalline structure, while their crystallinity and phase composition were influenced by the level of salt stress applied to the source biomass. Furthermore, biological evaluation revealed that ZnO NPs synthesized from salt-stressed duckweed exhibited enhanced antioxidant activity and greater antimicrobial efficacy against *Staphylococcus haemolyticus* than those produced from non-stressed plants.

Overall, this study demonstrates how stress-induced metabolic changes in plants can be exploited to tailor the synthesis and functional properties of metal oxide nanomaterials. By linking alterations in the phenolic profile with nanoparticle characteristics and biological performance, the authors present an environmentally sustainable approach to producing bioactive ZnO NPs while promoting the valorization of aquatic biomass within the framework of a circular bioeconomy. These findings underscore the potential of plant-mediated green nanotechnology for applications in biomedicine, environmental remediation, and other advanced technological fields.

Hinojosa-Nogueira et al. [Contribution 8] investigated the metabolism of beer-derived (poly)phenols in individuals with and without metabolic syndrome through a controlled dietary intervention study. The work provides valuable insights into how physiological status influences the absorption, biotransformation, and metabolic fate of dietary phenolic compounds.

The results demonstrated that metabolic health plays a key role in shaping (poly)phenol metabolic profiles. Healthy participants exhibited greater production of specific phase II conjugates and flavonoid-derived metabolites, whereas individuals with metabolic syndrome showed increased formation of microbial-derived catabolites, indicating alterations in intestinal microbial metabolism. These differences suggest that metabolic disturbances associated with metabolic syndrome, including oxidative stress and gut microbiota dysbiosis, can significantly influence the biotransformation of beer-derived (poly)phenols.

Overall, this study highlights the complex interplay between dietary (poly)phenols, host metabolic status, and gut microbiota in determining the bioavailability and biological activity of phenolic metabolites. The findings emphasize the importance of considering individual physiological characteristics when evaluating the health effects of dietary phenolic compounds and support the growing concept of personalized nutrition based on metabolic and microbial profiles.

4. Conclusions

The eight contributions in this collection reflect the scope and diversity of contemporary research on phenolic compounds and highlight their growing scientific significance. Together, the studies demonstrate how phenolic compounds' unique chemical properties continue to drive the development of innovative materials and technologies with broad application potential.

I express my sincere gratitude to all the authors for their high-quality contributions and unwavering dedication to advancing research in the field of phenolic complexes. I also thank the reviewers for their insightful and constructive evaluations and the *Molecules* editorial team for their support throughout the publication process. I hope that this SI will be a useful resource for researchers and encourage more investigations into chemistry, design, and uses of phenolic-based complexes.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

List of Contributions:

1. Hočevar, J.; Iskra, J.; Leonard, E. Phenolic Azobenzene as Ligand for Cation Complexation—Syntheses and Applications. *Molecules* **2025**, *30*, 2499. <https://doi.org/10.3390/molecules30122499>.
2. Tavera-Hernández, R.; Sánchez-Obregón, R.; Obregón-Mendoza, M.A.; Nieto-Camacho, A.; Ramírez-Apan, M.T.; Pérez-González, L.L.; Enríquez, R.G. Synthesis of First Copper Metal Complex of C=C Extended Curcuminoid Analogue: Structure, β -Cyclodextrin Association, and Biological Properties. *Molecules* **2025**, *30*, 3943. <https://doi.org/10.3390/molecules30193943>.
3. Racheva, P.V.; Saravanska, A.D.; Toncheva, G.K.; Kiradzhyska, D.D.; Milcheva, N.P.; Divarova, V.V.; Pencheva, I.P.; Stojnova, K.T.; Delchev, V.B.; Gavazov, K.B. A Semi-Micro Extraction Spectrophotometric Determination of Iron Using 4-Nitrocatechol and Xylometazoline Hydrochloride. *Molecules* **2025**, *30*, 899. <https://doi.org/10.3390/molecules30040899>.
4. Agabo-Martínez, A.; Gomez-Chang, E.; Hernández-Hipólito, E.; Estrada-Muñiz, E.; Escobedo-Martínez, C.; Obregón-Mendoza, M.A.; Enríquez, R.G.; Vega, L.; Romero, I. Anti-*Helicobacter pylori* Activity and Gastroprotective Effects of Diacetylcurcumin and Four Metal Derivatives. *Molecules* **2025**, *30*, 3849. <https://doi.org/10.3390/molecules30193849>.
5. Ildiz, E.; Yürümez Canpolat, E. Phenolic-Rich Wild Edible Macrofungi: Antimicrobial Activity and Antioxidant Potential. *Molecules* **2026**, *31*, 978. <https://doi.org/10.3390/molecules31060978>.
6. Zhang, S.; Zuo, K.; Zhang, L.; Zhang, C.; Shi, J. Preparation and Properties of Chitosan Complexes Consisting of *Artemisia argyi* Volatile Oil Nanoemulsion. *Molecules* **2025**, *30*, 585. <https://doi.org/10.3390/molecules30030585>.
7. Stamenković, N.; Nikolić, F.; Matić, A.; Antonić Reljin, D.; Milovančević, M.; Paunović, D.; Radulović, O. The Role of Phenolic Profile of Salt-Stressed Duckweed (*Lemna minor*) in Synthesis and Biological Activity of Green ZnO Nanoparticles. *Molecules* **2026**, *31*, 2326. <https://doi.org/10.3390/molecules31132326>.
8. Hinojosa-Nogueira, D.; Díaz-Perdigones, C.M.; García-López, M.J.; Marcos, A.; Portillo, M.P.; Lamuela-Raventós, R.M.; Subiri-Verdugo, A.; Nova, E.; Milton-Laskibar, I.; Galkina, P.; Tina-hones, F.J.; Moreno-Indias, I. Beer-Derived (Poly)phenol Metabolism in Individuals with and without Metabolic Syndrome: A Comparative Dietary Intervention. *Molecules* **2025**, *30*, 2932. <https://doi.org/10.3390/molecules30142932>.

References

- Chen, Z.; Świsłocka, R.; Chojińska, R.; Marszałek, K.; Dąbrowska, A.; Lewandowski, W.; Lewandowska, H. Exploring the Correlation Between the Molecular Structure and Biological Activities of Metal–Phenolic Compound Complexes: Research and Description of the Role of Metal Ions in Improving the Antioxidant Activities of Phenolic Compounds. *Int. J. Mol. Sci.* **2024**, *25*, 11775. [CrossRef] [PubMed]
- Merecz-Sadowska, A.; Kowalczyk, T.; Picot, L.; Tzortzakis, N.; Zajdel, R.; Sitarek, P. Coordination Chemistry of Phytochemicals: Enhancing Bioavailability and Stability of Flavonoids for Applications in Functional Foods and Medicine. *Coord. Chem. Rev.* **2026**, *562*, 217990. [CrossRef]
- Xue, S.; Tan, W.; Mao, S.; Pan, H.; Ye, X.; Donlao, N.; Tian, J. Polyphenol-Based Functional Materials: Structural Insights, Composite Strategies, and Biomedical Applications. *Adv. Sci.* **2025**, *12*, e08924. [CrossRef] [PubMed]
- Zhu, K.; Guo, M.; Li, W.; Zhang, X.; Chen, L.; Song, J. Supramolecular Coordination Platforms for Smart Theranostics: Leveraging the Dynamic Molecular Networks of Metal–Polyphenol Assemblies. *Aggregate* **2026**, *7*, e70394. [CrossRef]
- Lisjak, M.; Špoljarević, M.; Ravlić, J.; Lončarić, Z.; Galić, L. Phenolic Compounds and Antioxidant Activity: Analytical Methods and Current Knowledge—A Review. *Methods Protoc.* **2026**, *9*, 60. [CrossRef] [PubMed]
- Xu, T.; Xu, W.; Zhu, S.; Sun, H.; Liu, W.; Li, H.; Chen, F.; Sun, Z.; Chu, H. Engineering Coordination-Defined Metal–Phenolic Networks: From Molecular Self-Assembly to System-Level Platforms for Advanced Tumor Therapy. *Results Eng.* **2026**, *30*, 110087. [CrossRef]
- Li, L.; Pan, J.; Huang, M.; Sun, J.; Wang, C.; Xu, H. Metal–Phenolic Networks: A Promising Frontier in Cancer Theranostics. *Int. J. Nanomed.* **2024**, *19*, 11379–11395. [CrossRef] [PubMed]
- Wang, Z.; Zhu, X.; Fu, R.; Hu, H.; Ma, L.; Wang, Y.; Zou, A.; Xianyu, Y.; Guo, Y. Coordination-Driven Metal–Phenolic Network Complexes for Programmable Colorimetric Signal Transduction and Defined Nano–Bio Interfaces. *Small* **2026**, e74584. [CrossRef] [PubMed]
- Xia, N.; Liang, S.; Deng, D.; Chang, Y.; Yi, X. Metal–Phenolic Networks for Sensing Applications. *Biosensors* **2025**, *15*, 600. [CrossRef] [PubMed]
- Amorim, I.; Bento, F. Electrochemical Sensors Based on Transition Metal Materials for Phenolic Compound Detection. *Sensors* **2024**, *24*, 756. [CrossRef] [PubMed]
- Lin, G.; Zhao, L.; Jin, H.; Wang, S.; Wang, N.; Dai, M.; Lin, X. Designing Metal–Phenolic Networks in Biomedicine. *Appl. Mater. Today* **2025**, *45*, 102822. [CrossRef]
- Orita, T.; Ijiri, D.; Hou, D.-X.; Sakao, K. Creation of Zinc(II)-Complexed Green Tea and Its Effects on Gut Microbiota by Daily Green Tea Consumption. *Molecules* **2025**, *30*, 3191. [CrossRef] [PubMed]
- Chi, Z.; Chu, S.; Wang, B.; Zhang, Z.; Liu, G.; Wang, X. Advances in Metal Complex-Based Colorimetric Sensors. *Talanta* **2026**, *297*, 128591. [CrossRef] [PubMed]
- Zhang, X.; Jin, T.; Yang, C.; Xu, D.; Jia, H.; Xu, L. Azobenzene-Containing Photoresponsive Metal–Organic Cages. *Chin. Chem. Lett.* **2025**, *36*, 111135. [CrossRef]
- Jiang, J.; Yuan, X.; Hu, L. Recent Advances of Azobenzene-Based Photoresponsive Molecular Switches for Protein-Targeted Photopharmacology. *Molecules* **2026**, *31*, 1205. [CrossRef] [PubMed]
- Leonard, E.; Takeda, C.; Akitsu, T. Azobenzene-Containing Schiff-Bases—Syntheses and Dyes Applications. *Colorants* **2024**, *3*, 53–72. [CrossRef]
- Racheva, P.V.; Saravanska, A.D.; Kiradzhiyska, D.D.; Delchev, V.B.; Gavazov, K.B. A Novel Tris(Chromium(III))–4-Nitrocatechol Complex Extracted via an Eco-Friendly Ionic Liquid-Assisted Method. *Croat. Chem. Acta* **2025**, *98*, 215–222. [CrossRef]
- Kiradzhiyska, D.; Milcheva, N.; Ruzmanova, M.; Genç, F.; Racheva, P.; Gavazov, K. Ionic Liquid-Based Centrifuge-Less Cloud Point Extraction of a Copper(II)–4-Nitrocatechol Complex and Its Analytical Application. *Molecules* **2025**, *30*, 3287. [CrossRef] [PubMed]
- Mortada, W.I.; Zedan, H.E.; Khalifa, M.E. Spectrophotometric Determination of Trace Vanadium in Fresh Fruit Juice Samples by Ion Pair-Based Surfactant-Assisted Microextraction Procedure with Solidification of Floating Organic Drop. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2023**, *302*, 123107. [CrossRef] [PubMed]
- Gajdošová, A.; Racheva, P.; Kiradzhiyska, D.; Divarova, V.; Saravanska, A.; Šandrejová, J.; Gavazov, K. Centrifuge-Less Mixed Micelle-Mediated Cloud Point Extraction–Spectrophotometric Determination of Vanadium Using 4-Nitrocatechol and Cetylpyridinium Chloride. *Int. J. Mol. Sci.* **2025**, *26*, 5808. [CrossRef] [PubMed]
- Divarova, V.; Gajdošová, A.; Racheva, P.; Gavazov, K. An Ionic Liquid-Assisted Mixed Micelle-Mediated Centrifuge-Less Cloud Point Extraction Spectrophotometric Method for the Determination of Molybdenum(VI). *Int. J. Mol. Sci.* **2025**, *26*, 4597. [CrossRef] [PubMed]
- Panda, J.; Nongbet, A.; Avula, S.K.; Nayak, D.; Rustagi, S.; Panda, B.P.; Mohanta, Y.K. Macro-Fungi Mediated Nanoparticles for Sustainable Agriculture: Recent Advancement and Future Strategies. *Discov. Sustain.* **2025**, *6*, 375. [CrossRef]

23. Genet, M.B.; Jembere, A.L.; Kovács, N.; Maász, G.; Tombácz, E. Plant-Mediated Iron Oxide Nanomaterials and Their Nanocomposites for Environmental Remediation: A Review on Recent Advances, Mechanistic Insights, and Future Perspectives. *J. Environ. Chem. Eng.* **2026**, *14*, 124084. [CrossRef]
24. Martin, M.C.; Garay, A.G.; Pintos, B. Green Nanotechnology in Sustainable Agriculture: Plant-Based Synthesis of Metallic Nanoparticles for Crop Protection and Productivity. *Appl. Sci.* **2026**, *16*, 3867. [CrossRef]
25. Pourakbar, L.; Farnad, N.; Siavash Moghaddam, S.; Popović-Djordjević, J.; Maria Giuffrè, A. Antioxidant and Antifungal Activities of Green Synthesized Silver Nanoparticles in Comparison with Essential Oil and Extracts of *Origanum vulgare* Leaves. *AIMS Agric. Food* **2026**, *11*, 95–117. [CrossRef]
26. Sen, K.; Mondal, S. Extraction of Biomolecular Polyphenols for Nanoparticles Fabrication: Mechanistic Insights for Environmental Mitigation. *Inorg. Chem. Commun.* **2025**, *179*, 114682. [CrossRef]
27. Rukshan, R.; Rajapaksha, N.; Wijayawardana, S.; Thambiliyagodage, C.; Senevirathne, U.; Jayanetti, M.; Samarakoon, U. Fe₃O₄-Chitosan Nanocomposite as a pH-Responsive Delivery System for Enhanced Delivery of *Punica granatum* L. Polyphenols. *ACS Omega* **2025**, *10*, 50488–50510. [CrossRef] [PubMed]
28. Sahin, K.; Sahin Aktura, S.; Bahceci, I.; Yazici, Z.A.; Oskay, B.; Ejder, N.; Yurteri, E.; Bal Altuntas, D. Chitosan Coating Enhances the Antimicrobial Activity of *Punica granatum* L. Phenolic Compounds. *Life* **2025**, *15*, 1878. [CrossRef] [PubMed]
29. El-Ashaal, E.S.; Elshoky, H.A.; El-Sayed, N.M.; El-Kholany, E.A. Chitosan and Carboxymethyl Chitosan Nanocarriers Enhance Mango Seed Extract Stability and Antimicrobial Activity to Improve Strawberry Postharvest Quality. *Sci. Rep.* **2025**, *15*, 31384. [CrossRef] [PubMed]
30. Gaetano, V.; Gagliardi, A.; Giuliano, E.; Longo, E.; Cosco, D. Chitosan Nanoparticles Loaded with Polyphenols for Cosmeceutical Applications: A State-of-the-Art Review. *Pharmaceutics* **2025**, *17*, 1068. [CrossRef] [PubMed]
31. Jacob, A.; Nixon, R.; Thirumurthy, D.; Angel, S.; Haldar, D. Essential Oil Nano-Delivery Systems: Recent Developments and Emerging Applications. *Nat. Prod. Commun.* **2025**, *20*, 1934578X251390689. [CrossRef]
32. Wiacek, A.E. Chitosan, Chitosan Derivatives, Polysaccharides and Their Applications—2nd Edition. *Molecules* **2026**, *31*, 761. [CrossRef] [PubMed]
33. Gajdošová, A.; Racheva, P.; Saravanska, A.; Šandrejová, J.; Gavazov, K. A Sensitive Cloud Point Extraction–Spectrophotometric Determination of Vanadium Using Pyrogallol and Aliquat 336. *Int. J. Mol. Sci.* **2026**, *27*, 6279. [CrossRef]
34. Gavazov, K.B.; Racheva, P.V.; Milcheva, N.P.; Divarova, V.V.; Kiradzhiyska, D.D.; Genç, F.; Saravanska, A.D. Use of a Hydrophobic Azo Dye for the Centrifuge-Less Cloud Point Extraction–Spectrophotometric Determination of Cobalt. *Molecules* **2022**, *27*, 4725. [CrossRef] [PubMed]
35. Rudrapal, M.; de Oliveira, A.M.; Singh, R.P. Dietary Polyphenols Maintain Human Health through Modulation of Gut Microbiota. *Front. Pharmacol.* **2025**, *16*, 1710088. [CrossRef] [PubMed]
36. Liu, X.; Rodriguez-Mateos, A.; Gibson, R.; Alharbi, A. (Poly)Phenol–Gut Microbiota Interactions and Their Impact on Human Health. *Curr. Opin. Clin. Nutr. Metab. Care* **2025**, *28*, 316–322. [CrossRef] [PubMed]
37. De La Cruz, A.; Silva, T.G.; Verma, D.K.; López-Yerena, A.; Wong-Paz, J.; Aguilar, C.N.; Ramírez-Guzmán, N.; Torres-León, C. Phenolic Compounds in Human Health: From Biofunctionality and Bioavailability to Gut–Brain Interactions and Disease Prevention. *Discov. Mol.* **2026**, *3*, 23. [CrossRef]
38. Saad, A.M.M.; Mohammed, D.M.; Alkafaas, S.S.S.; Ghosh, S.; Negm, S.H.H.; Salem, H.M.; Fahmy, M.A.; Semary, H.E.; Ibrahim, E.H.; AbuQamar, S.F.F.; et al. Dietary Polyphenols and Human Health: Sources, Biological Activities, Nutritional and Immunological Aspects, and Bioavailability—A Comprehensive Review. *Front. Immunol.* **2025**, *16*, 1653378. [CrossRef] [PubMed]
39. Dzah, C.S. How to Increase Dietary Polyphenol Bioavailability: Understanding the Roles of Modulating Factors, Lifestyle and Gut Microbiota. *Food Humanit.* **2025**, *4*, 100602. [CrossRef]
40. Krezić, J.; Buljeta, I.; Pichler, A.; Kopjar, M. Stability of Polyphenols and Antioxidant Activity of Cellulose-Based Encapsulates Enriched with Tart Cherry Juice Polyphenols. *Molecules* **2026**, *31*, 2449. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Review

Phenolic Azobenzene as Ligand for Cation Complexation—Syntheses and Applications

Jan Hočevar ¹, Jernej Iskra ¹ and Estelle Leonard ^{2,*}
¹ University of Ljubljana, Faculty of Chemistry and Chemical Technology, Večna pot 113, 1000 Ljubljana, Slovenia; jernej.iskra@fkkt.uni-lj.si (J.I.)

² Université de Technologie de Compiègne, ESCOM, TIMR, F-60200 Compiègne, France

* Correspondence: e.leonard@escom.fr

Abstract: Phenolic azobenzenes have garnered significant attention as functional materials due to their ability to undergo reversible photoisomerization and their potential for cation complexation. This review aims to provide a comprehensive overview of the recent developments in the synthesis, properties, and applications of phenolic azobenzene derivatives in cation binding and complexation. This article explores various synthetic strategies for the preparation of phenolic azobenzenes. Additionally, the mechanisms of cation complexation, including the role of the phenolic hydroxyl group and the azobenzene scaffold, are discussed, along with insights into the coordination chemistry involved. This review further examines the diverse applications of phenolic azobenzene complexes in fields such as ion sensing, catalysis, and biological and DSSC applications.

Keywords: phenol; azobenzene; photoisomerization; colorimetric sensors; DSSC dyes; cation complexes

1. Introduction

1.1. General Presentation of Azobenzenes and Phenolic Compounds

1.1.1. Azobenzene Properties

Azobenzenes are organic molecules containing an azo ($-N=N-$) functional group linked to two benzene rings [1]. Their unique chemistry arises from their conjugated π -electron system, which affects their optical, electronic, and structural behaviour. One key chemical property of azobenzenes includes the fact that they exist in two isomeric forms, *trans* (E) and *cis* (Z), as shown in Figure 1.

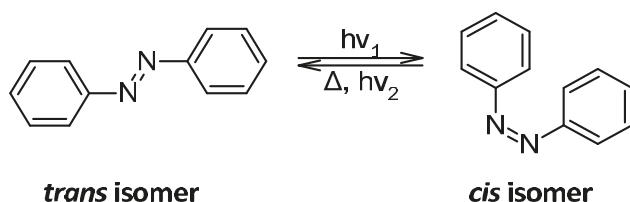


Figure 1. Two isomeric forms of azobenzenes.

The *trans* form is thermodynamically more stable, whereas the *cis* form has a higher energy and can revert to *trans* under thermal conditions [2]. The isomerization kinetics depend on substituents and solvent polarity. For example, electron-donating groups ($-OH$, $-OCH_3$) lower the *cis*-to-*trans* activation barrier [3]. They also affect the electronic distribution. Indeed, electron-donating groups (EDGs) (e.g., $-OH$, $-NH_2$) push electron

density towards the azo bond, stabilizing the *trans* form and shifting absorption maxima to longer wavelengths (bathochromic shift). However, electron-withdrawing groups (EWGs) (e.g., $-\text{NO}_2$, $-\text{CF}_3$) pull electron density away, destabilizing the π -system and affecting light absorption properties [4]. Azobenzenes can undergo redox reactions [5], particularly one-electron reduction, forming radical intermediates. This reduction typically converts the azo ($-\text{N}=\text{N}-$) bond into a hydrazo ($-\text{NH}-\text{NH}-$) linkage [6–9] under acidic or catalytic conditions. Azobenzenes act as ligands in metal complexes, coordinating with transition metals such as Fe, Ru, and Pd. Their ability to form charge transfer complexes makes them valuable in organometallic catalysis [10]. For example (Figure 2), in 2016, cyclometallated azobenzene palladium(II) complexes were synthesized, characterized using electronic absorption spectroscopy and electrochemical studies [11]. In 2008, dicyclopalladated complexes were synthesized using azobenzene derivatives, where the palladium centres form five-membered chelate rings through orthometallation. These complexes were characterized by X-ray crystallography and computational methods, providing insights into their structural and electronic properties [12]. A series of arene ruthenium complexes containing o-sulfonamide azobenzene ligands was also synthesized. These complexes exhibited uncommon coordination patterns with an exocyclic $\text{N}=\text{N}$ bond. Upon irradiation, they underwent *trans* $\rightarrow$ *cis* photoisomerization reversibly by thermal isomerization, with rates dependent on solvent, arene group, sulfonamide moiety, and azobenzene substitution [13].

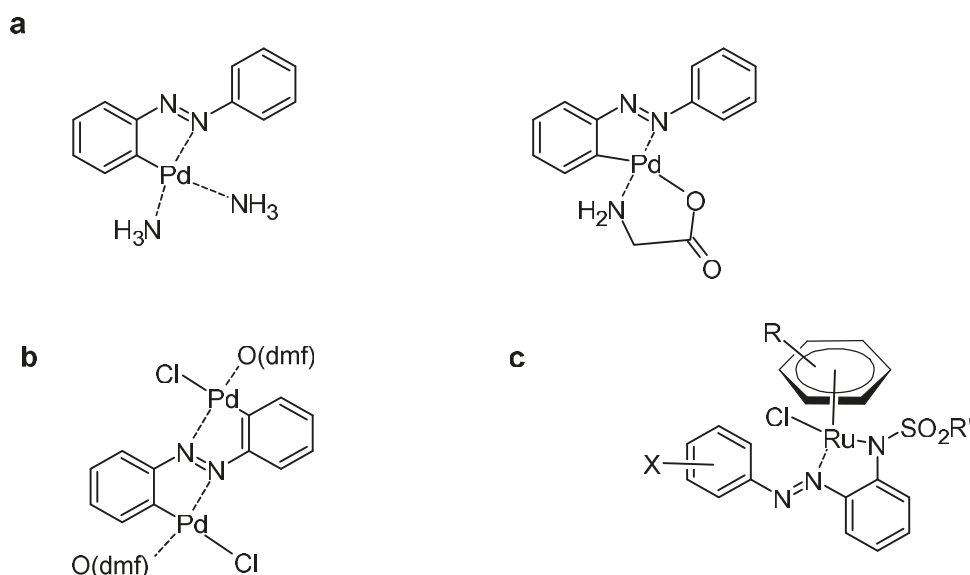


Figure 2. Examples of azobenzene metal complexes from (a) [11], (b) [12], and (c) [13].

1.1.2. Phenolic Properties

Phenolic compounds are aromatic molecules featuring one or more hydroxyl ($-\text{OH}$) groups attached to a benzene ring. Their chemical reactivity is largely determined by the electron-donating effects of the hydroxyl group and its ability to participate in various chemical transformations. Phenols are weak acids ($\text{pK}_a \sim 9\text{--}11$) and can donate protons to form phenoxide anions in basic solutions. The phenoxide ion ($-\text{O}^-$) is more reactive than its neutral form. Phenols can be easily oxidized to quinones [14,15] (e.g., hydroquinone to benzoquinone), and such transformations are crucial in redox reactions, dye chemistry, and electron transport mechanisms. The oxidation rate depends on substituents and environmental pH. The hydroxyl group in phenols participates in hydrogen bonding [16], influencing solubility and physical data (melting and boiling points, for example). Strong hydrogen bonds make phenols more soluble in polar solvents. Phenolic $-\text{OH}$ groups can coordinate with metal ions (Fe^{3+} , Cu^{2+}) [17], forming coloured metal complexes. For

example, Dalal Alhashmialameer and colleagues synthesized a series of iron(III) complexes supported by tetradentate amino-bis(phenolate) ligands. These complexes exhibit square pyramidal geometries, as confirmed by X-ray crystallography. They have been studied as catalysts for the formation of organic carbonates from CO₂ and epoxides. The Fe–O(phenolate) bond lengths range from approximately 1.8690(12) to 1.8805(12) Å, indicating strong iron–oxygen interactions [18]. Another example is detailed by Christopher M. Kozak and co-workers, who synthesized six new iron(III) diamine-bis(phenolate) complexes. These structures were elucidated through single-crystal X-ray diffraction (Figure 3) [19].

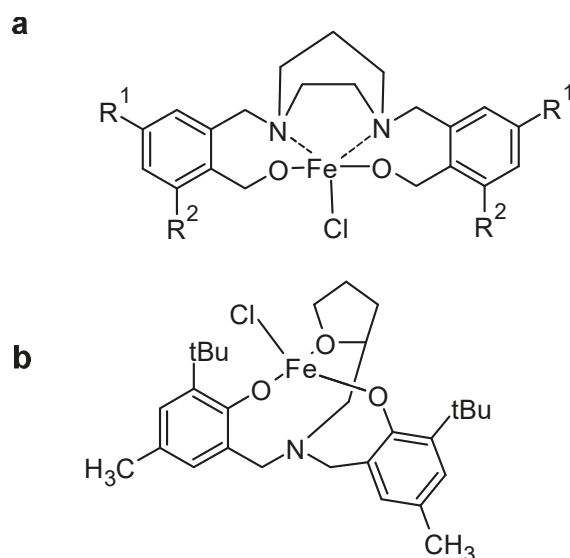


Figure 3. Phenolate metal coordinates from (a) ref [18] and (b) ref [19].

Finally, the main properties are summarized in (Table 1).

Table 1. Main properties of two parts of hydroxyazobenzenes.

Property	Azobenzenes	Phenolic Compounds
Acid or Base Behaviour	Stable but protonated under strong acid conditions	Weak acids, form phenoxide anions in base conditions
Electronic Effects	Azo (-N=N-) bond conjugation influences absorption	-OH is electron-donating, affects reactivity
Photoisomerization	UV-visible light-induced <i>trans-cis</i> switching	No isomerization, but light can degrade phenols
Redox Chemistry	Reduced to hydrazo (-NH-NH-) under mild conditions	Oxidized to quinones in oxidative media
Electrophilic Substitution	Substituent-dependent reactivity	Highly activated towards substitution reactions
Metal Coordination	Forms transition metal complexes	Coordinates metal ions (e.g., Fe ³⁺ , Cu ²⁺)

1.2. Objectives and Scope of This Review

This review focuses on phenolic azobenzenes, highlighting their reversible photoisomerization behaviour and utility in cation complexation. It aims to present a comprehensive summary of recent advancements in their synthesis, structural features, and functional

properties. This review explores diverse synthetic methodologies, emphasizing the role of the phenolic hydroxyl group and azobenzene scaffold in coordinating cations. Mechanistic insights into complexation processes are examined alongside their relevance to coordination chemistry. Furthermore, this review outlines the key applications of phenolic azobenzene–cation complexes, particularly in ion sensing, catalysis, biological systems, and dye-sensitized solar cells (DSSCs), thereby defining their significance as multifunctional materials.

2. Chemistry of Phenolic Azobenzenes

2.1. Structure and General Properties of Hydroxyazobenzenes

Hydroxyl-azobenzenes are organic compounds featuring both hydroxyl (-OH) and azo (-N=N-) functional groups attached to benzene rings. The position of the hydroxyl group significantly influences their properties. In *para*-hydroxyazobenzene, for example, ground-state keto/enol tautomerism is observed, affecting its photochemical behaviour [20]. Upon excitation, the *trans*-keto form can isomerize to the *cis* configuration, with the tautomeric equilibrium and isomerization dynamics being solvent-dependent. In aqueous solutions, *para*-hydroxyazobenzene exhibits considerable keto/enol tautomerism in the ground state, leading to specific excited-state behaviours. Indeed, the excited-state intramolecular proton transfer (ESIPT) in *para*-hydroxyazobenzene involves a photoinduced tautomerization between azophenol and quinone-hydrazone forms. Upon UV excitation, the molecule can undergo an ultrarapid intramolecular proton transfer. In the ground state, p-HAB predominantly exists in the enol form, stabilized by intramolecular hydrogen bonding. Upon excitation, the proton transfer to the azo nitrogen occurs, forming the keto tautomer in the excited state [21]. The ESIPT process in p-HAB is influenced by solvent polarity and pH. Polar solvents and acidic conditions can stabilize the keto form, enhancing the ESIPT process. Conversely, in nonpolar solvents or under basic conditions, the enol form is favoured, and ESIPT is less prominent [22].

These characteristics make hydroxyazobenzenes valuable in applications such as light-driven optical switching and photoactive monomers in elastomers for light-sensitive actuators [23]. The infrared (IR) spectra of hydroxyazobenzene compounds [24] exhibit characteristic absorption bands corresponding to different functional groups, including hydroxyl (-OH), azo (-N=N-), and aromatic (-C=C-) vibrations. The characteristic (N=N) IR absorption bands for hydroxyazobenzene are typically observed around 1400–1450 cm⁻¹, but these bands can shift depending on substituents and conjugation with the benzene rings. Other bands (OH, aromatic CH, and CO) are usually observed. However, the exact peak positions and intensities may vary depending on the substituents present on the benzene rings and whether the hydroxyl group participates in intramolecular hydrogen bonding (e.g., with the azo group). Moreover, in the case of *o*-hydroxyazobenzene, it has been established by polarized IR spectra in the region of 4000–400 cm⁻¹ that the molecule of 4-chloro-2'-hydroxy-4'-pentyloxyazobenzene undergoes a spontaneous ordering in thin layers of 10–20 μm [25]. The broad absorption in this region can be attributed to plane-to-plane intermolecular interactions, driven by resonance-assisted hydrogen bonding (RAHB) involving intramolecular O–H ··· N hydrogen bonds [26].

The UV–visible spectroscopy of hydroxyazobenzenes is a crucial technique for studying their electronic properties and photochemical behaviour. These compounds typically exhibit strong absorption in the visible region due to the electronic π – π^* transition of the conjugated azo (-N=N-) system, often accompanied by a weaker n– π^* transition. The presence of a hydroxyl (-OH) group at the *ortho* or *para* position to the azo group significantly influences the absorption spectra due to mesomeric effects and intramolecular interactions, particularly hydrogen bonding. In acidic or basic media, these compounds can undergo

bathochromic or hypsochromic shifts due to changes in electron density and tautomeric equilibrium (equilibrium between the azo and hydrazone forms). These properties make hydroxyazobenzenes particularly interesting for applications as dyes, pH indicators, or photochromic materials. For example, (E)-3,4,6-trichloro-2-(p-diazenil)-phenol (t-DZH) can be deprotonated in t-DZ (Figure 4), and this transformation has a strong impact on *trans* $\rightarrow$ *cis* isomerization [27]. Moreover, these hydroxyazobenzenes can also reversibly photogenerate tuneable pH drops in water up to one pH unit amplitude upon illumination at 365 nm (Figure 4, grey arrows, [28]).

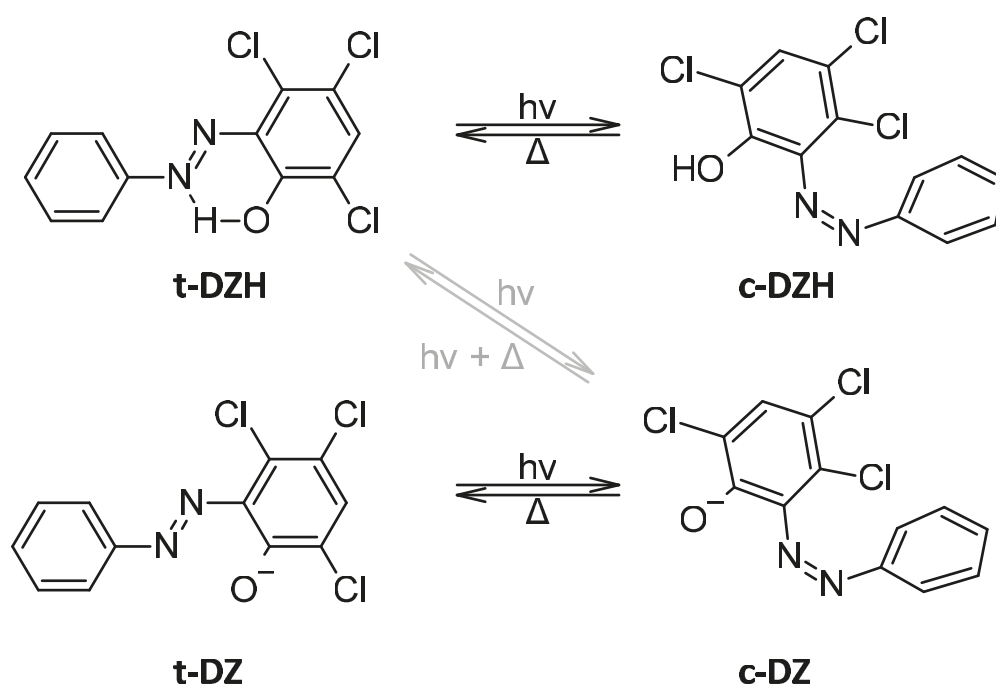


Figure 4. Molecular structures of t-DZH, c-DZH, t-DZ, and c-DZ with classical *trans* $\rightarrow$ *cis* isomerization (black arrows, [27]) or light-driven ionization (grey arrows, [28]).

2.2. Solvatochromism and Complexation Effects of Azo–Schiff Bases and Hydroxyl-Azobenzenes

Phenolic azo–Schiff bases are a specialized class of ligands that incorporate phenolic, azo, and imine ($\text{C}=\text{N}$) functional groups, offering multiple coordination sites for metal cation complexation [29]. The phenolic OH group, in particular, plays a crucial role by donating electron density to metal centres, often enhancing complex stability through hydrogen bonding or deprotonation to form phenolate ions [30]. This multi-dentate coordination capability allows phenolic azo–Schiff bases to form stable chelate complexes with a wide range of transition metal ions [31]. While azo dyes exist in azo or hydrazone tautomeric forms, 2-hydroxy Schiff bases exist in phenol-imine, hydrazone-imine, or keto-amine tautomers in solid state and solution. When complexation occurs with metal ions, tautomerization favours the phenol-imine isomer (Figure 5) [32].

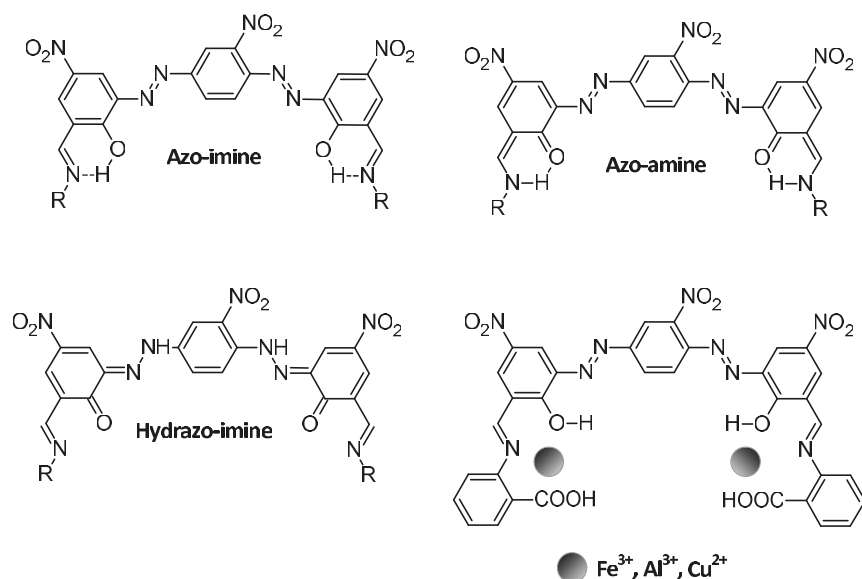


Figure 5. Proposed tautomerism and binding interaction of azo-Schiff base [32].

3. General Synthetic Methods for Azobenzenes and Hydroxyazobenzenes

Various synthetic strategies have been developed for the preparation of phenolic azobenzenes, each with specific advantages in terms of selectivity, efficiency, and compatibility with functional groups [33]. These methods usually involve either the coupling of phenolic compounds with azobenzene derivatives or the direct formation of the azo bond between anilines and phenols under controlled conditions [34]. The most commonly used methods for the synthesis of azobenzenes include the azo coupling reaction, the Mills reaction, the Wallach reaction, the oxidation of anilines, and the reduction of nitroaromatic compounds (Figure 6) [35]. While synthetic methods for symmetrically substituted azobenzene derivatives are well established, the development of efficient strategies for their asymmetrical counterparts remains an ongoing challenge [36].

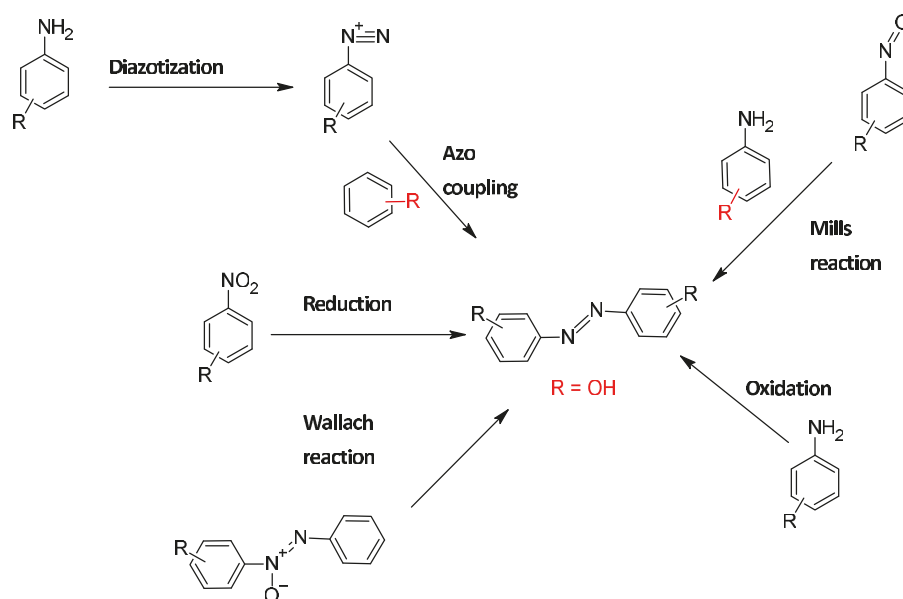


Figure 6. General synthetic methods for synthesis of azobenzenes.

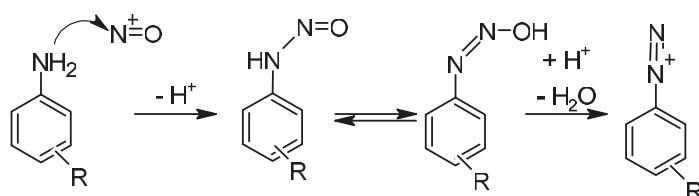
3.1. Azo Coupling Reaction

Azo coupling reactions are among the most commonly used methods for the synthesis of azobenzenes, involving an electrophilic aromatic substitution (S_EAr) reaction of a diazonium salt and an electron-rich arene [37]. The methodology usually begins with the diazotization of an aromatic primary amine using an in situ prepared nitrosonium ion, which is carried out under mild conditions at low temperature (0 °C to 5 °C) to form the diazonium salt (Figure 7) [38]. The electrophilicity of diazonium ions is relatively weak because their positive charge is delocalized. Therefore, phenols as electron-rich arenes are suitable substrates for the coupling reaction with aryl diazonium salts to form the azo compound.

a Synthesis of $N=O^+$



b Synthesis of diazonium salt



c Azo coupling reaction

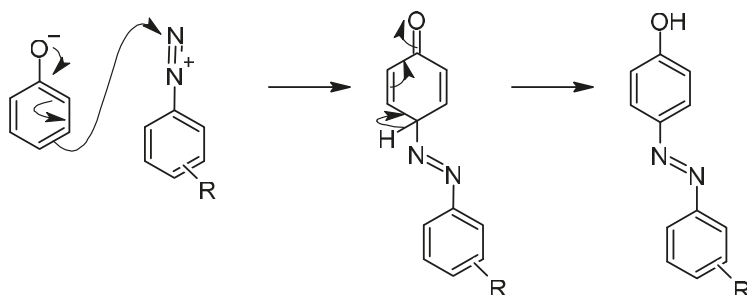


Figure 7. Mechanism of azo coupling reaction.

The substitution usually takes place at the para position, unless this is already occupied, in which case the ortho position is preferred. The reaction often requires short reaction times and generally leads to products of high purity (Figure 8) [39]. This process is extremely versatile and allows for the selective functionalization of both the amine and the nucleophilic component, making it suitable for a wide range of substrates (Figure 8) [40].

As can be seen from the diagram in Figure 8, the yield of azobenzene formation does not depend on the electronic properties of the substituents, and high yields of azobenzene products of 85% to 95% were obtained, irrespective of whether the substituent on the aniline is electron-withdrawing or electron-donating. However, this method requires careful temperature and pH control to ensure the stability of the diazonium salt and to avoid side reactions [41]. The side reactions related to azo coupling are mainly the decomposition of diazonium salt to phenols, chlorobenzene (if hydrochloric acid was used to form this salt), or to any functionalized benzene depending on the presence of nucleophilic moieties [42]. Moreover, the use of sodium nitrite can lead to the formation of nitrobenzene from the corresponding aniline [43]. This is why the acid and temperature used in diazotization not only adjust the pH of the reaction medium to slightly acidic or neutral conditions but also play a crucial role in determining the stability of the resulting

diazonium salt. Diazonium chloride salts, traditionally prepared from aniline, sodium nitrite, and hydrochloric acid, are unstable at room temperature and are normally prepared and used at 0–5 °C. However, diazonium compounds can be isolated as tetrafluoroborate or tosylate salts, which form stable solids at room temperature [44].

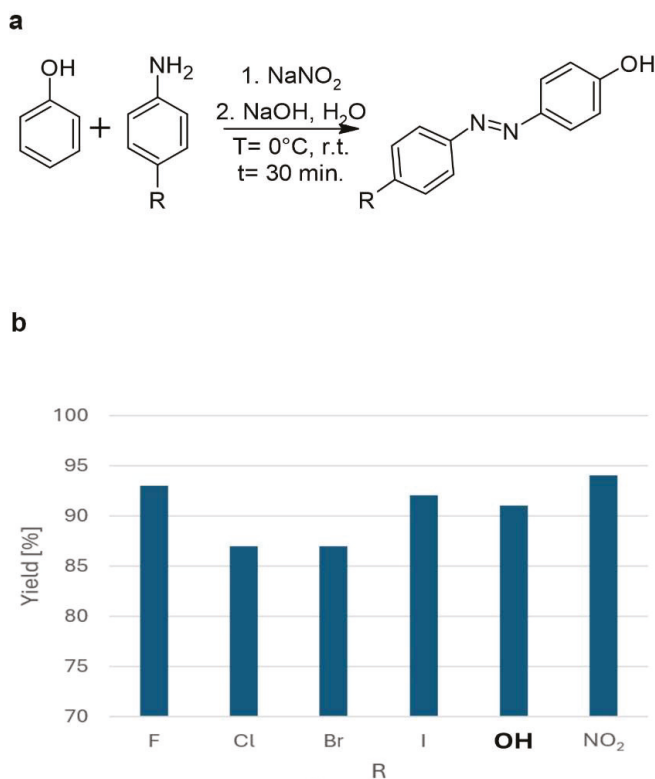


Figure 8. Yield of azobenzenes in coupling reaction of phenol with substituted anilines. (a) General pathway and (b) yields depending on R.

In the case of not performing the in situ conversion of aniline to azobenzene, it is also possible to isolate the diazonium salt, which can be further converted to the phenolic azobenzene via a reaction with a sodium acetate (NaOAc) base in aqueous medium (Table 2) [45].

Table 2. Synthesis of phenolic azobenzenes using diazonium salt and NaOAc as base.

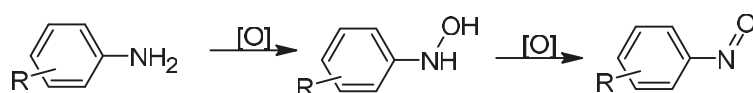
Solvent	Yield [%]
Methanol	46
Ethanol	42
Acetonitrile	40
Acetone	41
Dichloromethane	0
Methanol–H ₂ O = 1:1	80
Methanol–H ₂ O = 3:1	89
Methanol–H ₂ O = 9:1	63

As can be seen in Table 2, the use of a polar solvent is key to achieving higher conversion efficiencies. In fact, when a higher proportion of water is used, the efficiency ranges between 80% and 90% [45].

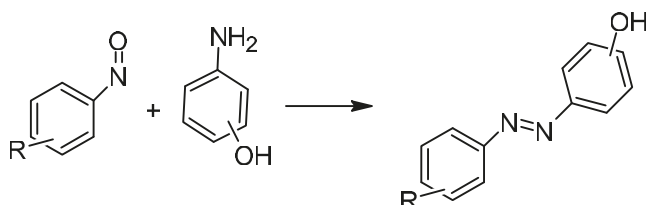
3.2. Baeyer–Mills Reaction

The Baeyer–Mills reaction is one of the most commonly used methods for accessing non-symmetric azobenzenes and is based on the condensation of nitrosobenzene derivatives with anilines [46]. This reactivity can be explained by the proposed mechanism, which involves the nucleophilic attack of the aniline on the nitrosobenzene derivatives in acidic or basic media (Figure 9) [47,48]. The Baeyer–Mills reaction consists of several steps. In the first step (Figure 9a), aniline is oxidized to a nitroso compound, whereby *N*-phenylhydroxylamine is formed as an intermediate. In the next step (Figure 9b), a reaction takes place between the nitroso compound and aniline, producing azobenzene. The use of suitable reagents and appropriate catalysts is crucial for this reaction, as otherwise, symmetrical azobenzenes are formed in situ (Figure 9c) or overoxidation takes place, leading to the formation of nitroaromatics (Figure 9d) [49].

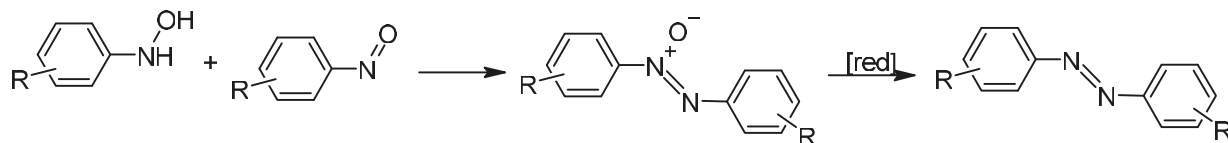
a Synthesis of nitroso compound



b Synthesis of azobenzene



c Example of side reaction : In situ preparation of homoazobenzenes



d Example of side-reaction : Formation of nitroaromatics

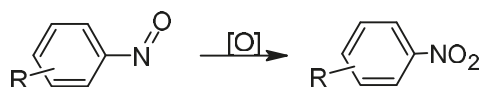


Figure 9. Mechanism of Baeyer–Mills reaction.

The reaction exhibits good selectivity, with control over the electronic properties of the reactants, allowing for the fine-tuning of the final product [46]. The presence

of electron-donating groups, such as hydroxyl groups, on the aniline ring can further influence the reactivity and regioselectivity of the process, making this method highly adaptable for the synthesis of functionalized phenolic azobenzenes (Table 3) [50]. Table 3 shows that electron-rich anilines perform poorly in Baeyer–Mills reactions because they have a high propensity to reduce nitrosoarene, which translates into higher yields of the azoxybenzene side products. Anilines with moderately electron-withdrawing and electron-donating substituents give excellent yields of the desired azobenzenes with a minimal formation of azoxybenzene. The yield of azobenzene decreases sharply when arylamines with strongly electron-withdrawing substituents are used, which is probably due to the lower nucleophilicity of the anilines [20]. The position of the functional group in relation to the amine group also affects the yield of azobenzene synthesis. The data presented in Table 3 show that the conversion of aniline (regardless of the nature of the substituent) in the Baeyer–Mills reaction is quite high, with the exception of the 2,6-difluoro and nitro functional groups, where the conversion rate is between 10% and 25%.

Table 3. The effects of the substituent on aniline on the formation of azobenzene.

R	Azobenzene yield [%]	Azoxybenzene yield [%]
<i>p</i> -NMe ₂	34	45
<i>p</i> -NH ₂	28	43
<i>o</i> -NH ₂	8	91
<i>p</i> -OMe	≥95	5
2,6-dimethoxy	46	35
<i>p</i> -NHCO ₂ ^t Bu	87	6
<i>p</i> -OH	34	69
<i>o</i> -OMe	82	13
<i>p</i> -Me	95	≤5
<i>o</i> -NHCO ₂ ^t Bu	85	12
<i>o</i> -Et	77	20
<i>p</i> -I	≥95	≤5
H	≥95	≤5
<i>o</i> -Br	49	8
<i>p</i> -CO ₂ Me	82	6
2,6-difluoro	12	≤5
<i>p</i> -CN	64	7
<i>p</i> -CF ₃	≥95	≤5
<i>o</i> -NO ₂	≤5	≤5
<i>p</i> -NO ₂	19	≤5

In cases where azoxybenzene is formed as the main product of the Baeyer–Mills reaction, the subsequent reduction of azoxybenzenes to azobenzenes occurs, and this is discussed in the following sections of this paper.

As can be seen from the above figures and the table, the key to the Baeyer–Mills reaction lies in the production of the nitroso compound. Generally, this is produced by the oxidation of aniline, but it should be noted that not all oxidation conditions allow for the production of a nitroso compound (Figure 10). The nitroso compound can be formed either with Oxone, which is also the most common process, or with another oxidizing agent such as hydrogen peroxide in the presence of a suitable catalyst [51]. Although H_2O_2 presents major problems in transport and handling, it is a green oxidizing agent, due to the formation of non-toxic and environmentally benign byproducts, besides presenting an excellent atom efficiency. In principle, Oxone provides better selectivity for the formation of nitroso compounds, since in the case of the use of H_2O_2 /catalyst (using classical catalysts such as SeO_2 , Na_2WO_4 , MTO), azoxybenzene is often formed directly. On the other hand, new catalytic systems have been developed which, in combination with H_2O_2 , allow for the highly selective conversion of aniline to nitroso compounds. One such catalyst is Na- $\beta\beta$ - Ln_4 (Figure 10a), a dimeric POM (polyoxometalate) composed of lanthanide-containing dilacunary Keggin subunits of the β -type that act as Lewis acids, enabling the full conversion of the substrate and total selectivity towards nitrosobenzene. The exact nature of the lanthanide centres affects not only catalytic activity but also the solubility of the catalyst during the reaction. Reaction rates decrease as the atomic number of lanthanide increases, but at the same time, less of the catalyst is transferred to the solution.

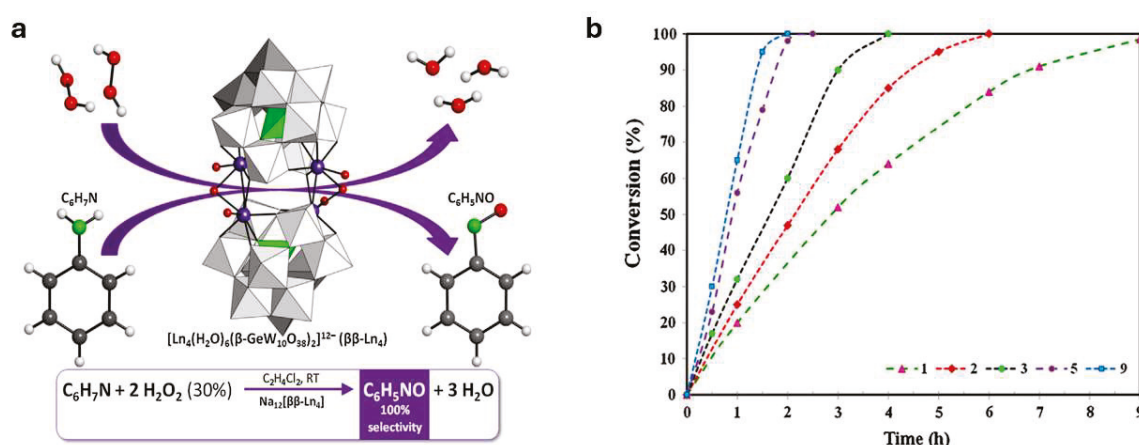


Figure 10. (a) Aniline-to-nitrosobenzene selective oxidation process and molecular structure of Na- $\beta\beta$ - Ln_4 catalysts and (b) aniline conversion at room temperature throughout nine consecutive runs. Reprinted with permission from Ref. [51]. Copyright 2015, Elsevier Inc.

Moreover, the catalyst has excellent recyclability properties. The selectivity of the catalyst towards nitrosobenzene remains constant (100%) even after nine consecutive reaction runs. Interestingly, the time required to obtain 100% conversion decreases in the successive reactions (Figure 10b) [51].

When a combination of KMnO_4 and FeSO_4 is used as an oxidizing agent (Figure 11), this reaction does not lead to the oxidation of aniline to nitrosobenzene. Instead, symmetrical azobenzene is formed directly [52,53].

With certain oxidizing agents, asymmetric azobenzenes can be formed directly and not via the synthesis of a nitroso intermediate [54].

In cases where nitroso compounds are not involved as intermediates in the reaction mechanism, the process can no longer be classified as a Baeyer–Mills reaction but rather as the oxidative coupling of anilines, leading to the formation of azobenzenes, as discussed in the following section of this review article.

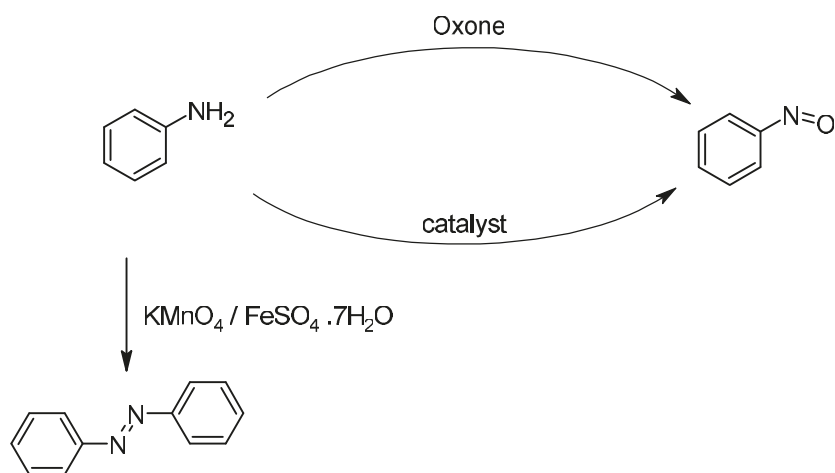


Figure 11. Oxidation of aniline to nitroso derivative or symmetrical azobenzenes using suitable oxidants.

3.3. Oxidation of Anilines

The essential difference between the Baeyer–Mills reaction mentioned above and the in situ formation of azobenzene by the oxidation of anilines is that in the latter case, the intermediate oxidized form of aniline is not isolated. It is precisely for this reason that symmetrical azobenzenes are formed in this type of synthesis.

The formation of azobenzenes by the oxidation of aniline is a fairly old process, originally based on electrolytic oxidation [53]. In general, these processes were characterized by synthesizing azobenzenes in lower yields than those of other processes. An example of electrolytic oxidation is the synthesis of azobenzene from trichloroaniline, which was carried out with a platinum (Pt) electrode in dimethylformamide and in the presence of pyridine [55].

It has been reported that various oxidizing agents can generate azo compounds from aromatic amines. When $\text{H}_2\text{O}_2/\text{Na}_2\text{WO}_4$ is used, the initial oxidation of aniline to the azodioxide and its subsequent reduction with Si_2Cl_6 take place. Azobenzene is obtained in very good yield [56].

A variety of metallic and non-metallic reagents, such as Ag_2O , AgO [57], AgMnO_4 [58], MnO_2 [59], KO_2 [60], NaBO_3 [61], $\text{Pb}(\text{OAc})_4$ [62], BaMnO_4 [63], $\text{Ce}(\text{OH})_3\text{O}_2\text{H}$ [64], NiO_2 , peroxides (such as hydrogen peroxide or *tert*-butyl hydroperoxide [65]), and even chemicals from electrochemical oxidation [66], were used either stoichiometrically or in excess to synthesize azobenzenes from aromatic amines. This literature review showed that the choice of oxidation reagent has a really big impact on the final yield of azobenzene synthesis. It turns out that the yields in the case of the use of specialties such as BaMnO_4 , MnO_2 , and AgMnO_4 are high and range between 70% and 90%, in contrast to, for example, NaBO_3 , $\text{Ce}(\text{OH})_3\text{O}_2\text{H}$, NiO_2 , and AgO , where the yields are mostly lower than 60%. Furthermore, it is also interesting to note that the yield is also affected by which manganese speciation is used as the oxidation reagent. For example, oxidation with barium manganate gives reproducible results without requiring further activation, while an oxidation reaction with manganese dioxide is not reproducible in many aspects. In addition to the above, BaMnO_4 allows for higher selectivity in oxidation reactions, while at the same time, they are faster and have higher reaction yields [63].

Gold nanoparticles on titanium dioxide (TiO_2) have also been shown to be a very efficient system for the conversion of aniline to azobenzenes, allowing for a very selective conversion to azobenzene (up to 98 %) [67].

Nowadays, the use of green oxidizing agents such as oxygen/air or hydrogen peroxide (H_2O_2) in particular is being researched for this type of oxidation [68], due to the formation

of non-toxic and environmentally benign byproducts. In this case, oxidation reactions are carried out using suitable catalysts such as $\text{Zr}(\text{OH})_4$, and the azobenzene formation efficiencies are between 70% and 90%. However, when oxygen is used as the oxidation reagent, the yields increase above 90%, as a more selective formation of azobenzene occurs with a relatively low proportion of nitrosobenzene (around 2%) and is not significantly dependent on the functional groups present on the aniline [65].

Although the oxidation process is often efficient, it must be carefully controlled to avoid overoxidation, which leads to undesirable byproducts. For example, excessive oxidation can lead to the formation of azoxy or nitro compounds, which are usually more stable and may not be suitable for subsequent reactions or applications. Therefore, the optimization of reaction conditions, including the amount of oxidant and reaction time, is critical to achieve high yields of the desired phenolic azobenzene products. For example, in the case of NaBO_3 , the temperature of the reaction mixture proves to be very important. It turns out that the yield of the reaction increases with increasing temperature. In addition to the above, the pH of the reaction mixture also has a major influence, i.e., the yield of the azobenzene formation reaction decreases with an increasing pH of the reaction mixture. In the case of metal peroxides, it turns out that the reaction times in the case of nickel(II) peroxide are much longer than those in the case of ceric trihydroxy hydroperoxide. In the case of the use of O_2 or peroxide as the oxidation reagent in combination with a catalyst, it turns out that reaction conditions such as reaction time and the concentration of the oxidation reagent do not significantly affect the yield of the azobenzene synthesis reaction itself. On the other hand, the choice of peroxide and solvent is very influential. It was found that only the reaction in acetic acid and in the presence of the oxidizing agent *tert*-butyl hydroperoxide is selective as far as the synthesis of azobenzenes is concerned. In the other cases (using H_2O_2 in combination with H_2O , methanol, and mesitylene as solvents), a nitroso compound or an azoxybenzene is formed. If azoxybenzene is formed in the reaction, it can also be converted to azobenzene with a suitable reducing agent (e.g., Si_2Cl_6). In the case of using oxygen as an oxidizing agent, mesitylene proves to be a significantly better solvent than water in terms of the selectivity of azobenzene formation. On the other hand, the reaction time and solvent do not significantly affect the yield of the azobenzene formation reaction in the case of AgO .

In contrast to the above oxidation processes, the synthesis of asymmetric azobenzene can be achieved in combination with air or oxygen at standard pressure (1 atm) and the use of a copper catalyst (e.g., CuBr or CuCl) (Figure 12 [54]). A similar reaction pathway can be observed in the case of manganese oxide using chlorobenzene as solvent, when in the presence of oxygen and heated to 160 °C [69].

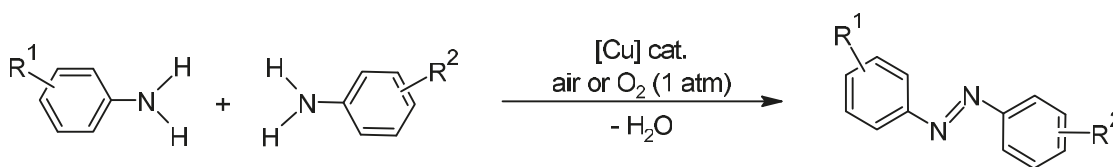


Figure 12. Oxidation of anilines to form asymmetrical azobenzene.

The above method of the oxidation of anilines and the formation of azobenzenes also allows for the synthesis of symmetrical azobenzenes in yields ranging from 60% to 100% depending on the functional groups on the aniline. In the case of symmetrical azobenzene synthesis, the reaction yields are lower, ranging between 40% and 70% depending on the functional groups on the aniline.

3.4. Reduction Reaction of Azoxybenzenes

As shown in the previous sections, overoxidation produces azoxybenzenes containing a N=N bond with an additional oxygen functionality ($-N=N(O)-$), which can be removed by controlled reduction to give the corresponding azo compounds, leaving the conjugated π -system intact.

The first way to reduce azoxybenzenes is to use various reducing reagents, including hydrogenation catalysts (e.g., palladium or platinum on carbon), metal-based reducing agents (such as zinc, iron, or tin in acidic media), and organic reducing agents such as hydrazine or sodium dithionite [70–72]. In addition, the reduction of azoxybenzenes to azobenzenes can also take place via photochemical conversion [73]. Upon direct irradiation, azoxybenzenes may undergo rearrangement to 2-hydroxyazobenzene and *cis-trans* isomerization. In contrast, being irradiated in the presence of a photosensitizer (such as acetophenone, benzophenone, thioxanthone, flavone, fluorenone, etc.) with suitable triplet excitation energy leads to a reduction to azobenzene. In the absence of sensitizers, only a change in the geometric configuration of azoxybenzene occurs, and 2-hydroxyazobenzene is formed. As the concentration of the sensitizer increases, the proportion of azobenzene formed also increases. If oxygen is also present in the reaction, it causes a decrease in the proportion of azobenzene formed. The following ketones were efficient photosensitizers to be used to reduce azoxybenzenes to azobenzene: acetophenone (triplet excitation energy $ET_1 = 74$ kcal/mol), benzaldehyde (72 kcal/mol), benzophenone (69 kcal/mol), thioxanthone (65 kcal/mol), phenylglyoxal (63 kcal/mol), and anthraquinone (62 kcal/mol). On the other hand, the following ketones were not efficient: flavone (62 kcal/mol), Michler's ketone (61 kcal/mol), 2-acetonaphthone (59 kcal/mol), biacetyl (55 kcal/mol), benzil (54 kcal/mol), and fluorenone (53 kcal/mol). Based on these results, the triplet state energy of the azoxybenzene intermediate is estimated to be approximately 62 kcal/mol. It is proposed that 2-hydroxyazobenzene and *cis*-azoxybenzene originate from the excited singlet state of azoxybenzene, whereas azobenzene forms via the triplet excited state [73].

The reduction of azoxybenzene is usually stepwise, with the oxygen atom being removed to form the azo product. The choice of reducing agent and reaction conditions has a significant impact on the efficiency, selectivity, and functional group tolerance of the process [74]. A process in which azoxyarenes are treated with hydrazine hydrate in the presence of aluminum in methanol under reflux or microwave irradiation is commonly used for such reactions (Figure 13). In general, the yields are quite high in the conversion of azoxybenzenes to azobenzenes (between 85% and 100%), and they are independent of the type of heating of the reaction mixture. On the other hand, the kinetics of the reaction depend mainly on the type of heating. In the case of microwaves, the reaction time is only 2 min, whereas in the case of reflux, it is about five times longer [71].

Another option for introducing a hydroxyl functional group into azobenzene is the Wallach rearrangement. In this reaction, the conversion of azoxybenzene to azobenzene occurs by the rearrangement of the hydroxy group to an azoxy functional group on the aromatic ring. Although the Wallach reaction is used less frequently than other synthesis methods, it is particularly suitable for the regioselective formation of phenolic azobenzenes [75]. Wallach rearrangement only takes place in strongly acidic solutions. It combines the loss of the oxygen bound to the azo group with the formation of a hydroxyl group in the para position to the azo bond in the benzene ring. In contrast to conventional azo coupling reactions based on diazonium salts or nitroso condensation, the Wallach reaction offers an alternative route to azobenzenes, especially in cases where the direct oxidation of aniline derivatives is difficult [76]. As shown in Figure 13, the main product of the Wallach rearrangement is the phenolic azobenzene, which has an -OH functional group at the para site. This rearrangement also produces byproducts such as *ortho*-hydroxyazobenzene,

azobenzene, and some polymeric materials from aniline and sulfonic acids in very small amounts [77]. The reaction of unsubstituted azoxybenzene at room conditions yields 72% *p*-hydroxyazobenzene and 28% azobenzene. In the Wallach rearrangement, sulphuric acid is generally used in concentrations of over 80% after heating to $T \geq 50\text{ }^{\circ}\text{C}$. With increasing acidity, a slight increase in the amounts of azobenzene and polymeric substances is observed. When azoxyazobenzene is heated to higher temperatures (245–250 $^{\circ}\text{C}$), azobenzene is formed as the main product with small amounts of *para*- and *ortho*-hydroxyazoxybenzene (Figure 13). In the case of Wallach rearrangement, the formation of a hydroxyl functional group can also promote the dehydration process, leading to the subsequent formation of the polymeric form of azobenzene [78]. In the case of unsubstituted azoxybenzene, 32% *p*-hydroxyazobenzene and 68% azobenzene are obtained. If the *para* position is occupied, a hydroxyl group forms in the *ortho* position (Figure 14) [79].

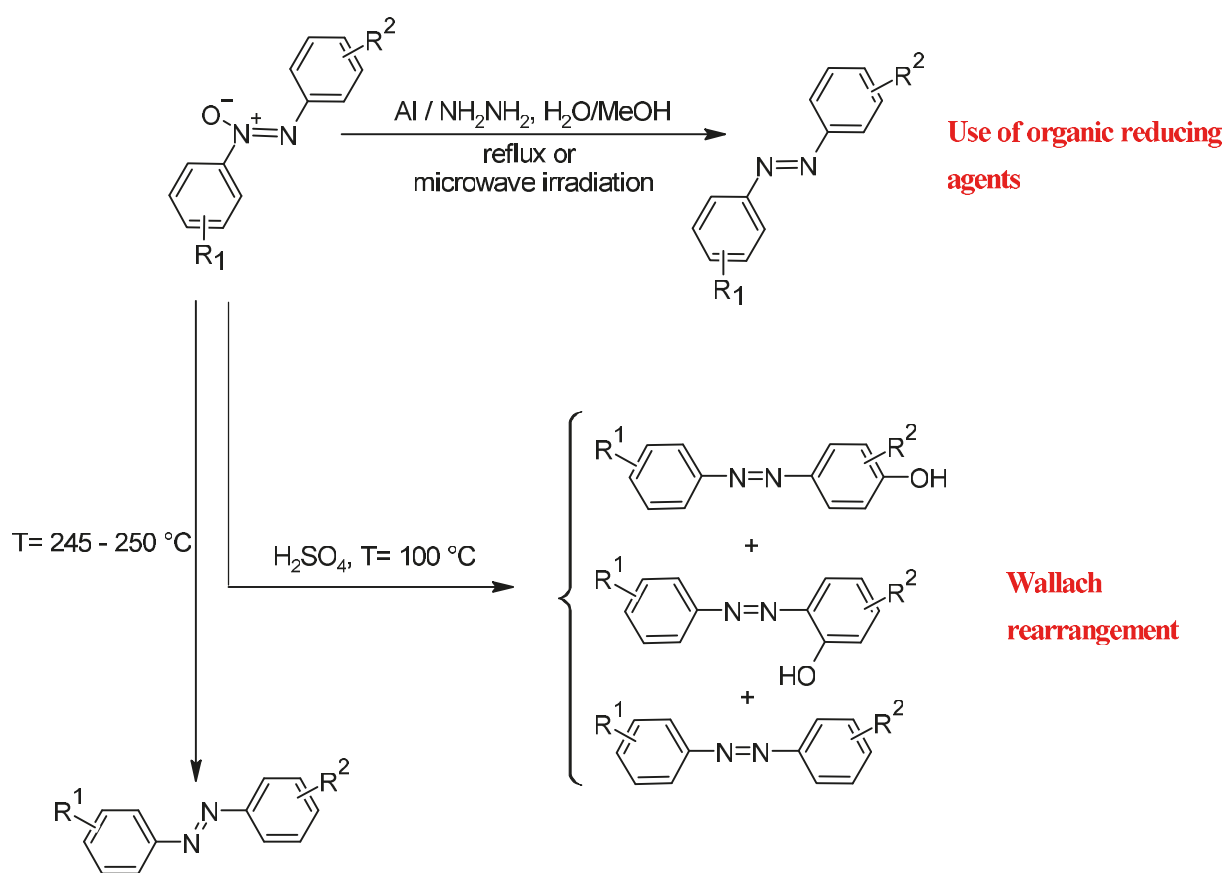


Figure 13. Reduction reaction of azoxybenzenes and Wallach rearrangement.

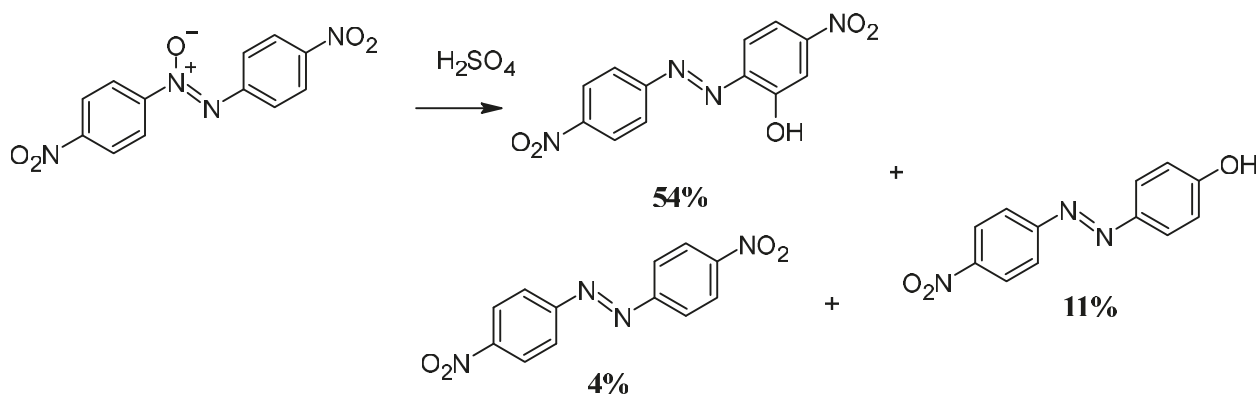


Figure 14. Wallach rearrangement in *para*-disubstituted azoxybenzene.

3.5. Reductive Coupling of Aromatic Nitro Derivatives

This reaction involves the selective reduction of nitroarenes to nitroso derivatives, which are starting substrates for the formation of azobenzene(-N=N-). The reaction leads to the production of symmetrically substituted azo derivatives [80]. Commonly used reducing agents include metal-based reagents such as zinc, iron, or tin in acidic or neutral media and catalytic hydrogenation with transition metal catalysts such as palladium, platinum, or ruthenium [81,82]. Nitroaromatics can also be reduced in the absence of a metal-based catalytic system using an alkaline medium or a combination of sodium hydroxide and ethanol [83]. In addition, glucose is often used as a reducing agent in alkaline media [84].

The reaction proceeds via the stepwise reduction of the nitro group, producing reactive intermediates such as nitroso and hydroxylamine species. These intermediates are then coupled, leading to the formation of the azo bond (Figure 15). The reduction process is very sensitive to the choice of reducing agent, catalyst, and reaction conditions, which significantly influence the selectivity of the reaction [85]. Controlling these parameters is important to prevent over-reduction to aniline derivatives [86]. The choice of metal has a major influence on the yield of the reaction in the synthesis of symmetrical azobenzene. Lithium or zinc is most commonly used as a reducing agent in conjunction with an active metal such as Mg, Zn, Al, In, Ti, or Nb. It can be seen that when the metals W, Mo, and Zr as activated metal are used, the reaction to form azobenzene from nitroaromatics does not take place at all [87]. The functional groups present on the nitroaromatic compound also have a major influence on the yield of the reaction. It turns out that they are the most likely to lower the yield of the azobenzene synthesis reaction. One of the main advantages of this method is its versatility, as it is suitable for a wide range of nitroaromatic compounds, including those with electron-donating or electron-withdrawing substituents. The use of metal-based reducing agents provides an efficient route for the selective generation of azo compounds, while catalytic hydrogenation offers a mild alternative with excellent scalability. In addition, the process can be adjusted to control the regioselectivity and functional group compatibility of the resulting azobenzene products.

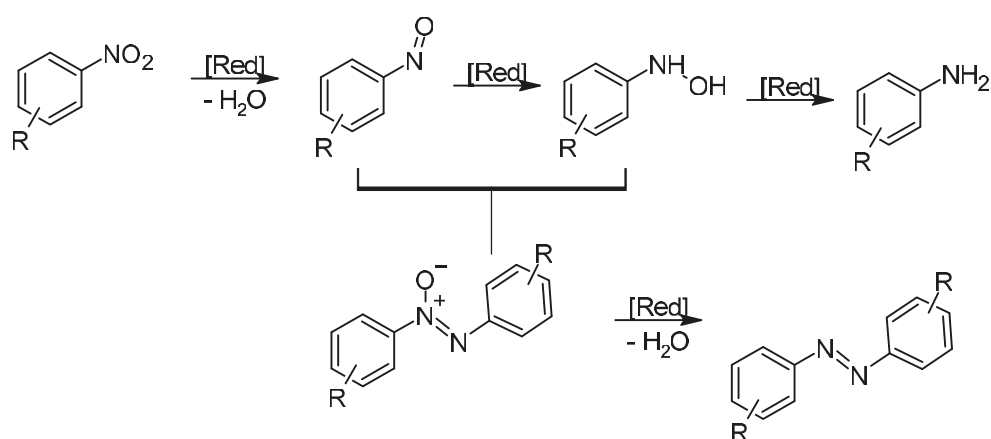


Figure 15. Reductive coupling of aromatic nitro derivatives.

In addition, azobenzenes can be prepared via the photochemically excited reduction of nitrobenzenes (Figure 16) [88].

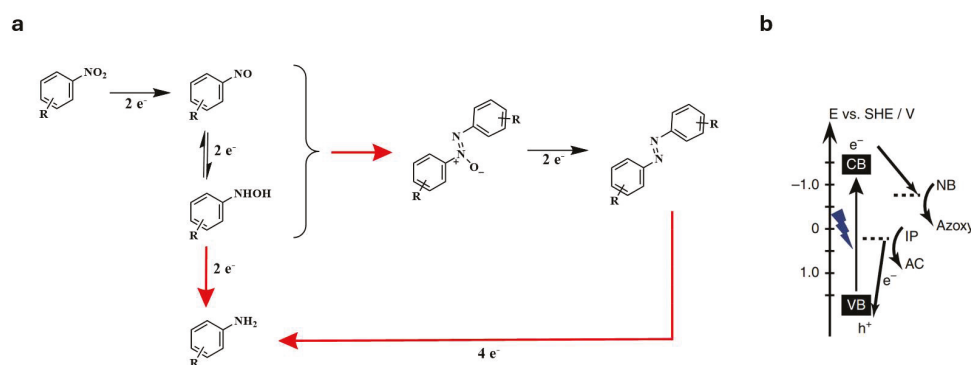


Figure 16. Photochemical process for reduction of nitrobenzenes to azobenzenes (a) and energy diagram of this conversion (b). Figure (b) reprinted with permission from Ref. [88] under Creative Commons CC BY license.

In the reaction shown above, the inexpensive graphitic C₃N₄ represents an efficient photocatalyst for the selective syntheses of a series of azo- and azoxy-aromatic compounds from their corresponding nitroaromatics when exposed to either purple (410 nm) or blue light (450 nm) excitation (Figure 16a). As can be seen in Figure 16b, the photoexcited e⁻ in the conduction band (CB) of g-C₃N₄ possesses sufficient energy to initiate the nitrobenzene reduction. Concurrently, isopropanol (IP) acts as a sacrificial electron donor, undergoing oxidation to acetone (AC), which generates two protons and releases two electrons that recombine with the holes (h⁺) in the valence band (VB).

The high efficiency and high selectivity towards azo- and azoxy-aromatic compounds can be attributed to the weakly bound photogenerated surface-adsorbed H-atoms and a favourable N-N coupling reaction. The selectivity in this type of reaction is shown by the low proportion of amine formed during the azobenzene synthesis reaction (up to 10%), irrespective of the functional groups present on the nitrobenzene. In addition, using 410 nm light, selective conversion to azobenzene and, at 450 nm, selective conversion to azoxybenzenes occur. The yields of the reactions are very high (between 90% and 100%) and do not significantly depend on the functional group on the nitrobenzene [88].

4. Complexation and Crystal Structures

In 1969, Baer et al. studied metal complexes of azo dyes [89]. Particularly, they added cupric chloride or nickel chloride in an aqueous solution of ethylenediamine to o-halide-o'-hydroxyazo compounds, and they obtained several compounds (Figure 17) analyzed by their melting point, their empirical formula, and also their λ_{max} value (nm). Their magnetic susceptibility was also determined. For example, the nickel complex was found to be diamagnetic, indicating that the complex has a square planar geometry around the nickel atom. But the copper complexes examined had a magnetic moment equal to or greater than the spin-only value of 1.73, suggesting that no significant interactions occur between copper atoms. The authors indicated that X-ray would have been required to establish the stereochemistry with certainty.

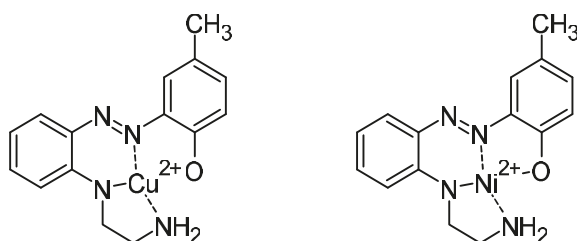


Figure 17. Example of complexes obtained in ref [89].

With this in mind, a relevant example is the complex chlorido[(E)-3-hydroxy-2-methyl-6-(quinolin-8-ylidiazenyl)phenolato]copper(II) monohydrate [90]. In this compound, the hydroxyazobenzene ligand is deprotonated at the ortho position and coordinated to the Cu(II) centre, forming a pseudo-square planar geometry. The crystal structure was determined by X-ray diffraction, revealing the following characteristic bond lengths:

- Cu–O: 1.917 Å.
- Cu–N: (quinoline): 2.008 Å.
- Cu–N: (azo): 1.945 Å.
- Cu–Cl: 2.280 Å.

The complex also shows a weak axial coordination between copper and a chlorine atom from a neighbouring molecule, with a Cu–Cl distance of 2.739 Å, suggesting an intermolecular interaction (Figure 18).

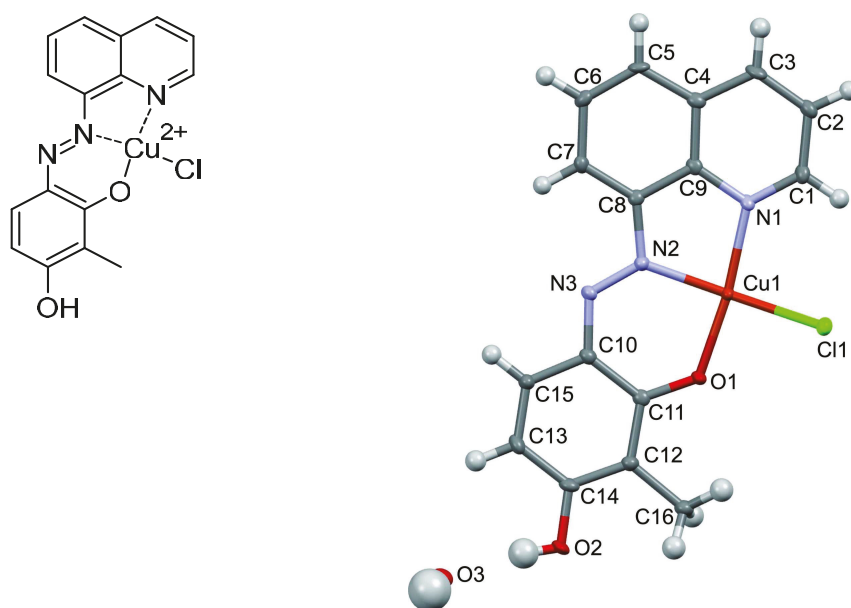


Figure 18. Copper complex and X-ray structure of chlorido[(E)-3-hydroxy-2-methyl-6-(quinolin-8-ylidiazenyl)phenolato]copper(II) monohydrate. Reprinted with permission from Ref. [90]. Copyright 2022, IUCr Journals.

Sometimes, complexation occurs within a synthetic pathway to obtain, after demetalization, ortho-OH azoaryl dyes. This was the case in 2017 where a dinuclear copper (II) complex was synthesized from copper diacetate and a hydrazone molecule by a Fenton reaction using H_2O_2 [91]. The crystal structure of this intermediate was determined in DMF by X-ray diffraction, revealing the following characteristic bond lengths (Figure 19):

- Cu1–O1: 1.944 Å.
- Cu1–O1A: 2.061 Å.
- Cu1–O4: 1.920 Å.
- Cu1–O6: 2.140 Å.
- Cu1–N2: 1.925 Å.

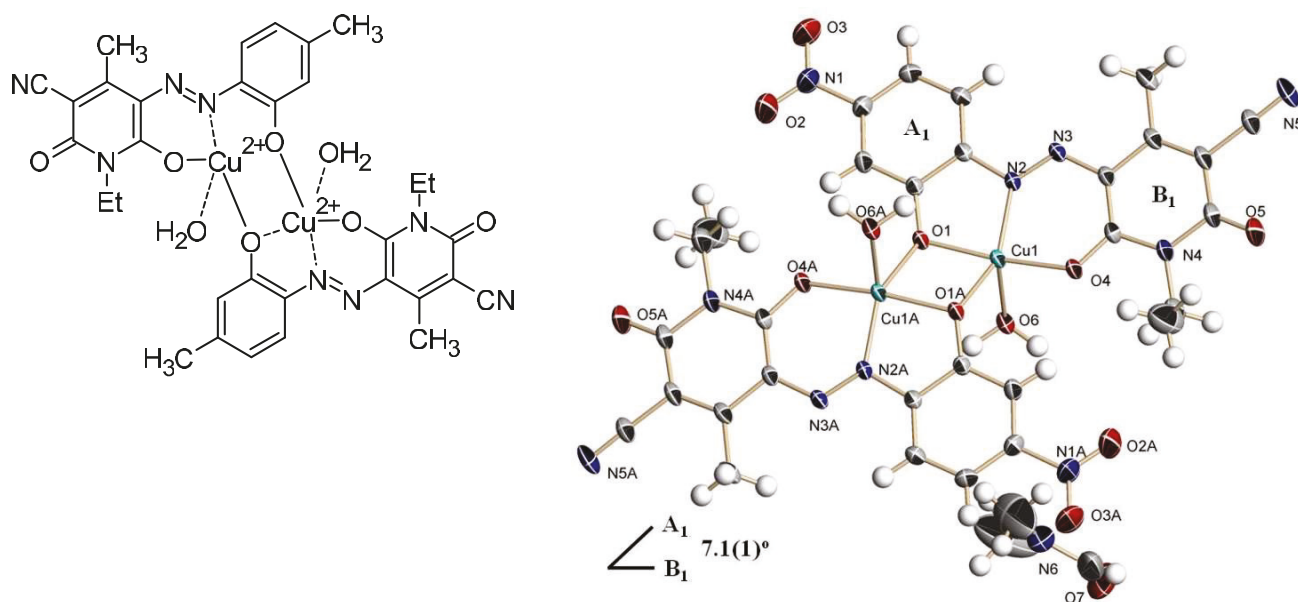


Figure 19. Copper complex and X-ray structure of intermediate. Reprinted with permission from Ref. [91]. Copyright 2017, Elsevier.

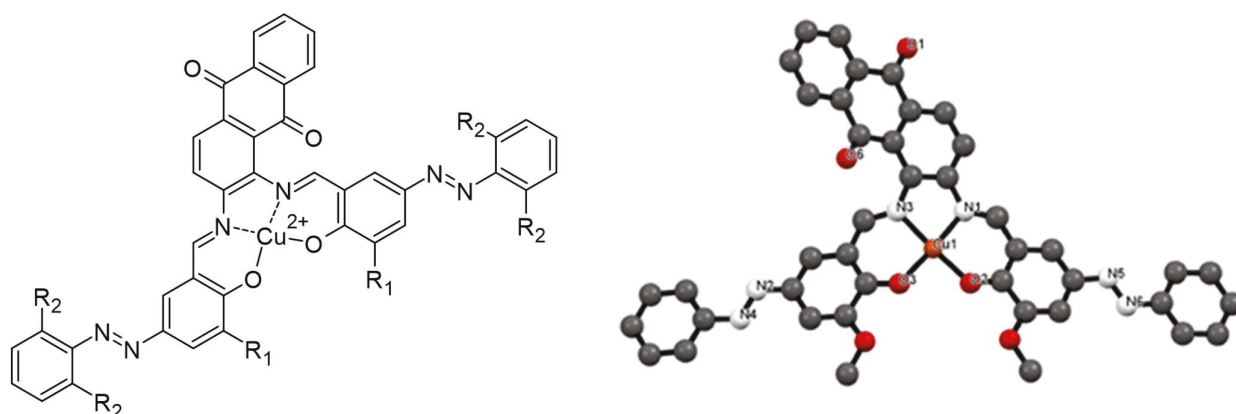
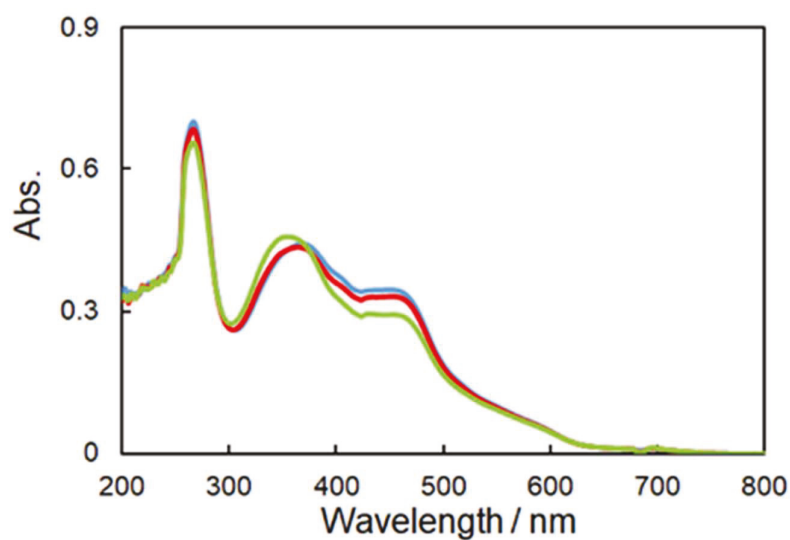
Schiff bases and especially (chiral) salen metal complexes can be employed in (asymmetric) catalysis because of their advantages in stereochemical tuning (with respect to chirality) and redox properties with proper metal ions [92]. These molecules (Figure 20) were synthesized and gave the following crystal structure:

Example for $R_1 = \text{OCH}_3$, $R_2 = \text{H}$:

- Cu1–O2: 1.854 Å.
- Cu1–O3: 1.885 Å.
- Cu1–N1: 1.957 Å.
- Cu1–N3: 1.913 Å.

Concerning photoisomerization capability, the change in UV-vis spectra due to the *trans*-to-*cis* photoisomerization of the azobenzene moiety ($R_1 = \text{OCH}_3$; $R_2 = \text{H}$) showed a shift in π - π^* bands to a shorter wavelength. The stable *trans* form was converted to the *cis* form by a metal-to-ligand charge transfer (MLCT) transition, where electrons move to the ligand, as well as other transitions. Moreover, the reversible *cis*-*trans* photoisomerization, which converts the *cis* to the *trans* form by visible light irradiation, was observed for all complexes (Figure 20).

Bi-metallic compounds can also be obtained by the reaction of an ethynylazobenzene and $[\text{RuCl}_2(\text{dppe})_2]$. This intermediate can react with anilines to obtain Schiff bases, and by the reaction of zinc salts in the presence of phenanthroline or bipyridine, complexes are formed (Figure 21) [93]. It is noticeable that luminescence is observed for all complexes as a result of the intra-ligand $\pi \rightarrow \pi^*$ transition and chelation enhanced fluorescence (CHEF) by the coordination of the imine group to Zn(II) in the complexes. The presence of azo chromophore with extended π -conjugation enhances the emission properties of the complexes and varies considerably with donor–acceptor substituents and the π -acidic character of the co-ligands.

a**b**

UV-vis spectra (DMSO solution) of 1 after UV light irradiation to yield cis form (Blue: 0 min; Red: 10 min; and Green: 20 min)

Figure 20. (a) Structures and (b) photoisomerization of example complex. Article is open access and distributed under terms and conditions of CC BY 4.0 licences [92].

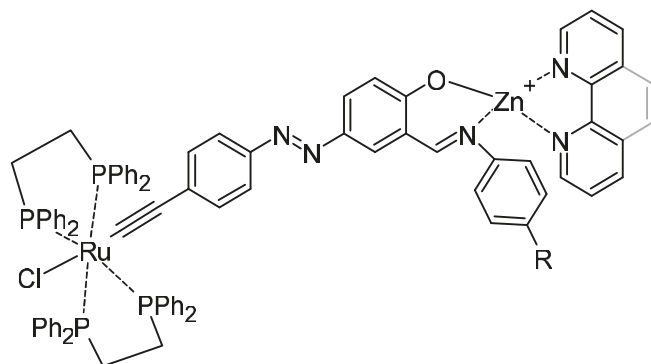


Figure 21. Azo bi-metallic complex.

5. Applications of Phenolic Azobenzene Complexes

5.1. Colorimetric Sensors

Hydroxyazobenzene compounds are a class of azo dyes known for their vivid colouration. The hydroxyl group plays a key role in metal ion binding through coordination chemistry, especially when the azo-OH moiety is in the *ortho* position of a Schiff base (Figure 22) [94]. When these molecules interact with metal cations, they form stable chelate complexes. These complexes alter the molecule's electron distribution, leading to a visible colour change. This property is exploited in colorimetric detection methods for various cations. UV-Vis spectrophotometry can quantify these changes based on absorbance shifts. Each metal ion produces a characteristic spectral change, enabling selective detection. Commonly detected cations include Cu^{2+} and Cr^{3+} [95], Cd^{2+} and Hg^{2+} [96], Co^{2+} , and Al^{3+} [97,98]. Detection is often pH-dependent as protonation affects the ligand binding sites. The simplicity of visual detection makes them suitable for rapid, low-cost analysis [94].

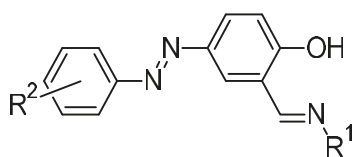


Figure 22. General structure of azo-Schiff bases.

In the domain of pollutant removal, it is also important to be able to perform selective cation detection [99–101]. With this in mind, azo-phenol-based receptors with an azo core linked to naphthol and variously substituted benzothiazole units were synthesized and investigated for their optical properties. Among them, the nitro-substituted derivative showed enhanced fluorescence upon interaction with Hg^{2+} ions, which enabled detection with the “naked eye” [102]. Spectroscopic analyses—including UV-Vis absorption, fluorescence emission, FT-IR, and ^1H NMR—revealed that the Hg^{2+} ion coordinates favourably with the phenolic oxygen, the azo nitrogen adjacent to the naphthol ring, and the thiazole nitrogen of the benzothiazole unit. This interaction occurs in a terdentate fashion (Figure 23). In addition, photoinduced and Hg^{2+} -induced proton transfer reactions supported the presence of *cis-trans* isomerism and azo-hydrazone tautomerism in these systems.

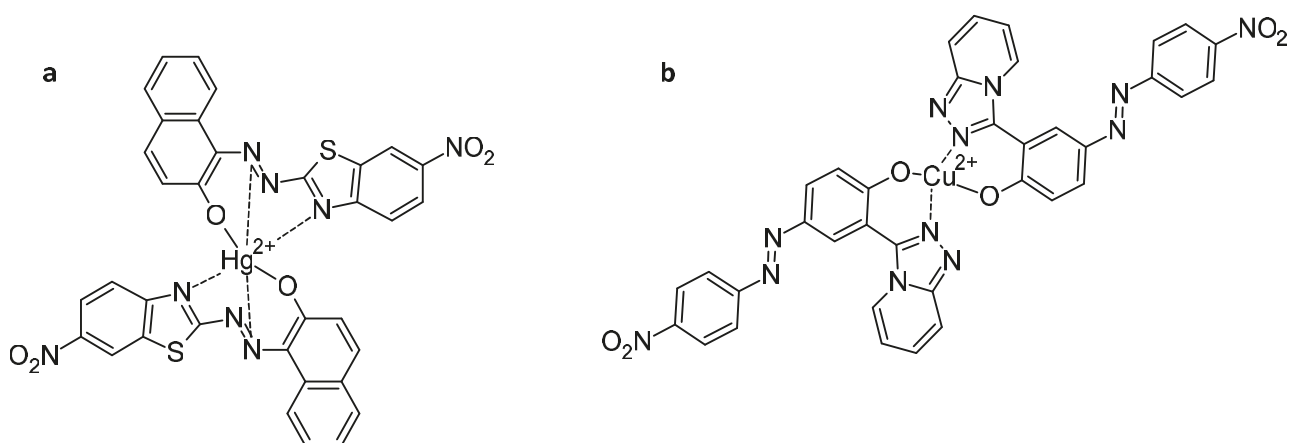


Figure 23. Azo phenol-based probe for (a) Hg^{2+} detection [102] and (b) Cu^{2+} detection [103].

Only recently (2025) was a naked-eye chemosensor developed that makes it possible to quickly and selectively detect copper cations in solution (Figure 24) [104].

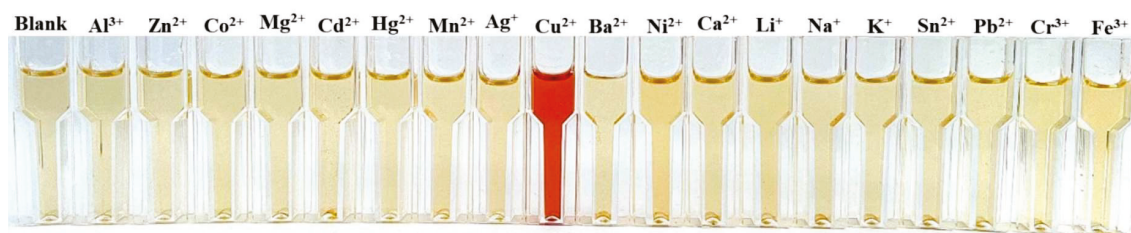


Figure 24. Selective chemosensing of copper cations. Reprinted with permission from Ref. [103]. Copyright 2025, Elsevier.

5.2. Smart Materials and DSSCs

Hydroxyazobenzene compounds exhibit promising potential as dyes for dye-sensitized solar cells (DSSCs) due to their ability to form a complex with metal cations. The hydroxyl groups, mainly supported by the Schiff base in their structure, facilitate coordination with metal ions such as Ti^{4+} , Cu^{2+} [103,105], Fe^{2+} [106], or Zn^{2+} [106], which enhances electronic communication with semiconductor surfaces.

This complexation often results in improved light-harvesting capabilities by extending the absorption range into the visible spectrum (Figure 25, Table 4). The resulting metal–dye complexes can also promote efficient charge separation and electron injection into the conduction band of TiO_2 . Moreover, hydroxyazobenzene dyes are synthetically tuneable, allowing for the optimization of photophysical and electrochemical properties. Indeed, their structural versatility supports the fine-tuning of absorption maxima and redox potentials as azo dyes can be synthesized with various functional groups with different absorption spectra, dipole moments, and conjugation lengths [107].

Additionally, these complexes can exhibit strong anchoring to TiO_2 surfaces [106]. As the hydroxyl group is complexed with a metal cation, it does not interfere with the other anchoring groups, as carboxylate, sulfonate, or pyridyl anchors, knowing that amongst these groups, OH yields the worst performance, with its inferior UV/vis absorption intensity on the TiO_2 substrate and the lowest DSSC performance conversion efficiency [108].

Table 4. Spectral and electrochemical properties of compounds 1–9.

	E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE (eV)	Absorption Range/ λ_{max} (nm)	Reference
1	−5.85	−3.13	2.72	387–679	[103]
2	−5.74	−3.11	2.73	440–727	[103]
3	−5.92	−3.18	2.74	472–752	[103]
4	−6.64	−4.02	2.62	511–790	[103]
5	−5.483	−3.429	2.234	368	[106]
6	−5.312	−3.785	1.527	387	[106]
7	−5.579	−3.279	2.282	352	[106]
8	−4.36	−3.73	0.63	450	[105]
9	−4.36	−3.83	0.53	402	[105]

Dye-sensitized solar cells (DSSCs) were constructed to evaluate the performance of hydroxyazobenzene molecules that complex cations under controlled laboratory conditions. These cells served as a testing platform to investigate the photoelectrochemical properties and potential of azobenzene-based dyes as light-harvesting materials. By producing DSSCs

with these compounds, the aim was to gain insight into their efficiency, stability, and suitability for solar energy conversion applications.

For example, two types of materials were tested (Figure 26). The first one was a polychelate [109], whereas the second one was a metal complex [110].

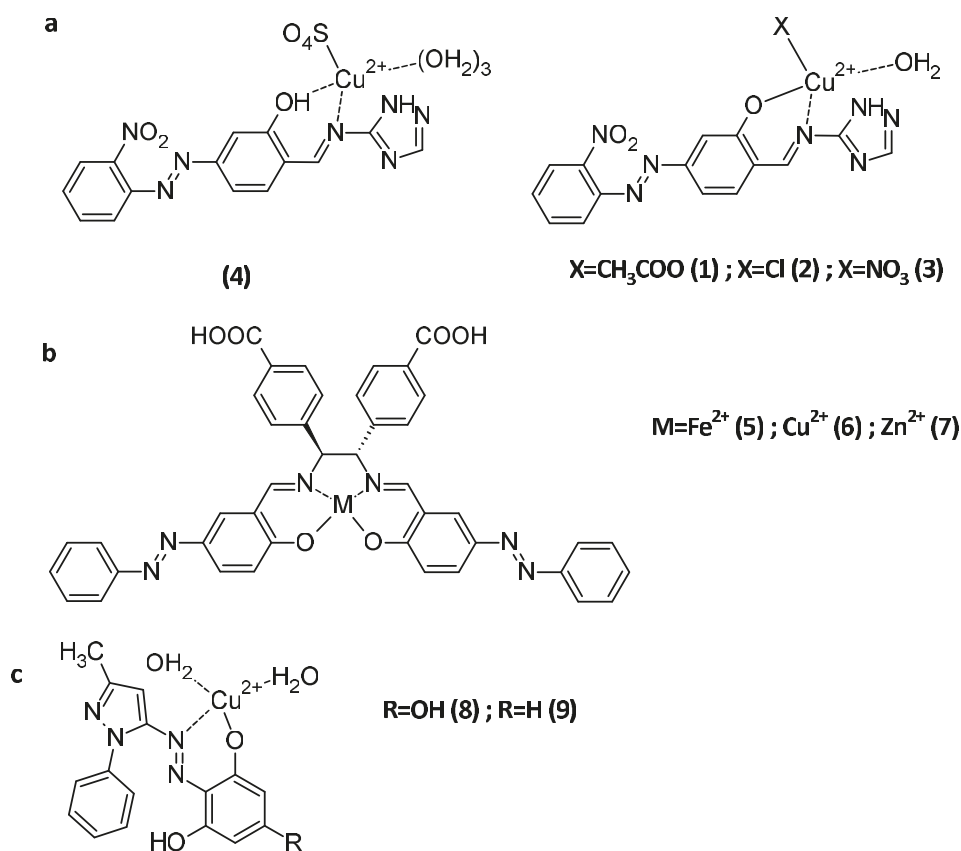


Figure 25. Molecules involved in DSSC applications (a) from ref. [103]; (b) from ref. [106]; (c) from ref. [106].

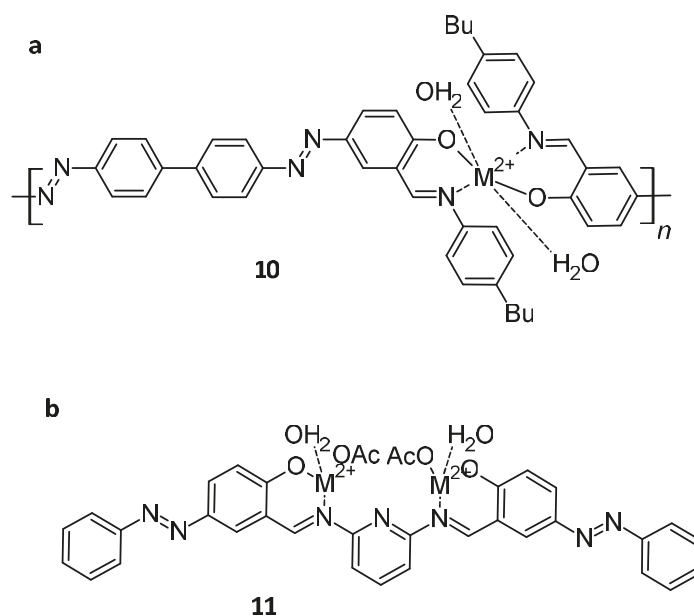


Figure 26. Complexes from (a) ref. [109] and (b) ref. [110].

The photovoltaic parameters of these devices were determined (Table 5). Open-circuit voltage (V_{OC}) is the maximum voltage a solar cell can provide to an external circuit, which

is derived from the splitting of hole and electron quasi-Fermi levels. In organic materials (OSC), disorder induces gap tail states. The relaxation of carriers into these tail states brings the electron quasi-Fermi level down and the hole quasi-Fermi level up and hence reduces V_{OC} [111]. In this context, compared to the reference N3 dye ($[Ru(dcbpy)_2(NCS)_2]$) [112], organometallic compounds 10 and 11 seem to have lower efficiency, but the results can vary a lot depending on the device used (full commercial device, light power, etc.).

Table 5. Photovoltaic parameters with V_{oc} : open-circuit voltage; FF: Fill factor; η : overall energy conversion yield.

Compound	V_{OC} , mV	FF	η , %	
I_3^-/I^-	724	0.70	8.0	[113]
N3	678	0.607	7.3	[112]
10				[109]
Cu_{2+}	400	0.54	2.05	
11				[110]
Co_{2+}	515	0.585	0.742	
Ni_{2+}	495	0.689	0.328	
Pd_{2+}	495	0.654	0.856	

5.3. Catalysis and Coordination Chemistry

The catalytic activity of Cu(II), Co(II), and Ni(II) azo-Schiff base complexes (Figure 27) was tested for the oxidation of various alkenes (cyclooctene, cyclohexene, styrene, α -methylstyrene, and norbornene). It was found that under the optimized reaction conditions, the copper(II) complex displayed 94% conversion for the oxidation of cyclooctene, and the cobalt(II) and nickel(II) complexes exhibited 90 and 85% conversions for the oxidation of α -methyl styrene, respectively. The authors demonstrated that back electron transfer from the complexes to TBHP (tert-butyl hydroperoxide, the oxidant used) breaks the O-O bond and forms tert-butoxyl radicals [114].

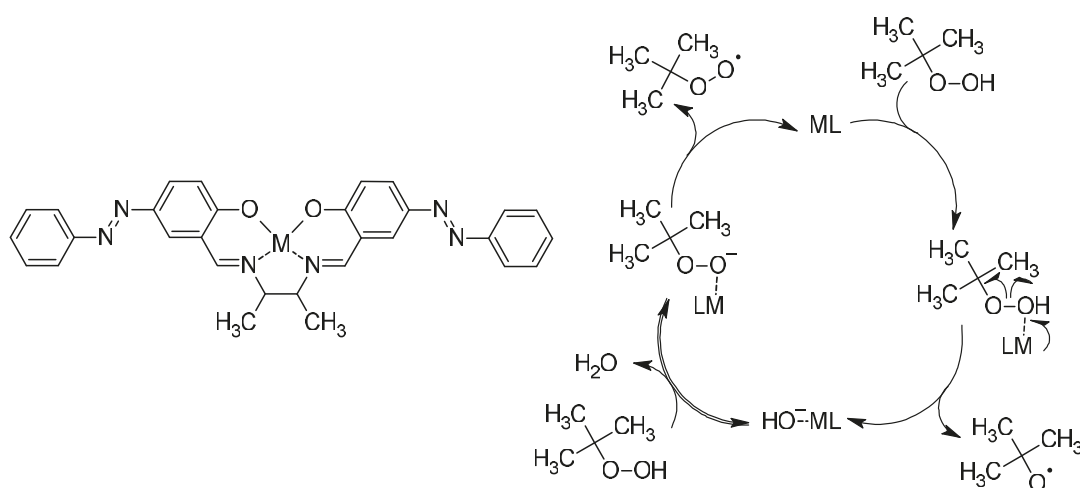
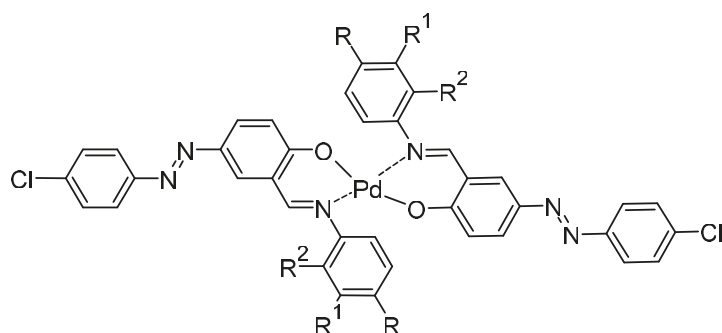


Figure 27. Azo-Schiff base catalyst and proposed mechanism of oxidant activation [114].

Very recently, the Suzuki–Miyaura coupling reactions of phenylboronic acid with aryl bromides were also investigated using Pd(II) complexes (Figure 28) [115].

Various aryl bromides were tested with these complexes, giving very high yields (Table 6), and in the case where 4-bromoacetophenone was used, the highest yield of 99% was obtained with catalysts 12–14, while the lowest efficiency was obtained when catalyst 17 was used (94%). But in the presence of 4-bromobenzaldehyde, the highest yields were

obtained with catalyst 15, while the lowest yield was observed when catalyst 14 was used with 86% efficiency. When an electron-donating group is present, catalysts 13 and 14 seem to work the best. So, there is not really any structure–activity relationship.



R=CH₃, R¹=H, R²=H (12) ; R=OMe, R¹=H, R²=H (13) ; R=OMe, R¹=OMe, R²=H (14)
R=H, R¹=H, R²=Cl (15) ; R=H, R¹=H, R²=Cl (16)

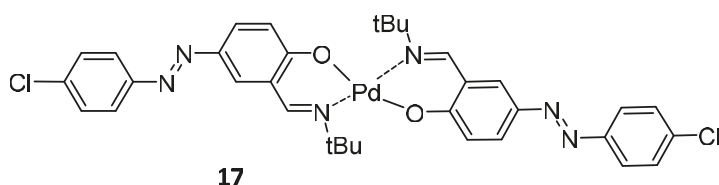


Figure 28. Some of the complexes used for Suzuki coupling [115].

Table 6. Results of Suzuki–Miyaura coupling reactions [115].

R	Catalyst	Yield (%)
CH ₃	12	95
	13	88
	14	97
	15	91
	16	95
	17	90
OMe	12	96
	13	98
	14	98
	15	96
	16	93
	17	97
C(O)CH ₃	12	99
	13	99
	14	99
	15	97
	16	98
	17	94
CHO	12	94
	13	92
	14	86
	15	95
	16	94
	17	94

For the N-alkylation reaction via hydrogen borrowing, nickel catalysis using hydrox-yazobenzene as a ligand was performed and gave access to the N-alkylation of a variety

of anilines with alcohols, with moderate to good yields. What is interesting is that the hydrogen atom transfer (HAT, Figure 29) to the reduced azo backbone for alcohol oxidation occurred, as opposed to a conventional mechanism [116].

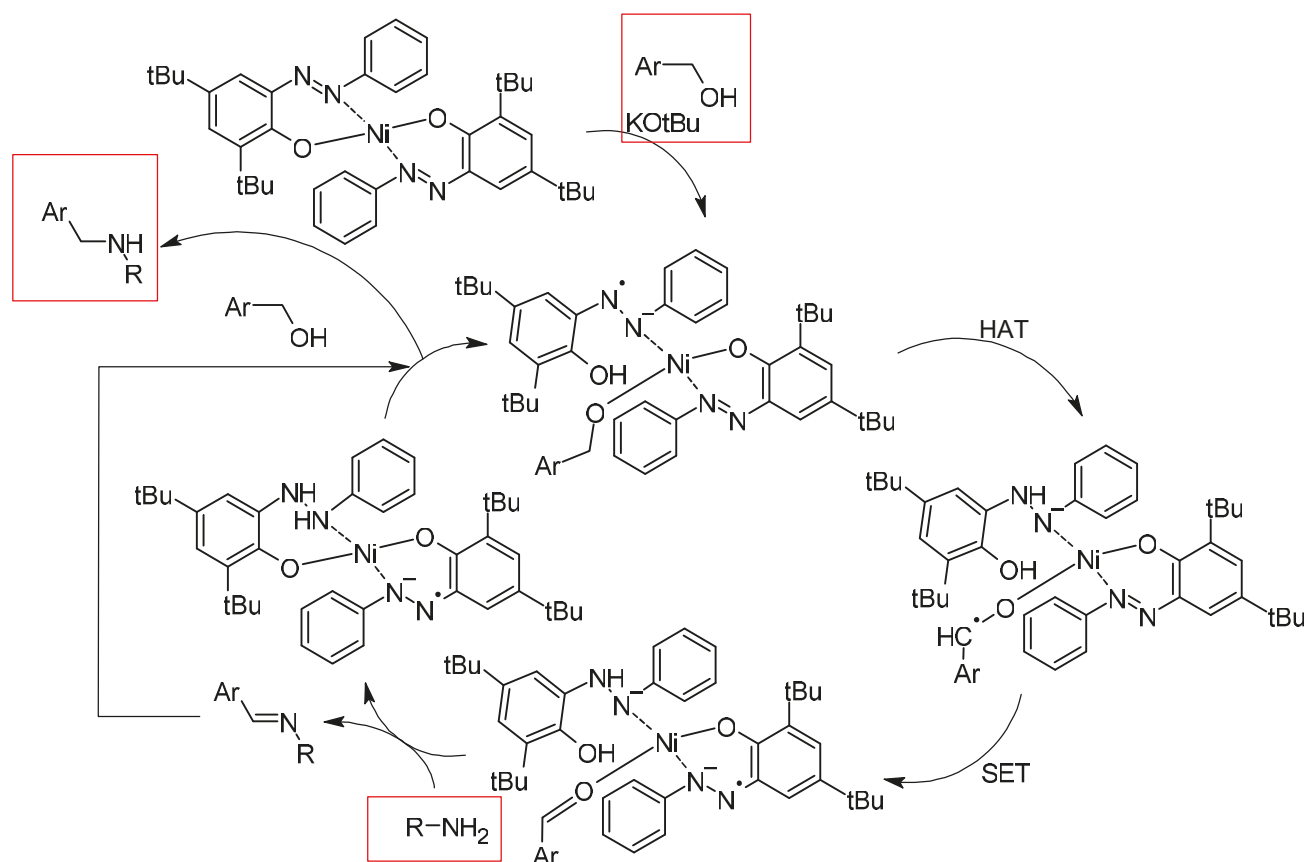


Figure 29. Proposed mechanism [116].

Bis(phenoxo-azo)titanium(IV) complexes were also used for the polymerization of styrene. It is interesting to note in this article that due to its poor σ -donating character, the N atom of the azo group forms long Ti–N bonds, which in turn lead to a more open coordination sphere around the Ti centre. Unfortunately, the bis(phenoxo-azo)titanium complexes do not undergo photoinduced E $\rightarrow$ Z isomerization in their chelating azobenzene ligand frameworks. We attribute this behaviour to the relatively strong Ti–N and Ti–O bonds, which cannot be broken in the course of the photoisomerization reaction [117].

When the diazo moieties are part of a porous organic polymer (POP), it can not only permit the complexation of metals such as zinc but also, with a high surface area, allow for the conversion of carbon dioxide. This is the case of Zn/HAzo-POP, which was able to catalyze the reaction between propylene oxide and CO₂ [118].

5.4. Biomedical and Environmental Applications

The MCF-7 cell line is a widely used human breast cancer model, originally isolated from a metastatic breast cancer patient in 1970. These cells are estrogen receptor-positive (ER⁺) and progesterone receptor-positive (PR⁺), making them an ideal system for studying hormone-dependent breast cancer [119]. MCF-7 cells exhibit an epithelial-like morphology and grow in response to estrogen, allowing researchers to investigate the mechanisms of cancer proliferation, hormone signalling, and drug sensitivity [120]. HeLa cells are the first immortal human cell line, derived in 1951 from the cervical cancer tumour of Henrietta Lacks [121]. These cells are remarkable for their ability to proliferate indefinitely under suitable laboratory conditions,

making them a cornerstone of biomedical research. HeLa cells have been used in countless scientific breakthroughs, including the development of the polio vaccine, cancer research, and studies on viruses and cell biology. However, they are highly genetically unstable, which can affect experimental outcomes. Despite ethical concerns about their origin, HeLa cells remain one of the most important tools in medical and biological research [122].

In this context, it is urgently needed to find anticancer agents and especially cancer-selective ones. Very recently, the cytotoxicity of functionalized hydroxyazobenzene copper(II) complexes (Figure 30) was investigated in the human breast cancer cell line MCF-7 and the human cervical cancer cell line HeLa, demonstrating selective cytotoxicity against cancer cells with a $GI_{50} < 10 \mu\text{g/mL}$ [123].

Another application, in which hydroxyazobenzene is coupled to rhodamine, is the fluorescence imaging of HeLa cells incubated with Cu^{2+} . This experiment confirms that this type of sensor has good cell permeability and can be useful and specific for Cu(II) sensing in living cells [124].

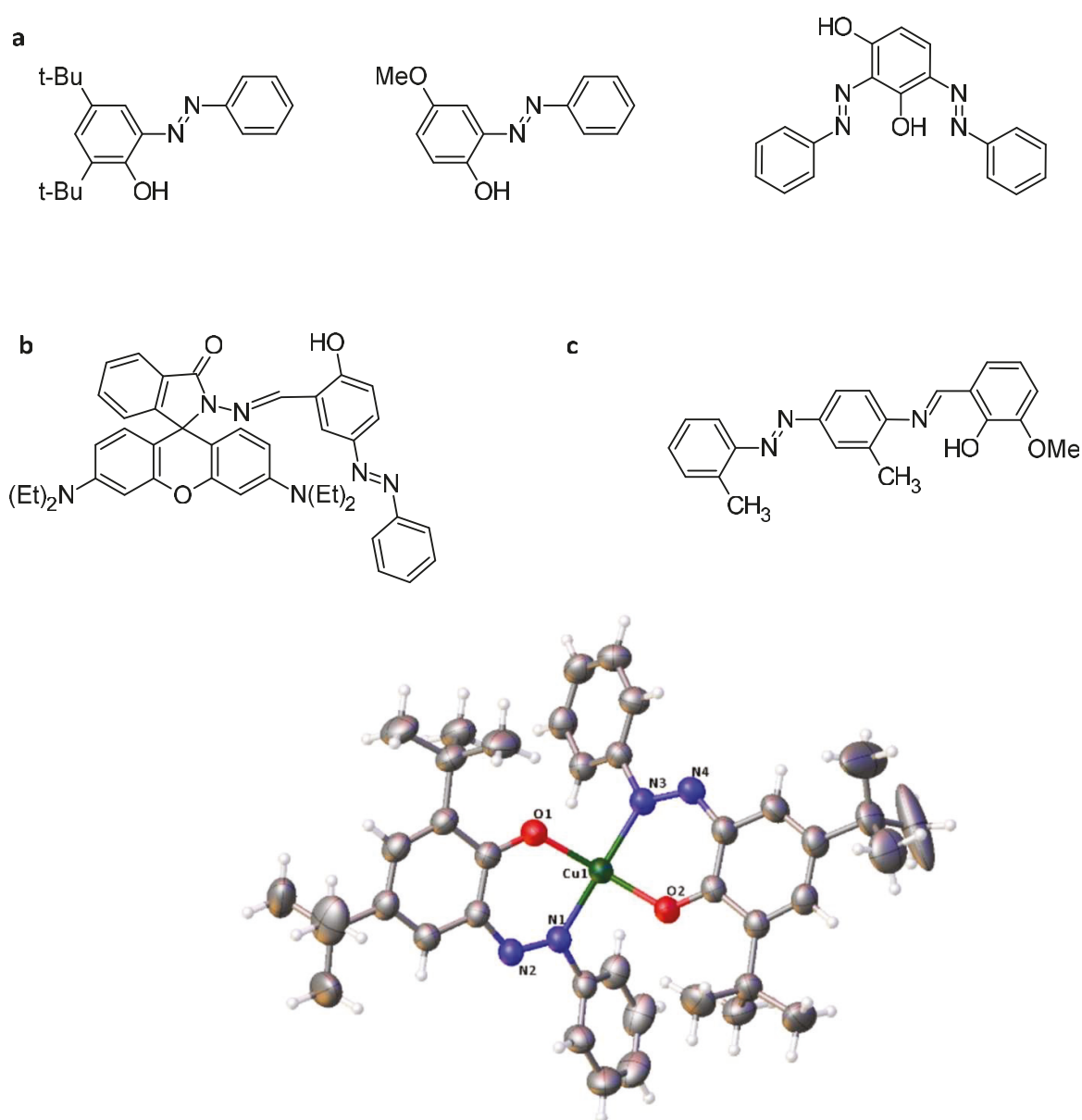


Figure 30. Structures of ligands (a) in ref. [123], (b) in ref. [124], and (c) in ref. [125] and ellipsoid representation of copper complex from (a) [123]. Reprinted with permission from Ref. [123]. Copyright (Elsevier) 2025.

In 2024, *o*-aminoazotoluene, which is capable of photoisomerization, and *o*-vanillin, a potent comutagen, were used to synthesize a new ligand which, upon complexation with copper(II), results in a new copper(II) complex—Cu(L)₂ [125]. This complex had excellent results against MCF-7 cell lines with an IC₅₀ of $4.2 \pm 0.53 \mu\text{M}$ (Figure 30).

In the field of antimicrobial agents, and especially against *Staphylococcus aureus* or *Pseudomonas aeruginosa*, it was shown that the stronger effect of complexes against the ligand alone could be due to the chelation of ligands with metal ions, facilitating the permeability of the drug to the cell's environment. For example, Schiff base azobenzenes complexed with cobalt, nickel, or copper ions (Figure 31) decrease the minimal inhibitory concentration (MIC) from approximately 350 mg/L for the ligand alone to less than 50 mg/L [126]. Zinc complexes can also have excellent MIC values against Gram-positive and Gram-negative bacteria, compared to chloramphenicol (Table 7, Figure 31) [127]. Copper and nickel complexes of the same ligands were also compared for their ZOI (zone of inhibition) against several bacteria, and the results show that depending on the ligand or the metal, the structure–activity relationship is quite impossible to obtain [128].

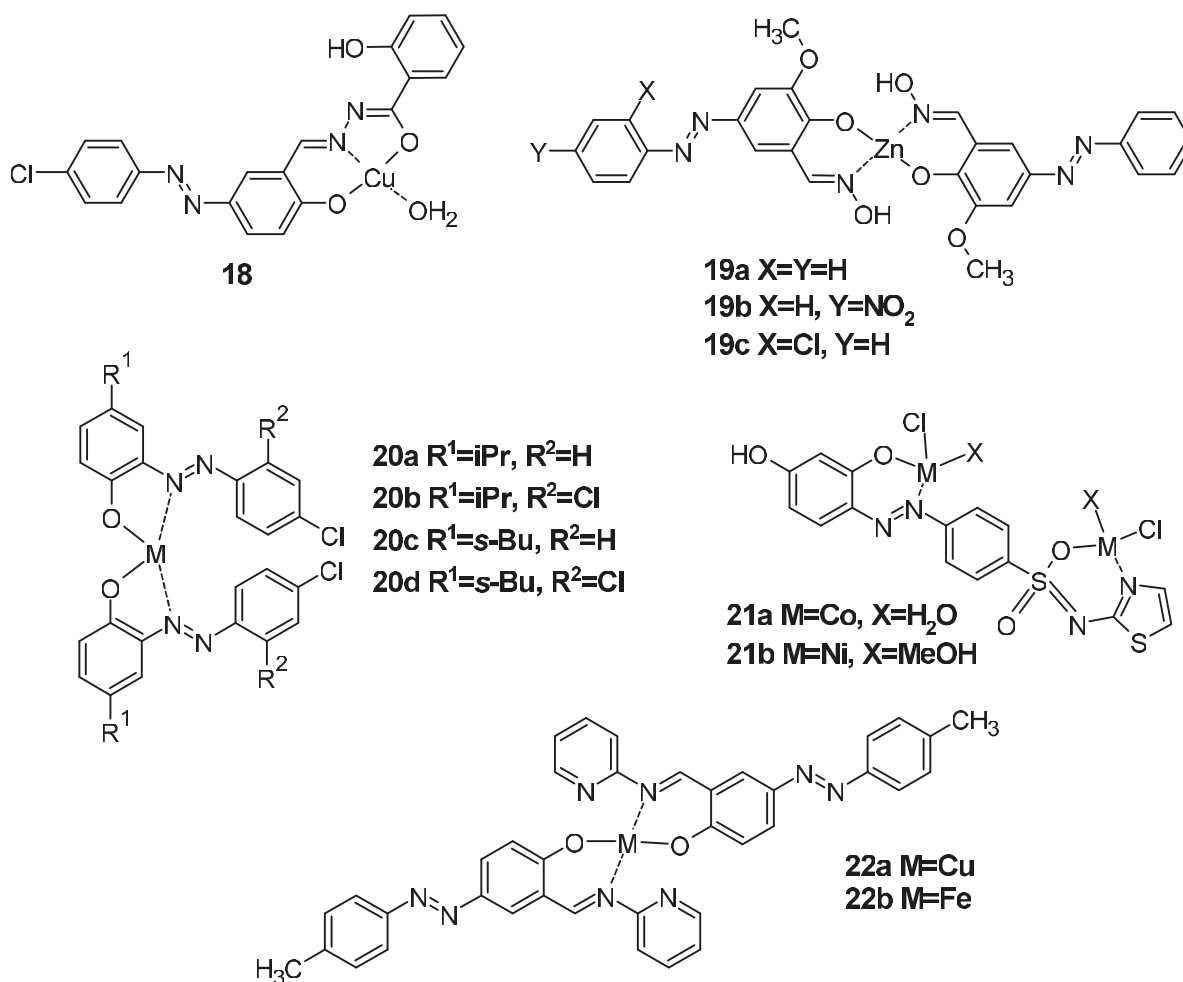


Figure 31. Structure of azo-Schiff base copper complex 18 [126], zinc complex 19 [127], copper and nickel complexes 20 [128], cobalt and nickel complexes 21 [129], and copper and iron 22 [130].

When the examined organisms were *Salmonella typhimurium* and *Staphylococcus aureus*, the results obtained showed that Ni(II) complex 21b (Table 7, Figure 31) displayed good activity towards *S. aureus*, within the 11.97 mm inhibition zone, whereas Co(II) complex 21a showed high efficiency contra *S. Typhimurium* within the 11.13 mm inhibition zone [129]. It is interesting to note that azo-azomethine complexes 22a and 22b exhibited

moderate antimicrobial activity against bacterial strains, but this activity was insignificant against fungi, revealing a good selectivity against pathogens [130].

Table 7. Compiled antimicrobial effects against bacteria from complexes **19–22**.

Micro-Organisms		ZOI	MIC	Ref
<i>Salmonella typhimurium</i>	19a	-	125 µM/mL	[127]
	19b	-	250 µM/mL	[127]
	19c	-	125 µM/mL	[127]
	21a	11.13 mm	-	[129]
<i>Staphylococcus aureus</i>	19a	-	7.81 µM/mL	[127]
	19b	-	15.63 µM/mL	[127]
	19c	-	3.90 µM/mL	[127]
	20a	15 mm (Cu), 12 mm (Ni)	-	[128]
	20b	17 mm (Cu), 13 mm (Ni)	-	[128]
	20c	17 mm (Cu), 12 mm (Ni)	-	[128]
	20d	16 mm (Cu), 14 mm (Ni)	-	[128]
	21b	11.97 mm	-	[129]
	22a	13 mm	128 µg/mL	[130]
	22b	11 mm	256 µg/mL	[130]
<i>Escherichia coli</i>	19a	-	62.5 µM/mL	[127]
	19b	-	250 µM/mL	[127]
	19c	-	62.5 µM/mL	[127]
	20a	15 mm (Cu), 20 mm (Ni)	-	[128]
	20b	19 mm (Cu), 14 mm (Ni)	-	[128]
	20c	20 (Cu)	-	[128]
	20d	12 mm (Cu), 14 mm (Ni)	-	[128]
	22a	15 mm	64 µg/mL	[130]
	22b	17 mm	256 µg/mL	[130]
<i>Bacillus subtilis</i>	19a	-	7.81 µM/mL	[127]
	19b	-	31.25 µM/mL	[127]
	19c	-	7.81 µM/mL	[127]
<i>Enterococcus faecalis</i>	20a	12 mm (Cu), 14 mm (Ni)	-	[128]
	20b	18 mm (Cu), 15 mm (Ni)	-	[128]
	20c	14 mm (Cu)	-	[128]
	20d	17 mm (Cu), 17 mm (Ni)	-	[128]
<i>Enterobacter cloacae</i>	20a	12 mm (Cu), 22 mm (Ni)	-	[128]
	20b	15 mm (Cu), 15 mm (Ni)	-	[128]
	20c	17 mm (Cu), 12 mm (Ni)	-	[128]
	20d	13 mm (Cu), 16 mm (Ni)	-	[128]
<i>Micrococcus luteus</i>	20a	13 mm (Cu), 14 mm (Ni)	-	[128]
	20b	21 mm (Cu), 17 mm (Ni)	-	[128]
	20c	19 mm (Cu), 14 mm (Ni)	-	[128]
	20d	18 mm (Cu), 16 mm (Ni)	-	[128]

6. Conclusions

Phenolic azobenzene derivatives represent a uniquely versatile class of compounds at the intersection of photochemistry, coordination chemistry, and materials science. Their dual functional nature—combining the reversible photoisomerization capability of the azobenzene core with the metal-binding propensity of the phenolic group—enables a wide range of applications, from colorimetric sensing and smart materials to catalysis, solar energy conversion, and biomedical fields. The diversity in synthetic strategies, including azo coupling, Baeyer–Mills reactions, and oxidation–reduction pathways, allows for fine structural tuning and functionalization, further broadening their applicability.

Particularly notable is their effectiveness as ligands in the formation of stable metal complexes, which exhibit enhanced photophysical, catalytic, and bioactive properties. These complexes have shown promise not only in environmental and industrial contexts but also in the development of anticancer and antimicrobial agents. As demonstrated in dye-sensitized solar cells (DSSCs), phenolic azobenzene–metal complexes contribute to improved light-harvesting and electron transfer efficiencies.

Future developments in this field could focus on expanding the functional diversity of these ligands, improving synthetic efficiencies, and deepening the understanding of

structure–activity relationships to design next-generation materials with tailored properties. Overall, phenolic azobenzenes stand out as highly adaptable and functional scaffolds for advancing molecular design across multiple scientific domains.

Author Contributions: J.H. writing, J.I. writing and correcting, E.L. writing and correcting and templating. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by TIMR Laboratory, UTC/ESCOM, and Slovenian Research and Innovation Agency (grant P1-0134).

Acknowledgments: The authors would like to acknowledge the Erasmus grant which allowed for the travelling of researchers and Students in Slovenia and France.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Griffiths, J., II. Photochemistry of Azobenzene and Its Derivatives. *Chem. Soc. Rev.* **1972**, *1*, 481–493. [CrossRef]
- Wang, X. trans-cis Isomerization. In *Azo Polymers: Synthesis, Functions and Applications*; Wang, X., Ed.; Springer: Berlin/Heidelberg, Germany, 2017; pp. 19–56. ISBN 978-3-662-53424-3.
- Aggarwal, A.; Rajyan, A.; Ramachandran, C.N. Substitution-Induced Band Shifts and Relative Population of cis-trans Azobenzene. *Theor. Chem. Acc.* **2024**, *143*, 62. [CrossRef]
- Chen, H.; Chen, W.; Lin, Y.; Xie, Y.; Liu, S.H.; Yin, J. Visible and Near-Infrared Light Activated Azo Dyes. *Chin. Chem. Lett.* **2021**, *32*, 2359–2368. [CrossRef]
- Chandrappa, S.; Vinaya, K.; Ramakrishnappa, T.; Rangappa, K. An Efficient Method for Aryl Nitro Reduction and Cleavage of Azo Compounds Using Iron Powder/Calcium Chloride. *Synlett* **2010**, *2010*, 3019–3022. [CrossRef]
- Gong, D.; Kong, D.; Li, Y.; Gao, C.; Zhao, L. Ni(II)-Catalyzed Transfer Hydrogenation of Azoarenes with $\text{NH}_3 \text{BH}_3$. *Org. Lett.* **2023**, *25*, 4198–4202. [CrossRef]
- Wang, W.; Wang, Y.; Yang, Y.; Xie, S.; Wang, Q.; Chen, W.; Wang, S.; Zhang, F.; Shao, Y. Cobalt-Catalyzed Borylative Reduction of Azobenzenes to Hydrazobenzenes via a Diborylated-Hydrazine Intermediate. *J. Org. Chem.* **2024**, *89*, 9265–9274. [CrossRef]
- Zhou, H.; Fan, R.; Yang, J.; Sun, X.; Liu, X.; Wang, X.-C. N, N-Diisopropylethylamine-Mediated Electrochemical Reduction of Azobenzenes in Dichloromethane. *J. Org. Chem.* **2022**, *87*, 14536–14543. [CrossRef]
- Gong, D.; Kong, D.; Xu, N.; Hua, Y.; Liu, B.; Xu, Z. Bidentate Ru(II)-NC Complex as a Catalyst for Semihydrogenation of Azoarenes to Hydrazoarenes with Ethanol. *Org. Lett.* **2022**, *24*, 7339–7343. [CrossRef]
- Léonard, E.; Mangin, F.; Villette, C.; Billamboz, M.; Len, C. Azobenzenes and Catalysis. *Catal. Sci. Technol.* **2016**, *6*, 379–398. [CrossRef]
- Nikolaeva, M.V.; Puzyk, A.M.; Puzyk, M.V. Azobenzene Pd(II) Complexes with N⁺N- and N⁺O-Type Ligands. *Opt. Spectrosc.* **2017**, *122*, 715–722. [CrossRef]
- Babić, D.; Ćurić, M.; Molčanov, K.; Ilc, G.; Plavec, J. Synthesis and Characterization of Dicyclopalladated Complexes of Azobenzene Derivatives by Experimental and Computational Methods. *Inorg. Chem.* **2008**, *47*, 10446–10454. [CrossRef] [PubMed]
- Deo, C.; Bogliotti, N.; Métivier, R.; Retailleau, P.; Xie, J. Photoswitchable Arene Ruthenium Complexes Containing O-Sulfonamide Azobenzene Ligands. *Organometallics* **2015**, *34*, 5775–5784. [CrossRef]
- Liu, F.; Dong, H.; Zhong, S.; Wu, X.; Wang, T.; Wang, X.; Liu, Y.; Zhu, M.; Lo, I.M.C.; Zhan, S.; et al. Selective Electrocatalytic Transformation of Highly Toxic Phenols in Wastewater to Para-Benzoquinone at Ambient Conditions. *Water Res.* **2024**, *251*, 121106. [CrossRef] [PubMed]
- Serpone, N.; Terzian, R.; Colarusso, P.; Minero, C.; Pelizzetti, E.; Hidaka, H. Sonochemical Oxidation of Phenol and Three of Its Intermediate Products in Aqueous Media: Catechol, Hydroquinone, and Benzoquinone. Kinetic and Mechanistic Aspects. *Res. Chem. Intermed.* **1993**, *18*, 183–202. [CrossRef]
- Trevisan, L.; Bond, A.D.; Hunter, C.A. Quantitative Measurement of Cooperativity in H-Bonded Networks. *J. Am. Chem. Soc.* **2022**, *144*, 19499–19507. [CrossRef]
- Geng, H.; Zhong, Q.-Z.; Li, J.; Lin, Z.; Cui, J.; Caruso, F.; Hao, J. Metal Ion-Directed Functional Metal–Phenolic Materials. *Chem. Rev.* **2022**, *122*, 11432–11473. [CrossRef]
- Alhashmialameer, D.; Collins, J.; Hattenhauer, K.; Kerton, F.M. Iron Amino-Bis(Phenolate) Complexes for the Formation of Organic Carbonates from CO_2 and Oxiranes. *Catal. Sci. Technol.* **2016**, *6*, 5364–5373. [CrossRef]
- Roy Chowdhury, R.K.; Crane, A.; Fowler, C.; Kwong, P.M.; Kozak, C. Iron(III) Amine-Bis(Phenolate) Complexes as Catalysts for the Coupling of Alkyl Halides with Aryl Grignard Reagents. *Chem. Commun.* **2008**, *1*, 94–96. [CrossRef]

20. Steinwand, S.; Halbritter, T.; Rastädter, D.; Ortiz-Sánchez, J.M.; Burghardt, I.; Heckel, A.; Wachtveitl, J. Ultrafast Spectroscopy of Hydroxy-Substituted Azobenzenes in Water. *Chem. Eur. J.* **2015**, *21*, 15720–15731. [CrossRef]
21. Guan, P.-J.; Cui, G.; Fang, Q. Computational Photochemistry of the Azobenzene Scaffold of Sudan I and Orange II Dyes: Excited-State Proton Transfer and Deactivation via Conical Intersections. *ChemPhysChem* **2015**, *16*, 805–811. [CrossRef]
22. Das, S.K.; Krishnamoorthy, G.; Dogra, S.K. Excited State Intramolecular Proton Transfer in 2-(2'-Hydroxyphenyl)-1H-Naphth-[2,3-d]-Imidazole: Effects of Solvents and pH. *Can. J. Chem.* **2000**, *78*, 191–205. [CrossRef]
23. García-Amorós, J.; Velasco, D. Recent Advances towards Azobenzene-Based Light-Driven Real-Time Information-Transmitting Materials. *Beilstein J. Org. Chem.* **2012**, *8*, 1003–1017. [CrossRef] [PubMed]
24. Colinese, D.; Ibbitson, D.; Stone, C. Dipole Moments and Infrared Spectra of a Series of 4'-Substituted 4-Hydroxyazobenzenes. *J. Chem. Soc. B Phys. Org.* **1971**, 570–574. [CrossRef]
25. Rospenk, M.; Majewska, P.; Czarnik-Matusiewicz, B.; Sobczyk, L. Polarized IR Spectra of Resonance Assisted Hydrogen Bond (RAHB) in 2-Hydroxyazobenzenes. *Chem. Phys.* **2006**, *326*, 458–464. [CrossRef]
26. Sahu, M.; Saha, M.B.; Bera, S.C. Azo-Hydrazo Tautomerization and Cis-Trans Isomerization of 2-Carboxy-2'-Hydroxy-4'-Methyl Azobenzene. *J. Photochem. Photobiol. A Chem.* **1995**, *89*, 19–24. [CrossRef]
27. Gentili, P.L.; Capaccioni, A.; Germani, R.; Fantacci, S. The Versatile Photo-Thermal Behaviour of a 2-Hydroxyazobenzene. *Molecules* **2023**, *28*, 1183. [CrossRef]
28. Emond, M.; Sun, J.; Grégoire, J.; Maurin, S.; Tribet, C.; Jullien, L. Photoinduced pH Drops in Water. *Phys. Chem. Chem. Phys.* **2011**, *13*, 6493–6499. [CrossRef]
29. Shaghghi, Z. Spectroscopic Properties of Some New Azo–Azomethine Ligands in the Presence of Cu²⁺, Pb²⁺, Hg²⁺, Co²⁺, Ni²⁺, Cd²⁺ and Zn²⁺ and Their Antioxidant Activity. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2014**, *131*, 67–71. [CrossRef]
30. Shaabani, B.; Shaghghi, Z.; Khandar, A.A. Optical Spectroscopy Studies of the Complexation of Bis(Azophenol)Calix[4]Arene Possessing Chromogenic Donors with Ni²⁺, Co²⁺, Cu²⁺, Pb²⁺ and Hg²⁺. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2012**, *98*, 81–85. [CrossRef]
31. Bal, S. A Novel Azo-Schiff Base Ligand and Its Cobalt, Copper, Nickel Complexes: Synthesis, Characterization, Antimicrobial, Catalytic and Electrochemical Features. *Univ. J. Sci. Technol. A Appl. Sci. Eng.* **2016**, *17*, 315–326. [CrossRef]
32. Özdemir, Ö. Synthesis of Novel Azo Linkage-Based Schiff Bases Including Anthranilic Acid and Hexanoic Acid Moieties: Investigation of Azo-Hydrazone and Phenol-Keto Tautomerism, Solvatochromism, and Ionochromism. *Turk. J. Chem.* **2019**, *43*, 266–285. [CrossRef]
33. Patel, A.R.; Patel, G.; Srivastava, A.; Banerjee, S. A Review on Traditional and Modern Methods for the Synthesis of Aromatic AzoCompounds. *Curr. Org. Chem.* **2023**, *27*, 1611–1628. [CrossRef]
34. Kurup, S.S.; Groysman, S. Catalytic Synthesis of Azoarenes via Metal-Mediated Nitrene Coupling. *Dalton Trans.* **2022**, *51*, 4577–4589. [CrossRef] [PubMed]
35. Jerca, F.A.; Jerca, V.V.; Hoogenboom, R. Advances and Opportunities in the Exciting World of Azobenzenes. *Nat. Rev. Chem.* **2022**, *6*, 51–69. [CrossRef]
36. Finck, L.; Oestreich, M. Synthesis of Non-Symmetric Azoarenes by Palladium-Catalyzed Cross-Coupling of Silicon-Masked Diazenyl Anions and (Hetero)Aryl Halides. *Angew. Chem. Int. Ed. Engl.* **2022**, *61*, e202210907. [CrossRef]
37. Benkhaya, S.; M'Rabet, S.; El Harfi, A. Classifications, Properties, Recent Synthesis and Applications of Azo Dyes. *Heliyon* **2020**, *6*, e03271. [CrossRef]
38. Eltaboni, F.; Bader, N.; El-Kailany, R.; Elsharif, N.; Ahmida, A. Issue 4 Chemistry and Applications of Azo Dyes: A Comprehensive Review. *J. Chem. Rev.* **2022**, *2*, 313–330. [CrossRef]
39. Merino, E. Synthesis of Azobenzenes: The Coloured Pieces of Molecular Materials. *Chem. Soc. Rev.* **2011**, *40*, 3835–3853. [CrossRef]
40. Rasheed, O.; Rasheed, O.; Raftery, J.; Quayle, P. A New Benzannulation Reaction of Azoaromatics. *Synlett* **2015**, *26*, 2806–2810. [CrossRef]
41. McCormack, A.T.; Stephens, J.C. The Continuous Flow Synthesis of Azos. *J. Flow Chem.* **2024**, *14*, 377–396. [CrossRef]
42. Xie, C.; Yuan, Y.; Wang, B.; Du, L. Research on the Decomposition Kinetics and Thermal Hazards of Aniline Diazonium Salt. *Thermochim. Acta* **2022**, *709*, 179156. [CrossRef]
43. Ikumi, A.; Nakamura, T. Method for Producing Nitrobenzene Compound. EP3015451B1, 17 January 2018.
44. Schotten, C.; Leprevost, S.K.; Yong, L.M.; Hughes, C.E.; Harris, K.D.M.; Browne, D.L. Comparison of the Thermal Stabilities of Diazonium Salts and Their Corresponding Triazenes. *Org. Process Res. Dev.* **2020**, *24*, 2336–2341. [CrossRef]
45. Liu, Z.; Fang, Y.; Liu, Y.; Fu, W.; Gan, X.; Gao, W.; Tang, B. One-Pot Difunctionalization of Aryldiazonium Salts for Synthesis of Para-Azophenols. *Front. Chem.* **2022**, *10*. [CrossRef] [PubMed]
46. Griwatz, J.H.; Campi, C.E.; Kunz, A.; Wegner, H.A. In-Situ Oxidation and Coupling of Anilines towards Unsymmetric Azobenzenes Using Flow Chemistry. *ChemSusChem* **2024**, *17*, e202301714. [CrossRef]
47. Griwatz, J.H.; Kunz, A.; Wegner, H.A. Continuous Flow Synthesis of Azobenzenes via Baeyer–Mills Reaction. *Beilstein J. Org. Chem.* **2022**, *18*, 781–787. [CrossRef]

48. Dommaschk, M.; Peters, M.; Gutzeit, F.; Schütt, C.; Näther, C.; Sönnichsen, F.D.; Tiwari, S.; Riedel, C.; Boretius, S.; Herges, R. Photoswitchable Magnetic Resonance Imaging Contrast by Improved Light-Driven Coordination-Induced Spin State Switch. *J. Am. Chem. Soc.* **2015**, *137*, 7552–7555. [CrossRef]
49. Priewisch, B.; Ruck-Braun, K. Efficient Preparation of Nitrosoarenes for the Synthesis of Azobenzenes. *J. Org. Chem.* **2005**, *70*, 2350–2352. [CrossRef]
50. Tombari, R.J.; Tuck, J.R.; Yardeny, N.; Gingrich, P.W.; Tantillo, D.J.; Olson, D.E. Calculated Oxidation Potentials Predict Reactivity in Baeyer–Mills Reactions. *Org. Biomol. Chem.* **2021**, *19*, 7575–7580. [CrossRef]
51. Trautwein, G.; El Bakkali, B.; Alcañiz-Monge, J.; Artetxe, B.; Reinoso, S.; Gutiérrez-Zorrilla, J.M. Dimeric Assemblies of Lanthanide-Stabilised Dilacunary Keggin Tungstogermanates: A New Class of Catalysts for the Selective Oxidation of Aniline. *J. Catal.* **2015**, *331*, 110–117. [CrossRef]
52. Monir, K.; Ghosh, M.; Mishra, S.; Majee, A.; Hajra, A. Phenyliodine(III) Diacetate (PIDA) Mediated Synthesis of Aromatic Azo Compounds through Oxidative Dehydrogenative Coupling of Anilines: Scope and Mechanism. *Eur. J. Org. Chem.* **2014**, *2014*, 1096–1102. [CrossRef]
53. Zhao, M.-Y.; Tang, Y.-F.; Han, G.-Z. Recent Advances in the Synthesis of Aromatic Azo Compounds. *Molecules* **2023**, *28*, 6741. [CrossRef] [PubMed]
54. Zhang, C.; Jiao, N. Copper-Catalyzed Aerobic Oxidative Dehydrogenative Coupling of Anilines Leading to Aromatic Azo Compounds Using Dioxygen as an Oxidant. *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 6174–6177. [CrossRef] [PubMed]
55. Wawzonek, S.; McIntyre, T.W. Electrolytic Preparation of Azobenzenes. *J. Electrochem. Soc.* **1972**, *119*, 1350. [CrossRef]
56. Olsen, H.; Snyder, J.P. Cis-Azoxyalkanes. 8. Concerted Thermal Cycloreversion of Unsaturated Azo N-Oxides. *J. Am. Chem. Soc.* **1977**, *99*, 1524–1536. [CrossRef]
57. Ortiz, B.; Villanueva, P.; Walls, F. Silver(II) Oxide as a Reagent. Reactions with Aromatic Amines and Miscellaneous Related Compounds. *J. Org. Chem.* **1972**, *37*, 2748–2750. [CrossRef]
58. Firouzabadi, H.; Vessal, B.; Naderi, M. Bispyridinesilver Permanganate[Ag(C₅H₅N)₂]MnO₄: An Efficient Oxidizing Reagent for Organic Substrates. *Tetrahedron Lett.* **1982**, *23*, 1847–1850. [CrossRef]
59. Laha, S.; Luthy, R.G. Oxidation of Aniline and Other Primary Aromatic Amines by Manganese Dioxide. *Environ. Sci. Technol.* **1990**, *24*, 363–373. [CrossRef]
60. Crank, G.; Makin, M. Oxidations of Aromatic Amines by Superoxide Ion. *Aust. J. Chem.* **1984**, *37*, 845. [CrossRef]
61. Mehta, S.M.; Vakilwala, M.V. Sodium Perborate as a Reagent in Organic Chemistry. I. Preparation of Azo-Compounds. *J. Am. Chem. Soc.* **1952**, *74*, 563–564. [CrossRef]
62. Baer, E.; Tosoni, A.L. Formation of Symmetric Azo-Compounds from Primary Aromatic Amines by Lead Tetraacetate. *J. Am. Chem. Soc.* **1956**, *78*, 2857–2858. [CrossRef]
63. Firouzabadi, H.; Mostafavipoor, Z. Barium Manganate. A Versatile Oxidant in Organic Synthesis. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 914–917. [CrossRef]
64. Firouzabadi, H.; Iranpoor, N. Ceric Trihydroxy Hydroperoxide Ce(OH)₃O₂H, A Regenerable, Mild, and A Versatile Reagent for the Oxidation of Organic Compounds. *Synth. Commun.* **1984**, *14*, 875–882. [CrossRef]
65. Qin, J.; Long, Y.; Sun, F.; Zhou, P.P.; Wang, W.D.; Luo, N.; Ma, J. Zr(OH)₄-Catalyzed Controllable Selective Oxidation of Anilines to Azoxybenzenes, Azobenzenes and Nitrosobenzenes. *Angew. Chem. Int. Ed. Engl.* **2022**, *61*, e202112907. [CrossRef] [PubMed]
66. Li, S.; Zhao, W.; Wang, L.; Jia, Y.; Cui, Q.; Wen, B.; Chen, X. Controllable Selective Oxidation of Anilines to Azoxybenzenes and Nitrobenzenes by Regulating the Base. *ACS Omega* **2024**, *9*, 39715–39723. [CrossRef]
67. Grirrane, A.; Corma, A.; García, H. Gold-Catalyzed Synthesis of Aromatic Azo Compounds from Anilines and Nitroaromatics. *Science* **2008**, *322*, 1661–1664. [CrossRef]
68. Deng, X.; Liu, X.; Xia, S.; Zhao, H.; Liu, Y.; Ding, Q.; Zhang, H. Selective Oxidation of Anilines to Azobenzenes by an Ag Nanoparticles Photocatalyst. *Colloids Surf. A Physicochem. Eng. Asp.* **2023**, *677*, 132352. [CrossRef]
69. Wang, M.; Ma, J.; Yu, M.; Zhang, Z.; Wang, F. Oxidative Coupling of Anilines to Azobenzenes Using Heterogeneous Manganese Oxide Catalysts. *Catal. Sci. Technol.* **2016**, *6*, 1940–1945. [CrossRef]
70. Furst, A.; Berlo, R.C.; Hooton, S. Hydrazine as a Reducing Agent for Organic Compounds (Catalytic Hydrazine Reductions). *Chem. Rev.* **1965**, *65*, 51–68. [CrossRef]
71. Nanjundaswamy, H.; Pasha, M. Facile, Product-Selective Reduction of Azoxyarenes into Azoarenes or Hydrazoarenes by Aluminum/Hydrazine Hydrate. *Synth. Commun.* **2005**, *35*, 2163–2168. [CrossRef]
72. Singh, S.K.; Srinivasa Reddy, M.; Mangle, M.; Ravi Ganesh, K. Cu(I)-Mediated Deoxygenation of N-Oxides to Amines. *Tetrahedron* **2007**, *63*, 126–130. [CrossRef]
73. Tanikaga, R. Photoreduction of Azoxybenzene to Azobenzene. *Bull. Chem. Soc. Jpn.* **1968**, *41*, 1664–1668. [CrossRef]
74. Saini, A.; Kumar, S.; Sandhu, J. An Efficient and General Method for the Deoxygenation of Organic N-Oxides Using Zn(OTf)₂ and Cu(OTf)₂. *Synlett* **2006**, *2006*, 395–398. [CrossRef]

75. Hamon, F.; Djedaini-Pilard, F.; Barbot, F.; Len, C. Azobenzenes—Synthesis and Carbohydrate Applications. *Tetrahedron* **2009**, *65*, 10105–10123. [CrossRef]
76. Mikheev, Y.A. Adjusting Mechanisms of the Wallach Rearrangement and Side Reactions. *Russ. J. Phys. Chem.* **2021**, *95*, 1803–1810. [CrossRef]
77. Lalitha, A.; Pitchumani, K.; Srinivasan, C. Thermal and Photochemical Wallach Rearrangement of Azoxybenzene in Zeolite Cages. *J. Mol. Catal. A Chem.* **2000**, *160*, 429–435. [CrossRef]
78. Gore, P.; Hughes, G. Studies of the Wallach Transformation. I. The Products of the Reaction. *Aust. J. Chem.* **1950**, *3*, 136. [CrossRef]
79. Shimao, I.; Oae, S. The Wallach Rearrangement of Some 4,4'-Disubstituted Azoxybenzenes. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 643–644. [CrossRef]
80. Shah, H.U.R.; Ahmad, K.; Naseem, H.A.; Parveen, S.; Ashfaq, M.; Aziz, T.; Shaheen, S.; Babras, A.; Shahzad, A. Synthetic Routes of Azo Derivatives: A Brief Overview. *J. Mol. Struct.* **2021**, *1244*, 131181. [CrossRef]
81. Yadav, G.D.; Mewada, R.K. Novelties of Azobenzene Synthesis via Selective Hydrogenation of Nitrobenzene over Nano-Fibrous Ag-OMS-2—Mechanism and Kinetics. *Chem. Eng. J.* **2013**, *221*, 500–511. [CrossRef]
82. Yang, X.; Liang, M.; Jia, J.; Wu, H. Insights into the Mechanism for the Catalytic Transfer Hydrogenation of Nitrobenzene to Azobenzene on Au (1 0 0) Surface under Alkaline Condition. *Appl. Surf. Sci.* **2021**, *568*, 150869. [CrossRef]
83. Gund, S.H.; Shelkar, R.S.; Nagarkar, J.M. An Efficient Catalyst-Free and Chemoselective Synthesis of Azobenzenes from Nitrobenzenes. *RSC Adv.* **2014**, *4*, 42947–42951. [CrossRef]
84. Opolonick, N. Reduction of Nitrobenzene with Dextrose in Alkaline Solutions. *Ind. Eng. Chem.* **1935**, *27*, 1045–1046. [CrossRef]
85. Liu, Z.; Huang, Y.; Xiao, Q.; Zhu, H. Selective Reduction of Nitroaromatics to Azoxy Compounds on Supported Ag–Cu Alloy Nanoparticles through Visible Light Irradiation. *Green Chem.* **2016**, *18*, 817–825. [CrossRef]
86. Guo, W.; Zheng, Y.; Xiang, W.; Zhang, Y. Advances in the Catalysis of Reduction of Nitroaromatics and Its Mechanism: A Tutorial Review. *RSC Sustain.* **2025**, *3*, 243–254. [CrossRef]
87. Pyo, S.-H.; Han, B.-H. Reduction of Nitroarenes by Activated Metals. *Bull. Korean Chem. Soc.* **1995**, *16*, 181–183. [CrossRef]
88. Dai, Y.; Li, C.; Shen, Y.; Lim, T.; Xu, J.; Li, Y.; Niemantsverdriet, H.; Besenbacher, F.; Lock, N.; Su, R. Light-Tuned Selective Photosynthesis of Azo- and Azoxy-Aromatics Using Graphitic C₃N₄. *Nat. Commun.* **2018**, *9*, 60. [CrossRef]
89. Liss, T.A.; Baer, D.R. Metal Complexes of Azo Dyes. I. Quadridentate Complexes from Bidentate Azo Compounds and Alkanediamines or Ethanolamine. *Inorg. Chem.* **1969**, *8*, 1328–1336. [CrossRef]
90. Kachi-Terajima, C.; Hagiwara, S. Synthesis, Crystal Structure and Photophysical Properties of Chlorido[(E)-3-Hydroxy-2-Methyl-6-(Quinolin-8-Yldiazanyl)Phenolato]Copper(II) Monohydrate. *Acta Crystallogr. E Cryst. Commun.* **2022**, *78*, 473–476. [CrossRef]
91. Qian, H.-F.; Feng, Y.-N.; Wang, Y.-Y.; Zhao, X.-L.; Huang, W. Preparation of Ortho Hydroxyl Azobenzene Dyes by Cu(II)/H₂O₂ Catalytic Oxidation and Complexation and the Following Demetallization. *Dyes Pigment.* **2017**, *146*, 37–46. [CrossRef]
92. Kajiwar, K.; Pradhan, S.; Haraguchi, T.; Sinha, C.; Parida, R.; Giri, S.; Roymahapatra, G.; Akitsu, T. Photo-Tunable Azobenzene-Anthraquinone Schiff Base Copper Complexes as Mediators for Laccase in Biofuel Cell Cathode. *Symmetry* **2020**, *12*, 797. [CrossRef]
93. Lolage, S.R.; Pawal, S.B.; Chavan, S.S. Azobenzene Based Zn(II)/Ru(II) Coordination-Organometallic Hybrid Complexes: Influence of π -Conjugation, Donor/Acceptor Substituent's and Coligands on Electrochemical, Luminescence and NLO Properties. *Opt. Mater.* **2017**, *67*, 162–171. [CrossRef]
94. Leonard, E.; Takeda, C.; Akitsu, T. Azobenzene-Containing Schiff-Bases—Syntheses and Dyes Applications. *Colorants* **2024**, *3*, 53–72. [CrossRef]
95. Mabhai, S.; Dolai, M.; Dey, S.K.; Dhara, A.; Choudhury, S.M.; Das, B.; Dey, S.; Jana, A. Rhodamine-Azobenzene Based Single Molecular Probe for Multiple Ions Sensing: Cu²⁺, Al³⁺, Cr³⁺ and Its Imaging in Human Lymphocyte Cells. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2019**, *219*, 319–332. [CrossRef] [PubMed]
96. Arabahmadi, R.; Orojloo, M.; Amani, S. Azo Schiff Bases as Colorimetric and Fluorescent Sensors for Recognition of F[−], Cd²⁺ and Hg²⁺ Ions. *Anal. Methods* **2014**, *6*, 7384–7393. [CrossRef]
97. Banerjee, S.; Brandão, P.; Saha, A. A Robust Fluorescent Chemosensor for Aluminium Ion Detection Based on a Schiff Base Ligand with an Azo Arm and Application in a Molecular Logic Gate. *RSC Adv.* **2016**, *6*, 101924–101936. [CrossRef]
98. Azadbakht, R.; Chidan, N.; Menati, S.; Koolivand, M. A New Azo-Schiff Base Dual-Mode Chemosensor: Colorimetric Detection of Cobalt Ions and Fluorometric Detection of Aluminum Ions in Aqueous Ethanol Solution. *J. Fluoresc.* **2023**, *33*, 527–538. [CrossRef]
99. Song, G.; Sun, R.; Du, J.; Chen, M.; Tian, Y. A Highly Selective, Colorimetric, and Environment-Sensitive Optical Potassium Ion Sensor. *Chem. Commun.* **2017**, *53*, 5602–5605. [CrossRef]
100. Paderni, D.; Macedi, E.; Lvova, L.; Ambrosi, G.; Formica, M.; Giorgi, L.; Paolesse, R.; Fusi, V. Selective Detection of Mg²⁺ for Sensing Applications in Drinking Water. *Chem. Eur. J.* **2022**, *28*, e202201062. [CrossRef]
101. Aglan, R.F.; Hamed, M.M.; Saleh, H.M. Selective and Sensitive Determination of Cd(II) Ions in Various Samples Using a Novel Modified Carbon Paste Electrode. *J. Anal. Sci. Technol.* **2019**, *10*, 7. [CrossRef]

102. Misra, A.; Shahid, M. Chromo and Fluorogenic Properties of Some Azo-Phenol Derivatives and Recognition of Hg²⁺ Ion in Aqueous Medium by Enhanced Fluorescence. *J. Phys. Chem. C* **2010**, *114*, 16726–16739. [CrossRef]
103. Shatir, T.M.; Aly, K.A.; Ebrahimum, M.M.; Saddeek, Y.B.; Ranjith Kumar, E. Linear and Non-Linear Optical and Dielectric Properties of Transition Metals Complexes Films Derived from Azo-Schiff Base for Photovoltaic Applications. *J. Mol. Liq.* **2024**, *401*, 124636. [CrossRef]
104. Poormoradkhan Melal, S.; Khalili, B.; Mahmoodi, N.O.; Pasandideh Nadamani, M. A Novel Azo-Triazolopyridine-Based Dual Naked-Eye Chemosensor for the Selective Detection of CN[−] and Cu²⁺ Ions. *J. Photochem. Photobiol. A Chem.* **2025**, *460*, 116140. [CrossRef]
105. Asiri, S.; Kassem, M.A.; Eltaher, M.A.; Saad, K.M. Novel Coordination Compounds Based on Copper Complexes with New Synthesized Schiff Bases and Azo-Dyes as Sensitizers for Dye-Sensitized Solar Cells: Spectral and Electrochemical Studies. *Int. J. Electrochem. Sci.* **2020**, *15*, 6508–6521. [CrossRef]
106. Tanaka, S.; Sato, H.; Ishida, Y.; Deng, Y.; Haraguchi, T.; Akitsu, T.; Sugiyama, M.; Hara, M.; Moon, D. Photo-Control of Adsorption of Dye Metal Complexes Incorporating Chiral Schiff Base Ligands Containing Azo-Groups on TiO₂. *J. Korean Chem. Soc.* **2018**, *62*, 328–332. [CrossRef]
107. Liu, Z.; Lu, G.-Y.; Ma, J. Tuning the Absorption Spectra and Nonlinear Optical Properties of D-π-A Azobenzene Derivatives by Changing the Dipole Moment and Conjugation Length: A Theoretical Study. *J. Phys. Org. Chem.* **2011**, *24*, 568–577. [CrossRef]
108. Zhang, L.; Cole, J.M.; Dai, C. Variation in Optoelectronic Properties of Azo Dye-Sensitized TiO₂ Semiconductor Interfaces with Different Adsorption Anchors: Carboxylate, Sulfonate, Hydroxyl and Pyridyl Groups. *ACS Appl. Mater. Interfaces* **2014**, *6*, 7535–7546. [CrossRef]
109. Al-Barody, S.M. Characterization and Thermal Study of Schiff-Base Monomers and Its Transition Metal Polychelates and Their Photovoltaic Performance on Dye Sensitized Solar Cells. *J. Struct. Chem.* **2018**, *59*, 53–63. [CrossRef]
110. Gencer Imer, A.; Syan, R.H.B.; Gülcan, M.; Ocak, Y.S.; Tombak, A. The Novel Pyridine Based Symmetrical Schiff Base Ligand and Its Transition Metal Complexes: Synthesis, Spectral Definitions and Application in Dye Sensitized Solar Cells (DSSCs). *J. Mater. Sci. Mater. Electron.* **2018**, *29*, 898–905. [CrossRef]
111. Qi, B.; Wang, J. Open-Circuit Voltage in Organic Solar Cells. *J. Mater. Chem.* **2012**, *22*, 24315–24325. [CrossRef]
112. Fetouh, H.A.; Dissouky, A.E.; Salem, H.A.; Fathy, M.; Anis, B.; Hady Kashyout, A.E. Synthesis, Characterization and Evaluation of New Alternative Ruthenium Complex for Dye Sensitized Solar Cells. *Sci. Rep.* **2024**, *14*, 16718. [CrossRef]
113. Li, J.; Yang, X.; Yu, Z.; Gurzadyan, G.G.; Cheng, M.; Zhang, F.; Cong, J.; Wang, W.; Wang, H.; Li, X.; et al. Efficient Dye-Sensitized Solar Cells with [Copper(6,6'-Dimethyl-2,2'-Bipyridine)₂]²⁺/1+ Redox Shuttle. *RSC Adv.* **2017**, *7*, 4611–4615. [CrossRef]
114. Lashanizadegan, M.; Asna Ashari, H.; Sarkheil, M.; Anafcheh, M.; Jahangiry, S. New Cu(II), Co(II) and Ni(II) Azo-Schiff Base Complexes: Synthesis, Characterization, Catalytic Oxidation of Alkenes and DFT Study. *Polyhedron* **2021**, *200*, 115148. [CrossRef]
115. Inan Duyar, A.; Sünbül, A.B.; Serin, S.; Ulu, Ö.D.; Ozdemir, I.; Ikiz, M.; Köse, M.; Ispir, E. Synthesis, Characterization, DFT Quantum Chemical Calculations and Catalytic Properties of Azobenzene-Bearing Schiff Base Palladium(II) Complexes for the Suzuki-Miyaura Cross-Coupling Reaction in Aqueous Solvent. *J. Mol. Struct.* **2025**, *1328*, 141296. [CrossRef]
116. Bains, A.K.; Kundu, A.; Yadav, S.; Adhikari, D. Borrowing Hydrogen-Mediated N-Alkylation Reactions by a Well-Defined Homogeneous Nickel Catalyst. *ACS Catal.* **2019**, *9*, 9051–9059. [CrossRef]
117. Tanaka, R.; Viehmann, P.; Hecht, S. Bis(Phenoxy-Azo)Titanium(IV) Complexes: Synthesis, Structure, and Catalytic Activity in Styrene Polymerization. *Organometallics* **2012**, *31*, 4216–4220. [CrossRef]
118. Ji, G.; Yang, Z.; Zhang, H.; Zhao, Y.; Yu, B.; Ma, Z.; Liu, Z. Hierarchically Mesoporous O-Hydroxyazobenzene Polymers: Synthesis and Their Applications in CO₂ Capture and Conversion. *Angew. Chem. Int. Ed.* **2016**, *55*, 9685–9689. [CrossRef]
119. Sweeney, E.E.; McDaniel, R.E.; Maximov, P.Y.; Fan, P.; Jordan, V.C. Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. *Horm. Mol. Biol. Clin. Investig.* **2012**, *9*, 143–163. [CrossRef]
120. Bullinger, D.; Neubauer, H.; Fehm, T.; Laufer, S.; Gleiter, C.H.; Kammerer, B. Metabolic Signature of Breast Cancer Cell Line MCF-7: Profiling of Modified Nucleosides via LC-IT MS Coupling. *BMC Biochem.* **2007**, *8*, 25. [CrossRef]
121. Baptiste, D.; Caviness-Ashe, N.; Josiah, N.; Commodore-Mensah, Y.; Arscott, J.; Wilson, P.R.; Starks, S. Henrietta Lacks and America's Dark History of Research Involving African Americans. *Nurs. Open* **2022**, *9*, 2236–2238. [CrossRef]
122. Varsha, D.S.; Nirmala, G.S.; Santhekadur, P.K. Our Shero HeLa and Her Immortal Life. *Reson* **2022**, *27*, 1731–1736. [CrossRef]
123. Rana, L.; Bisht, S.; Dheeraj; Biswas, M.; Garai, S. Copper (II) Complexes of Azo Phenolate Ligands: Synthesis, Crystal Structure, Functional Mimetic, and Anticancer Study. *Inorg. Chem. Commun.* **2025**, *177*, 114357. [CrossRef]
124. Li, Y.; Han, X.; Song, Y. An Azo-Phenol Derivative Probe: Colorimetric and “Turn-on” Fluorescent Detection of Copper(I) Ions and pH Value in Aqueous Solution. *RSC Adv.* **2017**, *7*, 20537–20541. [CrossRef]
125. Dev, S.; Mitra, D.; Sinha, C.; Das, G.; Murmu, N.; Maity, A.R.; Pandey, S. High Anticancer Activity in Short Response Time Exhibited by a New Azobenzene Derivative and Its Copper Complex. *Appl. Organom Chem.* **2024**, *38*, e7510. [CrossRef]
126. Pervaiz, M.; Sadiq, S.; Sadiq, A.; Younas, U.; Ashraf, A.; Saeed, Z.; Zuber, M.; Adnan, A. Azo-Schiff Base Derivatives of Transition Metal Complexes as Antimicrobial Agents. *Coord. Chem. Rev.* **2021**, *447*, 214128. [CrossRef]

127. Kasare, M.S.; Dhavan, P.P.; Jadhav, B.L.; Pawar, S.D. Synthesis of Azo Schiff Base Ligands and Their Ni(II), Cu(II) and Zn(II) Metal Complexes as Highly-Active Antibacterial Agents. *ChemistrySelect* **2019**, *4*, 10792–10797. [CrossRef]
128. Bal, S.; Bal, S.; Erener, A.; Halipci, H.; Akar, S. Synthesis, Thermal Stability, Electronic Features, and Antimicrobial Activity of Phenolic Azo Dyes and Their Ni(II) and Cu(II) Complexes. *Chem. Pap.* **2014**, *68*, 352–361. [CrossRef]
129. Saad, F.A.; El-Ghamry, H.A.; Kassem, M.A.; Khedr, A.M. Nano-Synthesis, Biological Efficiency and DNA Binding Affinity of New Homo-Binuclear Metal Complexes with Sulfa Azo Dye Based Ligand for Further Pharmaceutical Applications. *J. Inorg. Organomet. Polym.* **2019**, *29*, 1337–1348. [CrossRef]
130. Azam, M.; Al-Resayes, S.I.; Wabaidur, S.M.; Altaf, M.; Chaurasia, B.; Alam, M.; Shukla, S.N.; Gaur, P.; Albaqami, N.T.M.; Islam, M.S.; et al. Synthesis, Structural Characterization and Antimicrobial Activity of Cu(II) and Fe(III) Complexes Incorporating Azo-Azomethine Ligand. *Molecules* **2018**, *23*, 813. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

Preparation and Properties of Chitosan Complexes Consisting of *Artemisia argyi* Volatile Oil Nanoemulsion

Shun Zhang ^{1,2,3}, Kewei Zuo ^{1,2,3}, Lijun Zhang ^{4,5}, Chenlu Zhang ^{1,2,3,*} and Juan Shi ^{3,4,5,*}

¹ School of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong 723000, China; 15929874564@163.com (S.Z.); keweizuo@163.com (K.Z.)

² Shaanxi Province Key Laboratory of Bio-Resources, Hanzhong 723000, China

³ Qinba State Key Laboratory of Biological Resources and Ecological Environment (Incubation), Hanzhong 723000, China

⁴ School of Chemical and environmental Science, Shaanxi University of Technology, Hanzhong 723000, China; 18829368533@163.com

⁵ Shaanxi Provincial Key Laboratory of Catalysis Basis and Application, Hanzhong 723000, China

* Correspondence: chenluzhang@snut.edu.cn (C.Z.); hzhshijuan@126.com (J.S.); Tel.: +86-15891662100 (C.Z.); +86-15691621331 (J.S.)

Abstract: *Artemisia argyi* volatile oil (AAVO) is a kind of natural oil with abundant active components and remarkable medicinal and healthcare value. However, AAVO has low solubility, stability, and bioavailability. Here, to address these issues, a nanoemulsion system of *Artemisia argyi* volatile oil (AAVO-Ne) is constructed using phase transition titration, and the conditions are continuously optimized to combine it with chitosan, forming a chitosan composite of the volatile oil nanoemulsion (AAVO-NeCs). The structure was analyzed using Fourier transform infrared (FT-IR) spectroscopy, and the performance was evaluated through in vitro antibacterial tests, in vitro release experiments, and antioxidant assays. The results indicated that the typical characteristic absorption peaks of AAVO shifted in the AAVO-Ne spectrum and new absorption peaks appeared in the AAVO-NeCs, which implied that the formation of AAVO-NeCs involved not only a physical encapsulation process but also certain chemical interactions, thus enhancing the stability and bioactivity of the composites. Compared to AAVO, AAVO-NeCs exhibited a 1.87-fold increase in antibacterial activity against antibiotic-resistant bacteria. Meanwhile, the in vitro release study demonstrated that AAVO-NeCs exhibited a biphasic release pattern. Compared to AAVO-Ne and AAVO, AAVO-NeCs also showed a significant enhancement in antioxidant activity. Overall, AAVO-NeCs demonstrate improved solubility and efficacy of AAVO, as well as high-efficiency delivery, antibacterial, sustained-release, and antioxidant properties. These attributes position AAVO-NeCs as a promising candidate for applications in drug delivery, food preservation, and other fields, offering innovative solutions and contributing to the sustainable development of related industries.

Keywords: *Artemisia argyi* volatile oil; nanoemulsion; chitosan; antibacterial activity; in vitro release; antioxidant

1. Introduction

In recent years, the misuse of antibiotics has led to an increasing issue of bacterial resistance, which has become a major global public health threat. Monitoring by the China Antimicrobial Resistance Surveillance Network in the first half of 2024 revealed that the emergence of resistant strains continues to rise, highlighting the urgent need for the discovery of new antimicrobial agents [1,2]. Essential oils from natural plants, due

to their broad-spectrum antibacterial properties, show inhibitory effects against various bacteria, fungi, viruses, and parasites, and thus hold promise as effective antibacterial agents and antioxidants [3–5]. Studies by Sánchez et al. have demonstrated that essential oils from the *Ricinus* genus in Colombia exhibit significant antibacterial activity against methicillin-resistant *S. aureus*, and ampicillin-resistant and streptomycin-resistant *E. coli* [6]. Furthermore, essential oils from Cassia bark, oregano, thyme and carnation also display notable antimicrobial activity against resistant *E. coli* [7–9]. These findings highlight the tremendous potential of plant essential oils in addressing bacterial resistance, offering new perspectives and methods for the development of novel antimicrobial agents.

Artemisia argyi H. Lév. & Vaniot, commonly known as Mugwort, is a plant species belonging to the Asteraceae family and the *Artemisia* genus [10–14]. *Artemisia argyi*, a traditional Chinese medicinal herb, exhibits significant pharmacological potential through its main bioactive component, *Artemisia argyi* volatile oil (AAVO) (Table 1). This oil primarily contains compounds such as eucalyptol, camphor, borneol, and caryophyllene, which contribute to its diverse pharmacological activities. These activities include antiviral, antibacterial, bronchodilatory, anti-inflammatory, analgesic, and sedative effects, demonstrating the wide therapeutic potential of AAVO in various medical applications [15]. Currently, the extraction methods for AAVO primarily include steam distillation, organic solvent extraction, supercritical carbon dioxide extraction, and microwave extraction. Common methods used for antimicrobial testing include the agar disk diffusion method, colony counting method, minimum inhibitory concentration (MIC) method, and minimum bactericidal concentration (MBC) method [16–19]. Current research indicates that the AAVO extracted via steam distillation demonstrates inhibitory effects against both Gram-positive and Gram-negative bacteria, as well as fungi, as shown by the agar disk diffusion assay. The proposed mechanism of action suggests that the AAVO disrupts the cell wall and membrane of the tested microorganisms, leading to the leakage of lipids, proteins, and other molecules, as well as the release of alkaline phosphatase, ultimately resulting in cell death. This highlights the natural antimicrobial potential of AAVO [20,21].

Table 1. Abbreviations.

Abbreviations	Detailed Interpretation
AAVO	<i>Artemisia argyi</i> volatile oil
AAVO-Ne	<i>Artemisia argyi</i> volatile oil nanoemulsion
AAVO-NeCs	chitosan composite of <i>Artemisia argyi</i> volatile oil nanoemulsion
Ne	nanoemulsion
Cs	chitosan

AAVO has low water solubility, a high evaporation rate, and unstable physical properties, which result in its poor bioavailability. In recent years, nanodrug delivery systems have been increasingly applied to encapsulate essential oils, aiming to improve their physicochemical properties. Commonly used nanodelivery systems for essential oils include nanoemulsions (Ne), microemulsions, microcapsules, and liposomes [22]. Ne, as an advanced essential oil delivery system, has garnered significant attention in the scientific literature. It offers advantages such as simple preparation, small particle size, large specific surface area, and kinetic stability, and can encapsulate and directly load active compounds. This makes it a highly attractive novel approach for incorporating plant-derived active extracts into food and pharmaceutical formulations [23–25]. In the study of volatile oil nanoemulsion, it was found that the MIC and MBC of the nanoemulsion were significantly lower compared to the pure volatile oil, indicating that the nanoemulsion system can enhance the antimicrobial activity of the volatile oil [26]. Studies have indicated that

cinnamon essential oil nanoemulsion can be successfully synthesized using the water-in-oil method. By simulating the digestive process of the essential oil nanoemulsion in the gastrointestinal tract, the bioavailability of the essential oil in food and pharmaceuticals is enhanced. Both ABTS and DPPH radical scavenging assays, as well as the paper disc diffusion test, demonstrate that the nanoemulsion exhibits superior antioxidant and antimicrobial properties [27]. It can be concluded that by constructing an *Artemisia argyi* essential oil nanosuspension (AAVO-Ne), the solubility, bioavailability, and stability of AAVO can be significantly improved. This approach contributes to enhancing the therapeutic efficacy of AAVO and is an effective method to boost its performance [28–30].

Chitosan (Cs), a natural polysaccharide extracted from chitin, possesses various excellent physiological functions, such as biodegradability, biocompatibility, non-toxicity, and antimicrobial properties. Due to its wide range of applications, Cs has been successfully utilized in several fields, including food additives, textiles, cosmetics, and antimicrobial agents [31,32]. Studies have shown that encapsulating cinnamon essential oil into Cs nanolipid emulsions using ion gelation technology significantly enhances the oil's efficacy, demonstrating excellent performance in terms of encapsulation efficiency, oil loading, in vitro release, bioactivity, and antioxidant properties [33]. The direct loading of essential oils into Cs can significantly enhance their antibacterial properties, indicating the method's great potential for application in food preservation and antibacterial packaging [34,35]. However, research on the preparation of essential oils as nanoemulsions to alter their water solubility and bioavailability, and their subsequent complexation with Cs to enhance antibacterial properties, is relatively limited.

This study aims to prepare AAVO-Ne and incorporate Cs in order to significantly enhance their antibacterial, antioxidative, and controlled release properties. AAVO, as a natural active ingredient, exhibits various pharmacological activities, including antibacterial and antioxidative effects. However, its applications are limited due to issues such as poor stability and low water solubility. Ne technology can improve the stability and bioavailability of AAVO, while the addition of Cs is expected to further enhance its controlled release effect and antibacterial activity. By optimizing the preparation process of AAVO-Ne and combining the characteristics of Cs, this study aims to develop an efficient and stable AAVO-NeCs system. This approach not only provides a novel strategy for developing more effective natural antibacterial products, extending shelf life, and enhancing the efficacy of active ingredients in pharmaceuticals or cosmetics, but also facilitates the broader application of AAVO in hygiene, environmental, biomedical, and other fields.

2. Results and Discussions

2.1. Preparation and Quality Determination of AAVO-Ne

The phase inversion method in the preparation of nanoemulsions offers significant advantages, including low energy consumption, simple operation, cost-effectiveness, and strong environmental sustainability. Compared to high-energy methods, it does not require complex equipment and can prepare emulsions under mild conditions, making it suitable for the protection of volatile oil components [36]. During the preparation process, the role of surfactants is to cover a large interfacial area and reduce interfacial tension, thereby stabilizing the emulsion. If the concentration of surfactant is insufficient, it will not effectively lower the interfacial tension, leading to the breakdown of the emulsion structure and instability. However, when the surfactant concentration is too high, the intermolecular forces between emulsion droplets increase, promoting droplet collision and aggregation, ultimately leading to instability. Therefore, selecting an appropriate Km value is crucial for ensuring the stability of the emulsion and preventing droplet aggregation [37,38].

At different Km ratios, as shown in Table 2, the addition of varying amounts of AAVO results in the formation of different surfactant-to-cosurfactant ratios (SOR) in the AAVO-Ne system. Specifically, when the Km ratio is 4:1, AAVO-Ne forms effectively within the SOR range of 9:1 to 5:5. When the Km ratio is 3:1, AAVO-Ne forms within the SOR range of 9:1 to 6:4. At a Km ratio of 2:1, AAVO-Ne forms within the SOR range of 9:1 to 5:5. Furthermore, when Km is 1:1, AAVO-Ne can be obtained within the SOR range of 8:2 to 5:5. At Km = 1:2, AAVO-Ne formation is possible within the SOR range of 8:2 to 6:4. Similarly, when Km = 2:3, AAVO-Ne can also be successfully formed within the SOR range of 8:2 to 6:4.

Table 2. Different SOR AAVO-Ne formed at different Km values.

Km	SOR
4:1	9:1; 8:2; 7:3; 6:4; 5:5
3:1	9:1; 8:2; 7:3; 6:4
2:1	9:1; 8:2; 7:3; 6:4; 5:5
1:1	8:2; 7:3; 6:4; 5:5
1:2	8:2; 7:3; 6:4
2:3	8:2; 7:3; 6:4

As shown in Figure 1, the ternary phase diagram analysis indicates that the emulsion region has the largest area when the Km ratio is 4:1. Compared to other Km values, at this ratio, the surfactant and co-surfactant can effectively form a stable micelle structure [39]. Based on this result, we chose Km = 4:1 as the optimal mass ratio of surfactant to co-surfactant for preparing the AAVO-Ne system.

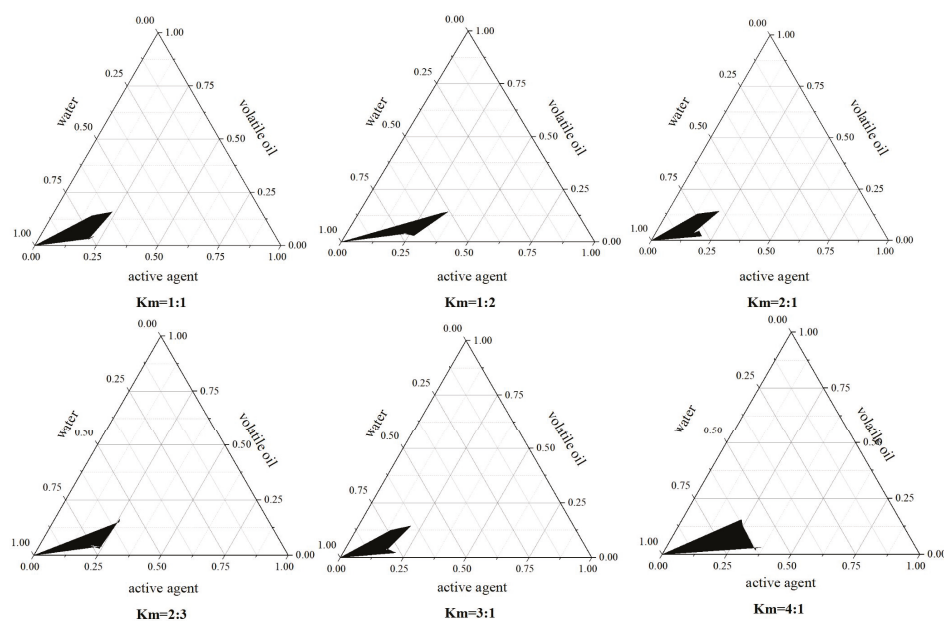


Figure 1. Quasi-ternary phase diagram of AAVO-Ne with different Km values (1:1, 1:2, 2:1, 2:3, 3:1, 4:1).

As shown in Table 3, under the optimal Km = 4:1 conditions, AAVO-Ne can be successfully prepared with surfactant-to-co-surfactant ratios (SOR) of 9:1, 8:2, 7:3, 6:4, and 5:5. However, when the SOR is 8:2 and 7:3, the resulting AAVO-Ne does not meet the required specifications due to noticeable turbidity and low transparency. In contrast, when the SOR is 9:1, 6:4, and 5:5, the emulsions are clear and transparent. Among these, the AAVO content reaches its maximum at an SOR of 5:5. Therefore, a surfactant-to-co-surfactant ratio of 5:5 is selected to optimize the preparation process of AAVO-Ne.

Table 3. Appearance and shape of AAVO-Ne.

Ratio	Appearance Shape
9:1	Clarification, clarity
8:2	Clarification, semi-clarity
7:3	Muddy
6:4	Clarification, clarity
5:5	Clarification, clarity

Based on the above screening results, the optimal composition for preparing AAVO-Ne is as follows: 19.88% Tween-80, 3.11% anhydrous ethanol, 16.15% AAVO, 60.86% distilled water. The AAVO-Ne prepared with these mass percentages is transparent, clear, stable, and shows no phase separation.

The structural type of AAVO-Ne was determined through dyeing experiments, which revealed that methylene blue diffused faster in AAVO-Ne than Sudan III, indicating that the prepared AAVO-Ne is of the oil-in-water (O/W) type. Additionally, the pH of the prepared AAVO-Ne was measured using precision pH test paper, yielding a result of 5.5 ± 0.5 , suggesting that the emulsion is mild and poses no safety risk to the skin. After one week of centrifugation, the AAVO-Ne showed no phase separation, emulsion breakdown, or crystallization, and there was no significant change in appearance. This indicates that the preparation process and stability of AAVO-Ne are suitable.

2.2. The Preparation of AAVO-NeCs

The preparation conditions of the AAVO-NeCs were optimized through a single-factor experiment, and the antibacterial activity of the AAVO-NeCs against *E. coli* was evaluated using the paper disc diffusion method. The antibacterial effect under different conditions was studied with the diameter of the inhibition zone as the indicator in Figure 2 (Supplementary Figure S2). In the pH adjustment experiment, the results of single-factor significance analysis indicated that the diameter of the inhibition zone was not linearly related to the pH value. Specifically, the AAVO-NeCs showed the best antibacterial effect at pH 2, with the inhibition zone diameter close to 20 mm. As the pH increased, the inhibition zone gradually shrank, although there was a slight recovery at pH 5 before it decreased again (Figure 2a). Comparing the inhibition effect of pure acetic acid solution on bacterial growth at different pH values, it was found that the inhibition zone diameter significantly increased when the pH was adjusted from 3 to 2, while at pH 4 and 5, the acid's inhibitory effect on bacterial growth was milder (Figure 2b). Some studies have also shown that under conditions of pH 2.0, *E. coli* requires the presence of glutamic acid, arginine, or lysine to survive, as these amino acids help neutralize the acidic intracellular environment through their decarboxylation [40]. Therefore, to exclude the impact of acidity on bacterial growth and to ensure the AAVO-NeCs is non-irritating to the skin, pH 5 was selected as the optimal condition.

The effect of temperature on the antibacterial activity of the AAVO-NeCs was also studied. The results showed that the AAVO-NeCs prepared at 20 °C had significantly higher antibacterial activity than those prepared at other temperatures. At 10 °C, 30 °C, 40 °C, and 50 °C, the antibacterial activity of the composite showed no significant differences (Figure 2c). This suggests that the antibacterial activity of the AAVO-NeCs is relatively stable across different temperatures, with the best effect observed at 20 °C.

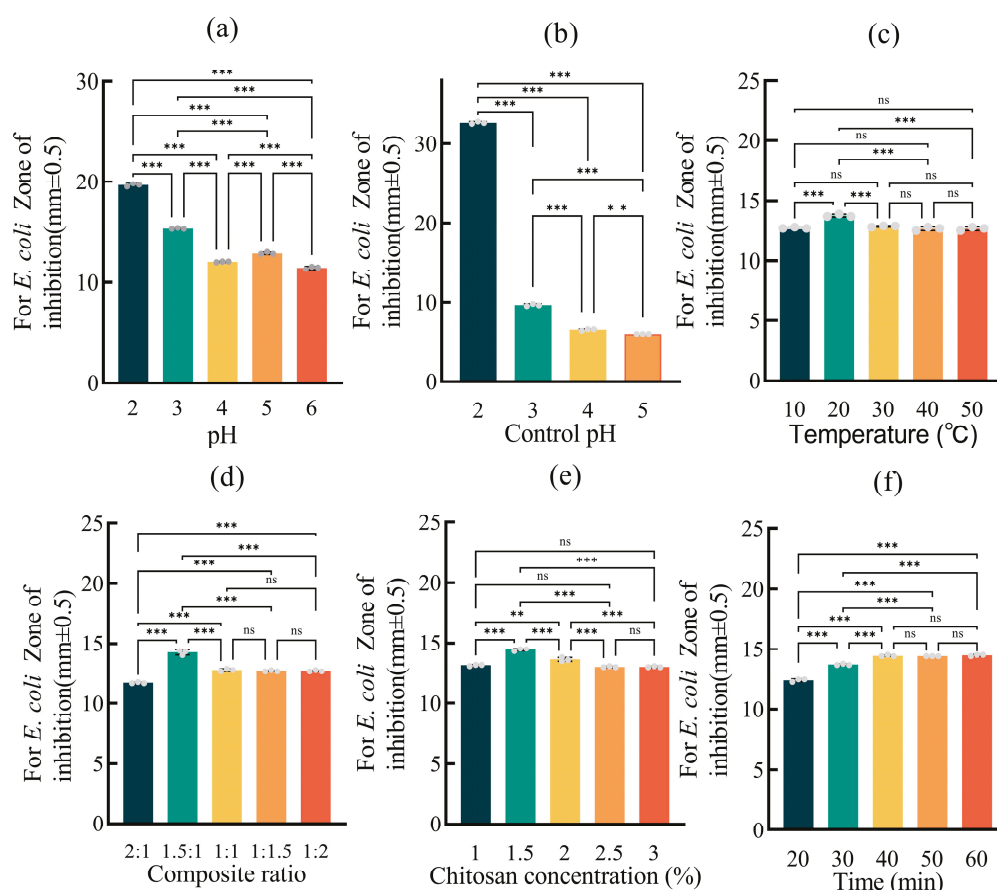


Figure 2. Analysis plot of AAVO-NeCs prepared from the one-factor experiment. (a) pH variation; (b) pH control (pure pH); (c) Temperature variation; (d) Variation of the complex ratio; (e) Variation of chitosan concentration; (f) Variation of complexation time. (**, $p < 0.01$; ***, $p < 0.001$, ns, no significance).

The effect of the ratio of the Cs acetic acid solution on AAVO-Ne and the Cs concentration on the antibacterial activity of the AAVO-NeCs was also investigated. It was found that as the ratio of AAVO-Ne and the Cs concentration increased, the antibacterial activity of the composite first increased and then decreased, stabilizing within a certain concentration range (Figure 2d,e). This result indicates that there is an optimal ratio between the AAVO-Ne and Cs concentrations and increasing the ratio does not necessarily improve the activity. Further analysis revealed that the composite exhibited the best antibacterial performance when the ratio of AAVO-Ne to Cs acetic acid solution was 1.5:1 and the Cs concentration was 1.5%.

Finally, the effect of ultrasound treatment time on the antibacterial activity of the AAVO-NeCs was studied. The results showed that within a certain time range, the antibacterial activity of the AAVO-NeCs increased with the extension of ultrasound treatment time and stabilized after 40 min. Significance analysis indicated that after 40 min, the antibacterial effect of the AAVO-NeCs was no longer significantly affected by further extension of the ultrasound time (Figure 2f). Therefore, 40 min was chosen as the optimal ultrasound treatment time.

In conclusion, the optimal preparation conditions for the composite of AAVO-Ne and Cs acetic acid solution are as follows: AAVO-Ne and Cs acetic acid solution mixed at a 1.5:1 ratio, pH adjusted to 5, temperature set at 20 °C, and ultrasound treatment for 40 min. Under these conditions, the AAVO-NeCs demonstrated the best antibacterial activity.

When the pH was adjusted to an alkaline level, the material was found to coagulate into flocculent precipitates due to structural changes induced by the alkaline environment.

This phenomenon can be attributed to the sensitivity of Cs solubility to pH. Under acidic conditions, the amino groups of Cs become protonated, thereby increasing its solubility, which facilitates its interaction with AAVO-Ne [41,42]. Therefore, a pH range of 2 to 6 was chosen for the study. Considering the effects of acidic substances on bacterial growth, and in order to ensure both the antibacterial efficacy and gentle, non-irritating properties of AAVO-NeCs on the skin, a final pH of 5 was selected for preparation.

Temperature also significantly influences the stability of AAVO-NeCs. Previous studies have shown that an increase in temperature leads to the degradation of Cs, while the components of AAVO may volatilize, thereby increasing the instability of AAVO-NeCs [43]. On the other hand, at very low temperatures, Cs may gel, causing AAVO molecules to aggregate, thus reducing the uniformity and stability of the emulsion. Furthermore, studies indicate that an increase in temperature can reduce the viscosity of Cs, likely due to the activation of molecules within a certain temperature range, which promotes effective collisions between molecules and the cleavage of glycosidic bonds. Therefore, AAVO-NeCs were prepared at 20 °C to maintain their stability and effectiveness, achieving optimal antibacterial performance.

The content of AAVO-Ne plays a crucial role in the properties of AAVO-NeCs, with different preparation results depending on the ratio. Research shows that too high a content of AAVO-Ne can lead to increased volatility, affecting the release rate and duration of the nanoemulsion, while too low a content may be insufficient for achieving the desired active effect. AAVO-Ne and Cs function complementarily. For instance, when Cs and pectin are used to prepare nanoemulsions for localized delivery of ethylhexyl triazine, it was found that low concentrations of Cs combined with varying percentages of pectin can enhance the stability of the nanoemulsion. This indicates that the stability of the nanoemulsion is closely related to the ratio of Cs and AAVO-Ne, and excessive use of one component can interfere with the function of the other, thereby reducing the overall effectiveness [44].

The concentration of Cs also has a significant impact on the viscosity and dispersion of AAVO-NeCs. High concentrations of Cs can increase the viscosity of the solution, affecting the uniform dispersion of AAVO-Ne and potentially leading to the formation of uneven AAVO-NeCs. Conversely, low concentrations of Cs may fail to form a sufficient network structure, resulting in insufficient strength of AAVO-NeCs, thereby affecting their stability [45,46].

Moreover, ultrasonic treatment has an important effect on the stability and shelf life of the nanoemulsion. Studies have shown that the ultrasonic treatment time significantly affects the particle size, turbidity, and dispersion of soybean protein isolate-phosphatidylcholine nanoemulsions. Extending the ultrasonic treatment time appropriately can improve the dispersion and stability of the emulsion [47]. However, excessive ultrasonic treatment time may lead to an increase in particle size, thereby altering the physical properties of AAVO-NeCs, such as viscosity and rheology, ultimately affecting the performance of the material. Considering both timeliness and performance, 40 min was selected as the optimal preparation time.

In conclusion, the preparation of AAVO-NeCs is influenced by factors such as the ratio of AAVO-Ne to Cs, ultrasonic treatment time, and Cs concentration. These factors interact with each other and collectively determine the stability and bioactivity of AAVO-NeCs.

2.3. FT-IR Analysis

FT-IR was used to test the structures of AAVO, AAVO-Ne, and AAVO-NeCs. The spectral analysis revealed distinct structural features in the characteristic absorption peaks of AAVO and AAVO-Ne. In the FT-IR spectrum of AAVO, as shown in Figure 3, the -OH stretching vibration peak around 3350 cm^{-1} , the -CH₂ stretching vibration peak near

2900 cm^{-1} , and the C=O absorption peak at 1670 cm^{-1} [48], indicate the presence of its main active ingredients and functional groups. Compared to AAVO, the FT-IR spectrum of AAVO-Ne shows slight shifts in the peak positions, suggesting that interactions between the AAVO and the surfactant lead to minor changes in the chemical structure. In the AAVO-NeCs spectrum, new absorption peaks appear, indicating that hydrogen bonding or other interactions might occur between the amino groups of Cs and components in AAVO. These changes suggest that the formation of AAVO-NeCs involves not only physical encapsulation but also possible chemical interactions, which enhance the stability and biological activity of the complex. In summary, the FT-IR analysis results show that there are certain structural changes in AAVO, AAVO-Ne, and AAVO-NeCs, and the formation of AAVO-NeCs significantly affects the molecular structure of the complex, contributing to its improved biological activity and drug-release properties.

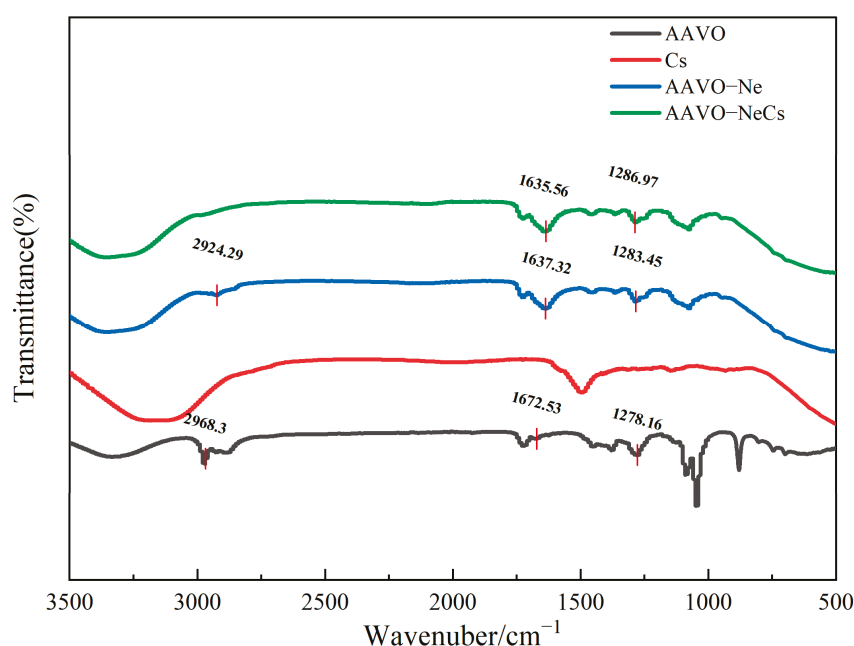


Figure 3. Infrared spectra of AAVO, AAVO-Ne and AAVO-NeCs.

2.4. Antibacterial Detection of AAVO, AAVO-Ne and AAVO-NeCs

Under the same concentration conditions, the antibacterial effects of AAVO, AAVO-Ne, and AAVO-NeCs on *S. aureus*, *E. coli*, *T-Salmonella* and *C. albicans* were tested using the paper disc diffusion method, with 1.5% Cs acetate solution and absolute ethanol as controls. The results are shown in Figure 4, indicating that all three materials had an inhibitory effect on the growth of the four bacterial strains, with varying degrees of inhibition depending on the bacterial species. Horizontal analysis reveals that, except for the control group, the inhibitory effects of the three materials on the four bacteria increase in sequence (Figure 4a–d). Compared to AAVO, the inhibitory effect of AAVO-NeCs on *T-Salmonella* increased from 10.7 mm to 20 mm, representing a 1.87-fold improvement (Figure 4c). Vertical analysis shows that AAVO-NeCs significantly enhanced the antibacterial activity against all four bacteria compared to AAVO. The effects of AAVO and AAVO-Ne on *S. aureus* and *C. albicans* were not significantly different, but AAVO-NeCs demonstrated an improved inhibitory effect on *T-Salmonella* (Figure 4e,f). This enhanced effect is primarily attributed to the natural antibacterial properties of Cs, which boost the antibacterial activity of AAVO. Additionally, the volatile compounds in AAVO are capable of effectively penetrating bacterial cell walls and interfering with metabolic processes, thus allowing AAVO-NeCs to overcome the resistance seen with conventional antimicrobial agents and exhibit stronger inhibition. In summary, AAVO-NeCs demonstrated a significant advantage in antibacterial

activity, particularly against *T-Salmonella*, showing a remarkable enhancement compared to AAVO alone. This suggests that AAVO-NeCs hold potential for application in antimicrobial fields, especially in combating antibiotic-resistant bacteria (Supplementary Figure S3) [49,50].

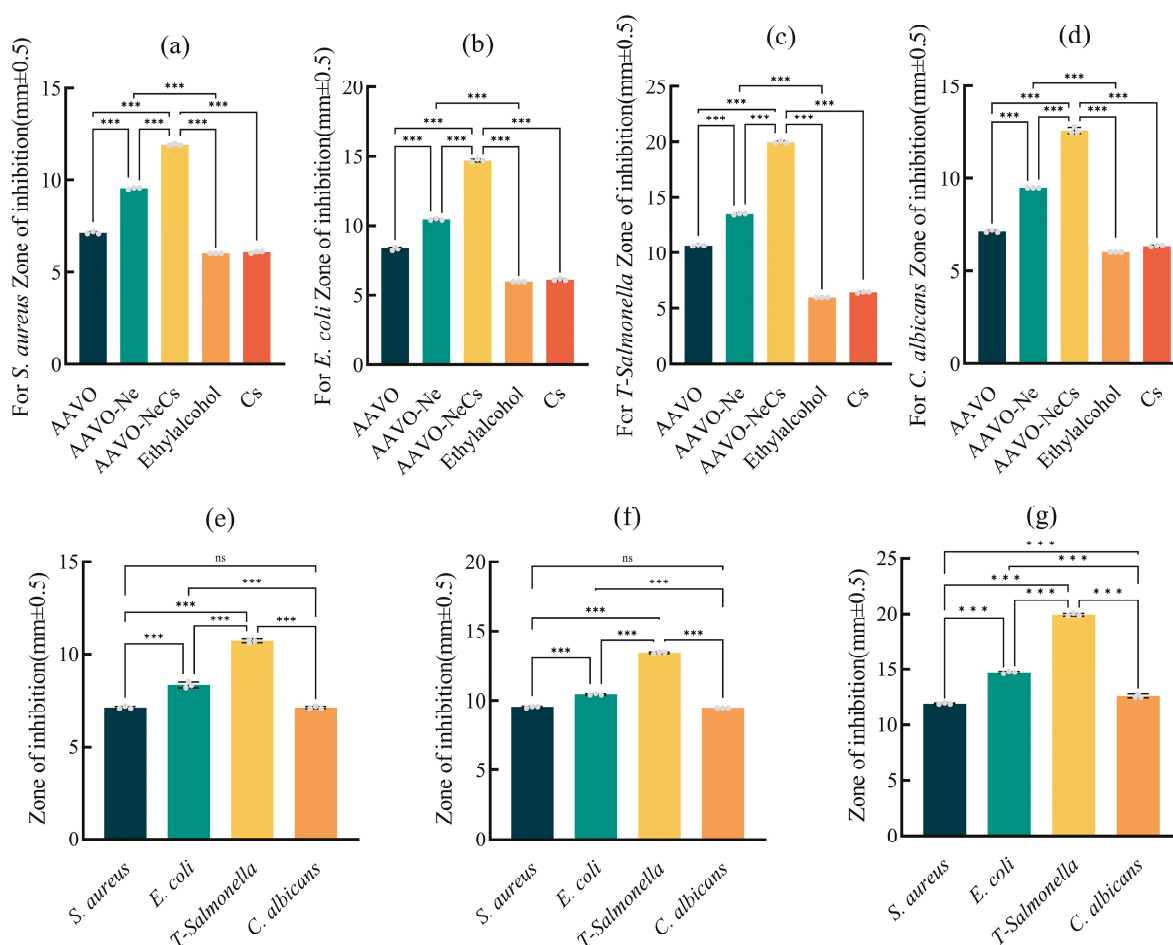


Figure 4. (a–d) Comparison analysis of the inhibitory effects of AAVO, AAVO-Ne, and AAVO-NeCs on the same type of bacteria. (e–g) Comparison analysis of the inhibitory effects of AAVO, AAVO-Ne, and AAVO-NeCs on different types of bacteria (***, $p < 0.001$, ns, no significance).

Through preliminary experiments and adjustments, it was observed that as the volume of the three materials increased, the turbidity of the suspension in the test tube gradually decreased until the solution became transparent. This indicates that all three materials exhibit antibacterial activity against *E. coli*, with the MIC values being 20.995, 17.765, and 4.307 $\mu\text{g/mL}$, respectively. The MIC of AAVO-NeCs was 4.8 times higher than that of AAVO, and the MIC of AAVO-NeCs was significantly higher compared to both AAVO and AAVO-Ne (Supplementary Figure S4).

To further investigate the antibacterial effects of the three materials on the test bacteria, colony counting was used to quantitatively assess the antibacterial activity over different time periods. As the exposure time of the bacteria to the materials increased, the number of colonies on the solid medium gradually decreased compared to the blank control group, eventually nearly disappearing. The calculation of the antibacterial activity of three materials against *E. coli* at different time intervals showed significant differences in their effectiveness at each time point. The results are shown in Figure 5, the antibacterial rates of AAVO at 5, 10, 20, 40, and 60 min were 2.51%, 7.45%, 16.84%, 61.26%, and 65.33%, respectively. In comparison, AAVO-Ne exhibited higher antibacterial rates at the same time points, with values of 14.82%, 24.11%, 39.52%, 63.09%, and 68.20%. In contrast, AAVO-

NeCs showed even more significant antibacterial effects, with antibacterial rates of 36.49%, 51.11%, 91.06%, 97.33%, and 98.94%. These results indicate that AAVO-NeCs outperformed the other two materials in terms of antibacterial performance, and its antibacterial effect gradually increased over time (Supplementary Figure S5).

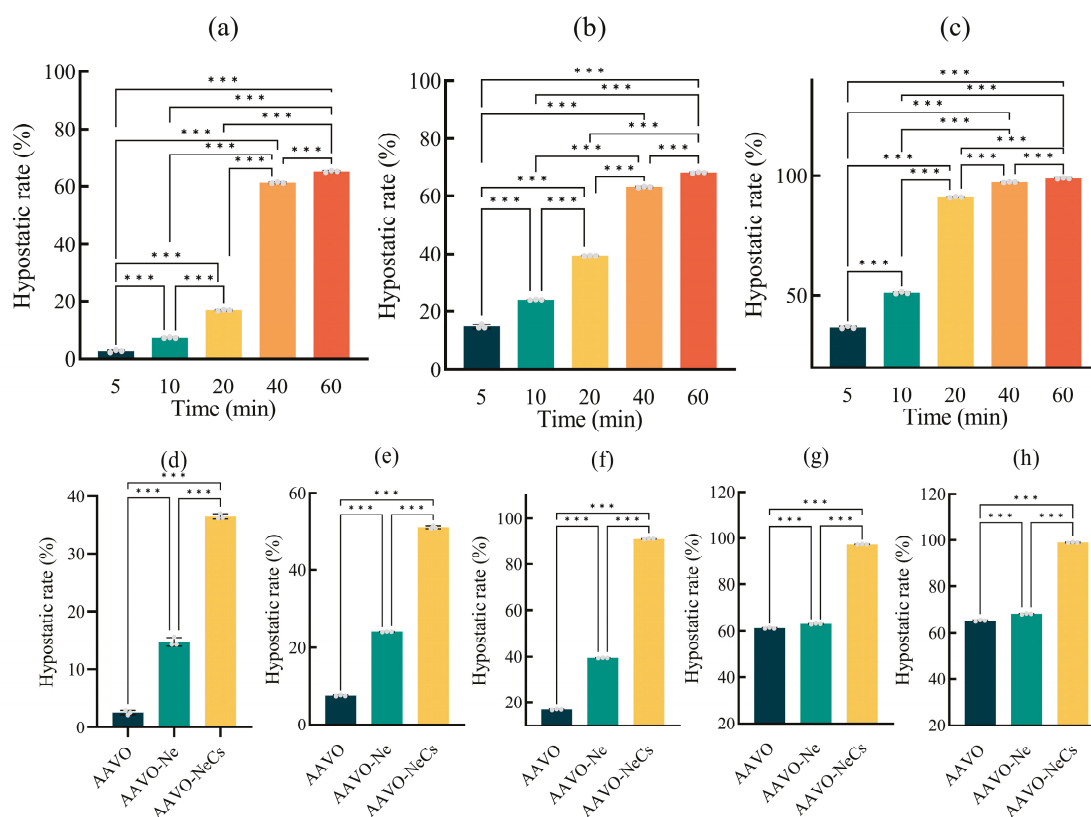


Figure 5. (a–c) The comparative analysis of the inhibition rates of AAVO, AAVO-Ne, and AAVO-NeCs against *E. coli* at different time intervals. (d–h) The comparative analysis of the inhibition rates of AAVO, AAVO-Ne, and AAVO-NeCs against *E. coli* at 5, 10, 20, 40, and 60 min, respectively. (***, $p < 0.001$).

A horizontal analysis of the antibacterial effects of the three materials at different exposure times shows that, as the exposure time increases, the inhibition rate against *E. coli* gradually increases (Figure 5a–c). However, the difference in their inhibitory effects is not particularly large. With the increase in exposure time, especially at 20 min, AAVO-NeCs achieved an inhibition rate of over 90%, while AAVO and AAVO-Ne did not reach this level even after 1 h. Compared to AAVO, the antibacterial rate at the same time point was 5.5 times higher, and compared to AAVO-Ne, it was 2.3 times higher. A vertical analysis of the antibacterial effects at the same time points shows that AAVO-NeCs consistently exhibited significantly higher antibacterial activity than both AAVO and AAVO-Ne (Figure 5d–h). This result is consistent with the findings from the paper disc diffusion test and MIC experiments, further confirming that AAVO-NeCs possess excellent antibacterial performance.

The results of the removal of *E. coli* biofilm on cucumber slices indicated that all three materials were able to remove the biofilm to varying degrees (Supplementary Figure S6). Among them, AAVO-NeCs exhibited the strongest ability to remove *E. coli* biofilm on the cucumber surface, and it is naturally safe, making it highly promising for use in food preservation and shelf-life extension.

In summary, the antibacterial activity of the three materials can be ranked as follows: AAVO-NeCs > AAVO-Ne > AAVO, further proving that AAVO-NeCs possesses excellent biological activity.

AAVO contains various bioactive compounds, such as phenols, aldehydes, alcohols, and ketones, which give AAVO its natural antibacterial, antifungal, and antiviral properties. These compounds can disrupt bacterial cell membranes, inhibit enzyme activity, and interfere with microbial metabolic processes. AAVO-Ne enhances the antimicrobial effect by increasing the specific surface area and improving bioavailability, allowing AAVO to more effectively penetrate bacteria and interact with them. At the same time, Cs protects AAVO-Ne from degradation by light, heat, and oxygen, prolonging its stability, controlling the release rate, improving its bioavailability, and enhancing antimicrobial activity. The progressive increase in antibacterial activity across the three materials can be attributed to the synergistic effect between AAVO-Ne and the AAVO-NeCs [51–53]. Previous studies have shown that the combination of AAVO-Ne and Cs can create a synergistic effect, enhancing the antimicrobial performance of the complex substance through the interaction of active ingredients [54]. The preparation of AAVO-Ne enhances the dispersibility of AAVO, allowing it to be evenly distributed within the system, which significantly improves its solubility. Additionally, Cs as a natural polysaccharide, possesses excellent biocompatibility and sustained-release properties. Cs can form a protective film that encapsulates the AAVO in AAVO-Ne, preventing its rapid degradation and inactivation in the environment, thus prolonging the drug's duration of action. Moreover, Cs has certain adhesive properties, which can enhance the drug's retention time on mucosal surfaces and further improve its absorption efficiency. These factors work synergistically to significantly increase the bioavailability of AAVO-NeCs. Therefore, the increasing antibacterial effect of AAVO in AAVO-Ne and AAVO-NeCs reflects the interaction between the surface characteristics of the materials and the active components. This further demonstrates that AAVO-NeCs effectively enhances the stability and bioavailability of AAVO, thereby significantly improving its overall antibacterial performance.

2.5. AAVO, AAVO-Ne and AAVO-NeCs In Vitro Release Assay

In the in vitro release experiment, as shown in Figure 6a the initial volatilization loss rates of the three materials exhibited abnormal behavior, especially within the first 12 h, where the loss rate of some materials even became negative. This phenomenon is likely due to the strong water absorption ability of AAVO, which may have absorbed moisture released during the volatilization process of AAVO-Ne and AAVO-NeCs, resulting in an increase in its own weight. After 24 h, the experimental behavior stabilized, and the volatilization loss rates of AAVO, AAVO-Ne, and AAVO-NeCs gradually decreased, showing the expected release trend. After 48 h, AAVO-NeCs exhibited a particularly significant release effect, with its loss rate being noticeably lower than that of AAVO and AAVO-Ne. By 60 h, the loss rate of AAVO-NeCs approached zero, indicating that this material has good in vitro sustained release performance.

Further analysis of the cumulative release rate of AAVO-NeCs reveals that it initially undergoes a rapid release phase, followed by a controlled-release phase, as shown in Figure 6b. This biphasic release pattern is consistent with the results of Singh et al. in their study on the in vitro release of Cs and cinnamon essential oil-based novel nanoemulsions [33]. The membrane structure formed by AAVO-NeCs effectively limits the rapid release of essential oils, and the formation of the membrane slows the diffusion rate of the essential oil molecules, resulting in a more stable release process. The combination of the hydrophilic and hydrophobic regions of Cs enables it to respond to changes in different environmental conditions (such as pH, temperature, etc.), thus achieving on-demand

release. This mechanism gives AAVO-NeCs excellent sustained-release and controlled-release properties, demonstrating its potential for widespread applications in drug delivery, food preservation, and cosmetics [55,56].

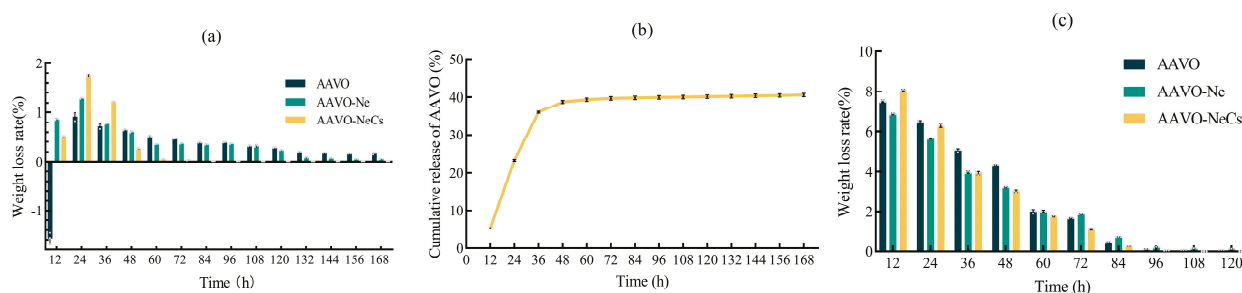


Figure 6. (a) The weight loss rate of AAVO, AAVO-Ne, and AAVO-NeCs at different time intervals; (b) in vitro release profile of AAVO-NeCs; (c) comparison of the weight loss rate of AAVO, AAVO-Ne, and AAVO-NeCs on freshly cut potato blocks.

2.6. Antioxidant Detection of AAVO, AAVO-Ne and AAVO-NeCs

After the harvest of potato cubes, the weight loss rate increases with the extension of storage time, primarily due to water loss. After harvesting, fruits and vegetables gradually lose mass due to water evaporation, and this water loss process may also lead to physiological metabolic disturbances, affecting their freshness. The results in Figure 6c showed that the three materials protected freshly cut potato pieces compared with the control group. In particular, after 36 h of storage, the protective effect of AAVO-NeCs becomes more pronounced. AAVO-NeCs can effectively prevent the antioxidant components in AAVO from being oxidized by forming a membrane structure, thus improving its stability. This is because AAVO is rich in natural antioxidant compounds, such as phenolic compounds, flavonoids, and terpenes, which can effectively scavenge free radicals and inhibit oxidation reactions. Additionally, Cs itself has certain bioactive and antioxidant properties, with active functional groups like amino and hydroxyl groups that can react with free radicals, slowing down the oxidation process. The combination of these two components creates a synergistic effect, significantly enhancing the overall antioxidant capacity [57,58].

3. Materials and Methods

3.1. Materials

Anhydrous ethanol (AR, Lianlonghua Pharmaceutical Chemical Co., Ltd., Tianjin, China); Tween-80 (CP, Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China); glacial acetic acid, sodium chloride (AR, Tianjin Tianli Chemical Reagents Co., Ltd., Tianjin, China); tryptone, agar, yeast extract powder (BR, Tianjin Tianli Chemical Reagents Co., Ltd., Tianjin, China); Chitosan (McLean Biochemical Technology Co., Ltd., Shanghai, China); *E. coli*, *S. aureus*, *C. albicans*, and *T-Salmonella* (Edible Fungi Research Institute, Shaanxi, China).

3.2. Preparation of AAVO

The *Artemisia argyi* leaves were sourced from the local market in Hanzhong City and identified by Professor Chenlu Zhang from the School of Biological Science and Engineering and Professor Juan Shi from the School of Chemistry and Environmental Science at Shaanxi University of Technology. The sample processing involved cleaning, trimming, drying at 35 °C for 30 min, pulverizing, and sieving through a 60-mesh screen. Several commonly reported extraction methods were evaluated, and one extraction method was selected for subsequent experiments based on yield calculation (Supplementary Figure S1) [59–61].

3.3. Preparation and Quality Assessment of AAVO-Ne

The AAVO-Ne system is constructed using phase transfer titration, a method that effectively controls the phase interface, enhancing the uniformity and stability of the nanoemulsion. This approach also improves its biocompatibility and drug-release properties. It offers high reaction efficiency with low energy consumption, making it more environmentally friendly. Additionally, it is applicable for the preparation of nanoemulsions with various active ingredients, demonstrating good adaptability [30].

In this study, Tween-80 was used as the surfactant and anhydrous ethanol as the co-surfactant, with various mass ratios (Km) (4:1, 3:1, 2:1, 1:1, 1:2, 2:3) prepared by thorough mixing. The mass ratio of AAVO to the surfactant–co-surfactant combination (SOR) was subsequently varied, with ratios of 9:1, 8:2, 7:3, 4:6, and 5:5, and the mixtures were thoroughly homogenized. Under the conditions of 20 °C and 650 rpm magnetic stirring, distilled water was added dropwise. The point of phase transition was determined when the emulsion transitioned from a clear to turbid appearance and then back to clear [62].

The quality of the emulsion was assessed based on transparency, clarity, and appropriate viscosity. A pseudo-ternary phase diagram was constructed using the mass fractions of the three components to determine the optimal Km value. Based on the optimal Km value, the effect of different mass ratios of the surfactant–co-surfactant combination and AAVO on the emulsion quality was evaluated to select the best formulation. The criteria for emulsion quality were clarity, transparency, appropriate viscosity, and no phase separation [63,64].

The structural type of the emulsion was identified using Sudan III and methylene blue staining methods. Specifically, 2 g of Sudan III and methylene blue were added to the emulsion, and the diffusion rates of both dyes were observed. A faster diffusion rate of Sudan III indicated that the emulsion was of the water-in-oil (W/O) type, while a faster diffusion rate of methylene blue suggested that the emulsion was of the oil-in-water (O/W) type. The pH of the prepared AAVO-Ne emulsion was measured using precision pH paper. One week later, we observed its appearance by centrifugation to assess its stability, with three parallel measurements conducted to ensure accuracy [65].

3.4. Preparation of AAVO-NeCs

Preparation conditions for the composite: A 1% *v/v* acetic acid aqueous solution was combined with varying amounts of Cs to obtain Cs solutions at different concentrations (1%, 1.5%, 2%, 2.5%, 3%). The mixture was stirred at 800 rpm using magnetic stirring until a transparent and homogeneous solution was achieved. The Cs solutions were then mixed with the AAVO-Ne at different mass ratios (2:1, 1.5:1, 1:1, 1:1.5, 1:2). The resulting mixtures were subjected to ultrasonic treatment for varying durations (20, 30, 40, 50, 60 min) at different temperatures (10, 20, 30, 40, 50 °C) and pH values (2, 3, 4, 5, 6). The optimal preparation conditions were determined based on the inhibitory activity against *E. coli* using the paper disk diffusion assay.

3.5. Fourier Transform Infrared Spectroscopy Analysis

AAVO, AAVO-Ne and AAVO-NeCs were characterized by Fourier transform infrared spectroscopy. (Model: VERTEX 70; Manufacturer: Bruker, Ettlingen, Germany; Main Test Range: 187,500 px^{-1} –9250 px^{-1} ; Resolution: 4 px^{-1}).

3.6. Antibacterial Experiment Research

3.6.1. Activation and Culture of Bacteria

Activation of microbial strains: To prepare the growth medium, 8 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride, and 30 g of agar were dissolved in 1 L of deionized water. The mixture was sterilized in an autoclave at 121 °C for 30 min. Under sterile

conditions, the hot medium was poured into pre-sterilized petri dishes and allowed to cool and solidify for later use. A sterile inoculation loop was used to transfer a small amount of bacterial stock solution onto the surface of Luria-Bertani (LB) agar plates, which were then incubated at 37 °C in a light-controlled incubator for 12 h. After incubation, individual bacterial colonies were selected and transferred into test tubes containing 5 mL of liquid medium. These tubes were then incubated at 37 °C with constant shaking for 12 h before being stored in a refrigerator for future use.

Bacterial cultivation: *E. coli*, *T-Salmonella*, *S. aureus* and *C. albicans* were cultured in LB medium at 37 °C. The bacterial suspensions were adjusted to a concentration of 10⁵ CFU (colony-forming units)/mL for subsequent antibacterial testing [66].

3.6.2. Antibacterial Experiment

Paper disc diffusion method experiment: In this experiment, *S. aureus*, *E. coli*, *T-Salmonella* and *C. albicans* were selected as test bacteria. The paper disc diffusion method was used to qualitatively evaluate the antibacterial properties of AAVO, AAVO-Ne, and AAVO-NeCs. All experimental materials were autoclaved at 121 °C for 20 min. AAVO, AAVO-Ne, and AAVO-NeCs were dissolved in 170 µg/mL solutions using absolute ethanol and stored for use. As controls, 1.5% Cs acetate solution and absolute ethanol were used. In the experiment, an appropriate amount of cultured test bacteria (400 µL) was evenly spread on the surface of solid agar media. Then, 6 mm diameter paper discs were placed on the agar surface, and 6 µL of different material solutions were applied to the holes in the discs. The plates were incubated at 37 °C for 12 h, and the antibacterial effects were recorded using a digital camera.

MIC experiment: *E. coli* was used as the test bacterium. No compound was added to the liquid medium for the blank control group. AAVO, AAVO-Ne, and AAVO-NeCs were added to the *E. coli* liquid medium at different concentrations in gradient steps and incubated at 37 °C on a shaking incubator for 12 h. The turbidity of the culture medium was observed, and the point at which the medium became clear was defined as the MIC.

Colony counting method experiment: In this experiment, *E. coli* was used as the test strain, and the colony counting method was employed to quantitatively analyze the antibacterial effects of AAVO, AAVO-Ne, and AAVO-NeCs. The three materials were mixed with bacterial suspension, and samples were taken at time points of 0, 5, 10, 30, 40, and 60 min. A 10 µL sample of the bacterial suspension was plated onto solid agar media and evenly spread. The plates were then incubated at 37 °C for 12 h, and the colony count was determined. The antibacterial rate was calculated using the following Formula (1):

$$A = \frac{B - C}{B} \times 100\% \quad (1)$$

where *A* is the antibacterial efficiency, *B* is the colony count of the control group, and *C* is the colony count after treatment with antibacterial materials at different time points.

E. coli clearance from fruit and vegetable surfaces experiment: Fresh cucumbers were selected, cut into 0.5 × 1 × 2 cm³ pieces, and sterilized under ultraviolet light to remove surface microorganisms. The cucumber pieces were immersed in a solution of *E. coli* and incubated at 35 °C for 24 h. After incubation, the cucumber pieces were removed and rinsed three times with sterile water. The pieces were then treated with AAVO, AAVO-Ne, and AAVO-NeCs and incubated in the culture chamber for another 24 h. After treatment, sterile water was added to the cucumber pieces, mixed well, and 10 µL of the supernatant was taken for viable bacterial counting using the plate colony counting method.

3.7. In Vitro Release Experiment Investigation

A precise amount of AAVO, AAVO-Ne, and AAVO-NeCs were weighed and placed in a drying oven at 60 °C for continuous evaporation over 7 days. Every 12 h, the samples were removed, weighed, and repositioned. The loss rate of the AAVO, AAVO-Ne, and AAVO-NeCs was calculated using Formulas (2)–(4) [67]. The cumulative release rate was determined according to Formula (5).

$$\text{AAVO loss rate} = \frac{m_J - m_{Jt}}{m_J} \times 100\% \quad (2)$$

$$\text{AAVO-Ne loss rate} = \frac{m_R - m_{Rt}}{m_R} \times 16.15\% \times 100\% \quad (3)$$

$$\text{AAVO-NeCs loss rate} = \frac{m_y - m_{yt}}{m_y} \times 16.15\% \times 60\% \times 100\% \quad (4)$$

$$\text{accumulated release rate} = \frac{\text{AAVO released per release}}{\text{The initial amount of AAVO added to the sample}} \quad (5)$$

In the formula, m_J and m_{Jt} are the initial mass/g of AAVO and the mass/g at t , respectively. The m_R and m_{Rt} are the initial mass/g and the mass/g at t of AAVO-Ne, respectively, and 16.15% is the mass fraction of AAVO in AAVO-Ne. m_y and m_{yt} are the initial mass/g of AAVO-NeCs and the mass/g at t , and 60% is the mass fraction of AAVO-Ne.

3.8. Antioxidant Experiment Investigation

Healthy, undamaged potatoes were selected from the market, cleaned with water, air-dried, and cut into $1 \times 1 \times 2 \text{ cm}^3$ pieces. These were randomly divided into four groups, with four pieces per group, ensuring no contact between them. Each group was coated with a solution of the same AAVO concentration using a brush, applying AAVO, AAVO-Ne, AAVO-NeCs, and an untreated control group. Once the coated surface dried naturally, the potato pieces were stored at room temperature. Starting on day 0, the weight of each group was measured every 12 h, and the weight loss rate was calculated using Formula (6) [68].

$$\text{weight loss rate} = \frac{m_k - m_{kt}}{m_k} \times 100\% \quad (6)$$

In the formula, m_k is the initial mass/g, and m_{kt} is the mass/g at time t .

3.9. Data Statistical Analysis

All experiments were conducted in triplicates, with results expressed as the mean $\pm$ standard error. Data analysis was performed using one-way ANOVA and tests in SPSS software (IBM SPSS Statistics 25). Statistical significance between means was calculated at a 95% confidence level ($p < 0.05$).

4. Conclusions

In this study, it was found that AAVO-Ne prepared with 19.88% Tween-80, 3.11% anhydrous ethanol, 16.15% AAVO, and 60.86% distilled water had the best performance. AAVO-NeCs were ultrasonically combined with 1.5% Cs acetic acid solution at a ratio of 1.5: 1 at 20 °C for 40 min. After adjusting the pH value to 5, the antibacterial effect of AAVO-NeCs was the best. The results of bacteriostatic experiment showed that the three materials had inhibitory effects on the growth of four kinds of bacteria, among which AAVO-NeCs had the best bacteriostatic effect and AAVO had the worst bacteriostatic effect. The results of filter paper diffusion method showed that the three materials had the best

antibacterial effect on drug-resistant *Salmonella*. In addition, the results of an in vitro release experiment, antioxidant experiment and bacteriostatic experiment were consistent, and the prepared AAVO-NeCs showed unique advantages. It can be seen that the AAVO-NeCs prepared in the experiment can realize the solubilization, synergism, efficient transportation, bacteriostasis, sustained release and controlled release, and antioxidant properties of VVAO.

Although we successfully prepared AAVO-related complexes with strong antibacterial activity, the molecular mechanism of how AAVO-related complexes are antibacterial, as well as their effects on bacterial cell membranes and interference with metabolic pathways, needs to be further explored. Meanwhile, AAVO-related complexes are inextricably linked to food preservation, so the effects of different concentrations and combinations of AAVO complexes on food preservation need to be further systematically evaluated.

With the increasing attention on natural medicines and green therapies, the development and application of AAVO have gained more focus. AAVO-NeCs, due to their improved bioavailability, enhanced stability, and sustained release properties, can effectively enhance their inhibitory effects against bacteria, fungi, and viruses, making them particularly advantageous in pharmaceutical formulations. This offers significant prospects for treating infectious diseases, skin care, and anti-inflammatory pain relief. The natural components of AAVO are well-compatible with the skin, and the Ne form can enhance its permeability, contributing to more effective local treatment and further boosting its clinical application value. In addition, AAVO-NeCs can also be applied in health foods, cosmetics, air fresheners, and other fields, demonstrating unique advantages in improving health and beauty effects.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules30030585/s1>, Figure S1: Comparison chart of extraction AAVO using three methods; Figure S2: Exploration of preparation conditions for AAVO-NeCs. Figure S3: Inhibition of four bacteria by three materials. Figure S4: Exploration of minimum inhibitory concentration. Figure S5: Colony count experiment. Figure S6: Bacterial removal effect on fruit and vegetable surfaces experiment.

Author Contributions: Conceptualization, S.Z., K.Z., C.Z. and J.S.; Data curation, S.Z.; Formal analysis, S.Z., K.Z. and L.Z.; Funding acquisition, C.Z. and J.S.; Investigation, S.Z. and L.Z.; Methodology, S.Z., K.Z., L.Z., C.Z. and J.S.; Project administration, C.Z. and J.S.; Resources, S.Z.; Software, S.Z. and K.Z.; Supervision, C.Z. and J.S.; Validation, S.Z., K.Z. and L.Z.; Visualization, C.Z. and J.S.; Writing—original draft, S.Z. and L.Z.; Writing—review and editing, C.Z. and J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Shaanxi Provincial Key Research and Development Programme (2022SF-223); Shaanxi University of Technology Qinba Biological Resources and Ecological Environment Provincial-Ministry Joint State Key Laboratory (Cultivation) ‘City-University Co-construction’ Research Special Project (SXC-2111; SXZC-2301; SXJ-2106).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are included in this manuscript.

Acknowledgments: The authors thank the Shaanxi University of Technology for providing the experimental equipment.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Qin, X.; Ding, L.; Hao, M.; Li, P.; Hu, F.; Wang, M. Antimicrobial resistance of clinical bacterial isolates in China: Current status and trends. *JAC-Antimicrob. Resist.* **2024**, *6*, dlae052. [CrossRef] [PubMed]
2. Tang, Y.; Chen, X.; Liu, C.; Xue, T.; Li, Y.; Gao, J. Policy text analysis of antimicrobial resistance governance in China: A focus on national-level policies. *Int. J. Antimicrob. Agents* **2024**, *64*, 107286. [CrossRef] [PubMed]
3. Leja, K.; Drożdżyńska, A.; Majcher, M.; Kowalczewski, P.; Czarczyk, K. Influence of sub-inhibitory concentration of selected plant essential oils on the physical and biochemical properties of *Pseudomonas orientalis*. *Open Chem.* **2019**, *17*, 492–505. [CrossRef]
4. Al-Maqtari, Q.A.; Rehman, A.; Mahdi, A.A.; Al-Ansi, W.; Wei, M.; Yanyu, Z.; Phyto, H.M.; Galeboe, O.; Yao, W. Application of essential oils as preservatives in food systems: Challenges and future prospectives—A review. *Phytochem. Rev.* **2021**, *21*, 1209–1246. [CrossRef]
5. Huang, X.; Lao, Y.; Pan, Y.; Chen, Y.; Zhao, H.; Gong, L.; Xie, N.; Mo, C.-H. Synergistic Antimicrobial effectiveness of plant essential oil and Its application in seafood preservation: A review. *Molecules* **2021**, *26*, 307. [CrossRef]
6. Sánchez, I.C.; Caro, J.A.S.; Galeano, E.; Alzate, F.; Ossa-Giraldo, A.C. Essential oils from Colombian *Croton* spp. exhibit antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and ampicillin- and streptomycin-resistant *Escherichia coli*. *Sci. Rep.* **2024**, *14*, 36043. [CrossRef]
7. Samy, S.; Mohammed, A.; Hussain, A.; Soad, K.; Fayez, M.; Mona, W.; Nashwa, H. Anti-Salmonella and Antibiofilm Potency of *Salvia officinalis* L. Essential Oil against Antibiotic-Resistant *Salmonella enterica*. *Antibiotics* **2020**, *11*, 489.
8. Naik, A.; Premanath, R. Anti-quorum sensing activity of essential oils against multidrug-resistant *Klebsiella pneumoniae*: A novel approach to control bacterial virulence. *Braz. J. Microbiol.* **2024**, *55*, 3909–3920. [CrossRef]
9. Alibi, S.; Selma, B.; Ramos-Vivas, J.; Smach, M.; Touati, R.; Boukadida, J.; Mansour, H. Anti-oxidant, antibacterial, anti-biofilm, and anti-quorum sensing activities of four essential oils against multidrug-resistant bacterial clinical isolates. *Curr. Res. Transl. Med.* **2020**, *68*, 59–66. [CrossRef]
10. Abad, J.M.; Bedoya, M.L.; Apaza, L.; Bermejo, P. The *Artemisia* L. Genus: A Review of bioactive essential oils. *Molecules* **2012**, *17*, 2542–2566. [CrossRef]
11. Asfaw, N.; Demissew, S. Essential oil composition of four *Artemisia* species from Ethiopia. *Bull. Chem. Soc. Ethiop.* **2015**, *29*, 123. [CrossRef]
12. Szopa, A.; Pajor, J.; Klin, P.; Rzepiela, A.; Elansary, H.O.; Al-Mana, F.A.; Mattar, M.A.; Ekiert, H. *Artemisia absinthium* L.—Importance in the history of medicine, the latest advances in phytochemistry and therapeutical, cosmetological and culinary uses. *Plants* **2020**, *9*, 1063. [CrossRef] [PubMed]
13. Bisht, D.; Kumar, D.; Kumar, D.; Kama, D.; Chellappan, D.K. Phytochemistry and pharmacological activity of the genus *artemisia*. *Arch. Pharmacol. Res.* **2021**, *44*, 439–474. [CrossRef] [PubMed]
14. Ekiert, H.; Knut, E.; Świątkowska, J.; Klin, P.; Rzepiela, A.; Tomczyk, M.; Szopa, A. *Artemisia abrotanum* L. (Southern Wormwood)—History, current knowledge on the chemistry, biological activity, traditional use and possible new pharmaceutical and cosmetological applications. *Molecules* **2021**, *26*, 2503. [CrossRef]
15. Wei, J.; Guo, T.; Xue, M.; Ma, R.; Wang, Y.; Chen, Z. *Artemisia argyi* H. Lév. & Vaniot: A comprehensive review on traditional uses, phytochemistry, and pharmacological activities. *Phytochem. Rev.* **2024**, *23*, 821–862.
16. Msaada, K.; Salem, N.; Bachrouch, O.; Bousselmi, S.; Tammar, S.; Alfaify, A.; Marzouk, B. Chemical composition and antioxidant and antimicrobial activities of wormwood (*Artemisia absinthium* L.) essential oils and Phenolics. *J. Chem.* **2015**, *2015*, 804658. [CrossRef]
17. Li, Y.; Xia, L.; Vazquez, T.F.J.; Song, S.X. Optimization of supercritical CO₂ extraction of essential oil from *Artemisia annua* L. by means of response surface methodology. *J. Essent. Oil Bear. Plants* **2017**, *20*, 314–327. [CrossRef]
18. Liu, T.-T.; Wu, H.-B.; Zhang, J. Wormwood (*Artemisia absinthium* L.) as a promising nematocidal and antifungal agent: Chemical composition, comparison of extraction techniques and bioassay-guided isolation. *Ind. Crop. Prod.* **2019**, *133*, 295–303. [CrossRef]
19. Guan, X.; Ge, D.; Li, S.; Huang, K.; Liu, J.; Li, F. Chemical composition and antimicrobial activities of *Artemisia argyi* Lévl. et vant essential oils extracted by simultaneous distillation-extraction, subcritical extraction and hydrodistillation. *Molecules* **2019**, *24*, 483. [CrossRef]
20. Xiang, F.; Bai, J.; Tan, X.; Chen, T.; Yang, W.; He, F. Antimicrobial activities and mechanism of the essential oil from *Artemisia argyi* Lévl. et Van. var. *argyi* cv. Qiai. *Ind. Crops Prod.* **2018**, *125*, 582–587. [CrossRef]
21. Li, D.; Wang, R.; You, M.; Chen, N.; Sun, L.; Chen, N. The antimicrobial effect and mechanism of the *Artemisia argyi* essential oil against bacteria and fungus. *Braz. J. Microbiol.* **2023**, *55*, 727–735. [CrossRef]
22. Wang, S.; Zhou, Z.; Yang, S.; Guo, Z.; Le, T.; Wu, J.; Guan, Z.; Liu, W.; Wu, W. Research Progress in Essential Oil Nanodelivery Systems. *Curr. Nanosci.* **2024**, *20*, 1–22. [CrossRef]
23. Wang, L.; Li, T.X.; Xin, B.J.; Liu, Y.; Zhang, F.L. Preparation and characterization of wormwood-oil-contained microcapsules. *J. Microencapsul.* **2020**, *37*, 324–331. [CrossRef] [PubMed]

24. Barradas, N.T.; Silva, E.H.D.G.K. Nanoemulsions of essential oils to improve solubility, stability and permeability: A review. *Environ. Chem. Lett.* **2020**, *19*, 1153–1171. [CrossRef]
25. Rahmani, Z.; Karimi, M.; Saffari, I.; Mirzaei, H.; Nejati, M.; Chaleshtori, R.S. Nanoemulsion and nanoencapsulation of a hydroethanolic extract of Nettle (*Urtica dioica*) and Wormwood (*Artemisia absinthium*): Comparison of antibacterial and anticancer activity. *Front. Chem.* **2024**, *12*, 1266573. [CrossRef]
26. Dana, Z.Z.; Lu, H.; Patrick, J.C.; Paula, B. Cold plasma inactivation of internalised bacteria and biofilms for *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes* and *Escherichia coli*. *Int. J. Food Microbiol.* **2015**, *210*, 53–61.
27. Liang, D.Y.; Wang, C.J.; Luo, X.M.; Wang, Z.; Kong, F.S.; Bi, Y.G. Preparation, characterization and properties of cinnamon essential oil nano-emulsion formed by different emulsifiers. *J. Drug Deliv. Sci. Technol.* **2023**, *86*, 104638. [CrossRef]
28. Sieniawska, E.; Świątek, Ł.; Wota, M.; Rajtar, B.; Polz-Dacewicz, M. Microemulsions of essentials oils—Increase of solubility and antioxidant activity or cytotoxicity? *Food Chem. Toxicol.* **2019**, *129*, 115–124. [CrossRef]
29. Dávila-Rodríguez, M.; López-Malo, A.; Palou, E.; Ramírez-Corona, N.; Jiménez-Munguía, M.T. Essential oils microemulsions prepared with high-frequency ultrasound: Physical properties and antimicrobial activity. *J. Food Sci. Technol.* **2020**, *57*, 4133–4142. [CrossRef]
30. Pavoni, L.; Perinelli, D.R.; Bonacucina, G.; Cespi, M.; Palmieri, G.F. An Overview of Micro- and Nanoemulsions as Vehicles for Essential Oils: Formulation, Preparation and Stability. *Nanomaterials* **2020**, *10*, 135. [CrossRef]
31. Elsabee, M.Z.; Abdou, E.S. Chitosan based edible films and coatings: A review. *Mater. Sci. Eng. C* **2013**, *33*, 1819–1841. [CrossRef] [PubMed]
32. Silva, A.O.; Cunha, R.S.; Hotza, D.; Machado, R.A.F. Chitosan as a matrix of nanocomposites: A review on nanostructures, processes, properties, and applications. *Carbohydr. Polym.* **2021**, *272*, 118472. [CrossRef] [PubMed]
33. Singh, B.K.; Chaudhari, A.K.; Das, S.; Tiwari, S.; Dubey, N.K. Preparation and characterization of a novel nanoemulsion consisting of chitosan and *Cinnamomum tamala* essential oil and its effect on shelf-life lengthening of stored millets. *Pestic. Biochem. Physiol.* **2022**, *187*, 105214. [CrossRef] [PubMed]
34. Zhang, X.; Ismail, B.B.; Cheng, H.; Jin, T.Z.; Qian, M.; Arabi, S.A.; Liu, D.; Guo, M. Emerging chitosan-essential oil films and coatings for food preservation—A review of advances and applications. *Carbohydr. Polym.* **2021**, *273*, 118616. [CrossRef]
35. Juthamas, T.; Pornchai, R.; Chitsiri, R. Synergistic Antimicrobial Activities of Thai Household Essential Oils in Chitosan Film. *Polymers* **2021**, *13*, 1519. [CrossRef]
36. Liu, Q.; Huang, H.; Chen, H.; Lin, J.; Wang, Q. Food-Grade Nanoemulsions: Preparation, Stability and Application in Encapsulation of Bioactive Compounds. *Molecules* **2019**, *24*, 4242. [CrossRef]
37. Almeida, F.; Corrêa, M.; Zaera, A.M.; Garrigues, T.; Isaac, V. Influence of different surfactants on development of nanoemulsion containing fixed oil from an Amazon palm species. *Colloids Surf. A Physicochem. Eng. Asp.* **2022**, *643*, 128721. [CrossRef]
38. Zhang, M.; Cao, Q.; Yuan, Y.; Guo, X.; Pan, D.; Xie, R.; Ju, X.; Liu, Z.; Wang, W.; Chu, L. Functional nanoemulsions: Controllable low-energy nanoemulsification and advanced biomedical application. *Chin. Chem. Lett.* **2023**, *35*, 108710. [CrossRef]
39. Raman, K.P.; Vijaya, L.A. Optimization and Solubilization study of nanoemulsion budesonide and constructing pseudoternary phase diagram. *Asian J. Pharm. Clin. Res.* **2019**, *12*, 551–553.
40. Park, J.; Kim, J.; Kim, K. Acid resistance and acid adaptation responses of foodborne *Escherichia coli* isolates. *J. Food Saf.* **2017**, *37*, e12352. [CrossRef]
41. Yang, J.; Dahlström, C.; Edlund, H.; Lindman, B.; Norgren, M. pH-responsive cellulose–chitosan nanocomposite films with slow release of chitosan. *Cellulose* **2019**, *26*, 3763–3776. [CrossRef]
42. Richa; Choudhury, A.R. pH mediated rheological modulation of chitosan hydrogels. *Int. J. Biol. Macromol.* **2020**, *156*, 591–597. [CrossRef] [PubMed]
43. Zhang, Z.; Dong, W.; Huang, Y. Oxidative degradation of chitosan by Fe-MCM-41 heterogeneous Fenton-like system. *Sci. Rep.* **2024**, *14*, 25972. [CrossRef]
44. Chen, Y.; Li, Z.; Yao, M.; Zhang, Y.; Zhu, Q.; Yang, Q. Study on the preparation of gelatin-chitosan-cinnamon essential oil natural film coating and its antibacterial effect. *J. Light Ind.* **2021**, *36*, 9–17.
45. Pudziulyte, L.; Siauruseviciute, A.; Morkuniene, R.; Lazauskas, R.; Bernatoniene, J. Influence of Technological Factors on the Quality of Chitosan Microcapsules with *Boswellia serata* L. Essential Oil. *Pharmaceutics* **2022**, *14*, 1259. [CrossRef]
46. Sobral, P.J.A.; Gebremariam, G.; Drudi, F.; Pinheiro, A.C.D.A.S.; Romani, S.; Rocculi, P.; Rosa, M.D. Rheological and Viscoelastic Properties of Chitosan Solutions Prepared with Different Chitosan or Acetic Acid Concentrations. *Foods* **2022**, *11*, 2692. [CrossRef] [PubMed]
47. Teng, F.; He, M.; Xu, J.; Chen, F.; Wu, C.; Wang, Z.; Li, Y. Effect of ultrasonication on the stability and storage of a soy protein isolate-phosphatidylcholine nanoemulsions. *Sci. Rep.* **2020**, *10*, 14010. [CrossRef] [PubMed]
48. Tang, X.; Guo, X.; Duo, Y.; Qian, X. Preparation and Characterization of a One-Step Electrospun Poly(Lactic Acid)/Wormwood Oil Antibacterial Nanofiber Membrane. *Polymers* **2023**, *15*, 3585. [CrossRef]

49. Nasaj, M.; Chehelgerdi, M.; Asghari, B.; Ahmadih-Yazdi, A.; Asgari, M.; Kabiri-Samani, S.; Sharifi, E.; Arabestani, M. Factors influencing the antimicrobial mechanism of chitosan action and its derivatives: A review. *Int. J. Biol. Macromol.* **2024**, *277*, 134321. [CrossRef]
50. Wu, K.; Yan, Z.; Wu, Z.; Li, J.; Zhong, W.; Ding, L.; Zhong, T.; Jiang, T. Recent Advances in the Preparation, Antibacterial Mechanisms, and Applications of Chitosan. *J. Funct. Biomater.* **2024**, *15*, 318. [CrossRef]
51. Zhang, F.; Ramachandran, G.; Mothana, R.A.; Noman, O.M.; Alobaid, W.A.; Rajivgandhi, G.; Manoharan, N. Anti-bacterial activity of chitosan loaded plant essential oil against multi drug resistant *K. pneumoniae*. *Saudi J. Biol. Sci.* **2020**, *27*, 3449–3455. [CrossRef] [PubMed]
52. Barbosa, R.; Villarreal, A.; Rodriguez, C.; De Leon, H.; Gilkerson, R.; Lozano, K. Aloe Vera extract-based composite nanofibers for wound dressing applications. *Mater. Sci. Eng. C* **2021**, *124*, 112061. [CrossRef] [PubMed]
53. Ghosh, S.; Nandi, S.; Basu, T. Nano-Antibacterials Using Medicinal Plant Components: An Overview. *Front. Microbiol.* **2022**, *12*, 768739. [CrossRef]
54. Basumatary, I.B.; Mukherjee, A.; Kumar, S. Chitosan-based composite films containing eugenol nanoemulsion, ZnO nanoparticles and Aloe vera gel for active food packaging. *Int. J. Biol. Macromol.* **2023**, *242*, 124826. [CrossRef]
55. Peers, S.; Montebault, A.; Ladavière, C. Chitosan hydrogels for sustained drug delivery. *J. Control. Release* **2020**, *326*, 150–163. [CrossRef]
56. Huang, Z.; Wang, Y.; Tan, S.; Gao, J.; Du, L. Preparation, Characterization, and Antibacterial Activity Evaluation of Chitosan-Cinnamon Essential Oil Microcapsules. *J. Cellul. Sci. Technol.* **2024**, *32*, 14–22.
57. El-Hack, A.E.M.; El-Saadony, T.M.; Shafi, E.M.; Zabermaawi, N.M.; Arif, M.; Batiha, G.E.; Khafaga, A.F.; El-Hakim, Y.M.A.; Al-Sagheer, A.A. Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review. *Int. J. Biol. Macromol.* **2020**, *164*, 2726–2744. [CrossRef]
58. Georgeta, L.C.; Gabriela, C.T.; Maria, D.; Mousa, S.; Maria, D.O. Preparation and Antimicrobial Activity of Chitosan and Its Derivatives: A Concise Review. *Molecules* **2021**, *26*, 3694. [CrossRef]
59. Nguyen, H.T.; Radácsi, P.; Gosztola, B.; Rajhárt, P.; Németh, Z. Accumulation and composition of essential oil due to plant development and organs in wormwood (*Artemisia absinthium* L.). *Ind. Crop. Prod.* **2018**, *123*, 232–237. [CrossRef]
60. Wang, L.H. Isolation of Active Antibacterial Components from Mugwort Leaves and Preparation and Application of Starch Nanofibres. Ph.D. Thesis, Tianjin Polytechnic University, Tianjin, China, 2022.
61. Wan, T.; Liu, J.; Li, J.; Fan, K. Therapeutic effect of *Artemisia argyi* essential oil nasal drops on avian infectious bronchitis. *J. Appl. Anim. Res.* **2024**, *52*, 2317892. [CrossRef]
62. Hien, L.T.M.; Khoa, T.D.; Dao, D.T.A. Characterization of black pepper essential oil nanoemulsion fabricated by emulsion phase inversion method. *J. Food Process. Preserv.* **2021**, *46*, e16207. [CrossRef]
63. Scheicher, B.; Lorenzer, C.; Gegenbauer, K.; Partlic, J.; Andreae, F.; Kirsch, A.H.; Rosenkranz, A.R.; Werzer, O.; Zimmer, A. Manufacturing of a Secretoneurin Drug Delivery System with Self-Assembled Protamine Nanoparticles by Titration. *PLoS ONE* **2017**, *11*, e0164149. [CrossRef] [PubMed]
64. Song, X.Q.; Wang, J.Y.; Li, S.T.; Wang, Y.F. Formation of sachinchi oil microemulsion systems: effects of non-ionic surfactants, short-chain alcohols, straight-chain esters and essential oils. *J. Sci. Food Agric.* **2021**, *102*, 3572–3580. [CrossRef] [PubMed]
65. Liang, C.-X.; Qi, D.-L.; Zhang, L.-N.; Lu, P.; Liu, Z.-D. Preparation and evaluation of a water-in-oil nanoemulsion drug delivery system loaded with salidroside. *Chin. J. Nat. Med.* **2021**, *19*, 231–240. [CrossRef]
66. Shi, J.; Zheng, J.; Liang, B.; Song, F.; Wang, J.; Guo, S.; Ge, H.; Gao, Y.; Zhang, T. Silver-decorated amino-modified Fe₃O₄@SiO₂@mTiO₂ core-shell nanocomposites with catalytic and antimicrobial bifunctional activity. *Colloids Surf. A Physicochem. Eng. Asp.* **2023**, *668*, 131402. [CrossRef]
67. Wang, M.D.; Xie, Y.L.; Li, Q.; Yang, Y.H.; Ma, W.B.; Sun, S.M. Study on the stability of *Artemisia argyi* essential oil Pickering emulsion. *J. Henan Univ. Technol. Nat. Sci. Ed.* **2022**, *43*, 67–72,79.
68. Wu, S. Extending shelf-life of fresh-cut potato with cactus *Opuntia dillenii* polysaccharide-based edible coatings. *Int. J. Biol. Macromol.* **2019**, *130*, 640–644. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

A Semi-Micro Extraction Spectrophotometric Determination of Iron Using 4-Nitrocatechol and Xylometazoline Hydrochloride

Petya V. Racheva ¹, Antoaneta D. Saravanska ¹, Galya K. Toncheva ², Denitsa D. Kiradzhiyska ¹, Nikolina P. Milcheva ¹, Vidka V. Divarova ¹, Ina P. Pencheva ¹, Kirila T. Stojnova ², Vassil B. Delchev ³ and Kiril B. Gavazov ^{1,*}

¹ Department of Chemical Sciences, Faculty of Pharmacy, Medical University of Plovdiv, 120 Buxton Bros Str., 4004 Plovdiv, Bulgaria; petya.racheva@mu-plovdiv.bg (P.V.R.); antoaneta.saravanska@mu-plovdiv.bg (A.D.S.); denitsa.kiradzhiyska@mu-plovdiv.bg (D.D.K.); nikolina.milcheva@mu-plovdiv.bg (N.P.M.); vidka.divarova@mu-plovdiv.bg (V.V.D.); 21301028@mu-plovdiv.bg (I.P.P.)

² Department of General and Inorganic Chemistry with Methodology of Chemistry Education, Faculty of Chemistry, University of Plovdiv 'Paisii Hilendarski', 24 Tsar Assen St., 4000 Plovdiv, Bulgaria; stojnova@uni-plovdiv.bg (K.T.S.)

³ Department of Physical Chemistry, Faculty of Chemistry, University of Plovdiv 'Paisii Hilendarski', 24 Tsar Assen St., 4000 Plovdiv, Bulgaria; vdelchev@uni-plovdiv.net

* Correspondence: kiril.gavazov@mu-plovdiv.bg

Abstract: A chromogenic solvent extraction system for Fe(III) based on 4-nitrocatechol (4NC) and xylometazoline hydrochloride (XMH) was investigated. The optimum conditions for extraction spectrophotometric determination of iron were found. Under these conditions, the formula of the extracted complex was $(XMH^+)_3[Fe(4NC)_3]$ and the apparent molar absorption coefficient at $\lambda_{max} = 464 \text{ nm}$ was $1.9 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ (4-fold enrichment). To validate the aforementioned formula and gather information about the spin state of the central Fe(III) ion, a combined experimental-theoretical approach was employed. This approach entailed the experimental determination of the 4NC:Fe and XMH:Fe molar ratios and the optimization of potential color-bearing structures utilizing the B3LYP/6-311G computational chemistry method. The effect of foreign ions was thoroughly examined, and a sensitive, simple, and inexpensive analytical procedure was proposed, which was successfully applied for determining iron in pharmaceutical and industrial samples.

Keywords: iron(III); spectrophotometric determination; 1,2-dihydroxy-4-nitrobenzene; xylometazoline hydrochloride; liquid-liquid extraction; B3LYP calculations

1. Introduction

Iron is among the most extensively studied elements, with a long history in metallurgy, construction, catalysis, warfare, and numerous applications in everyday life. It is a critical component in a number of essential biological processes, including oxygen transport, electron transfer, and DNA synthesis or repair [1]. Although its homeostasis is tightly regulated, an imbalance—either a deficiency or an overload—can occur [2–5].

Iron can exist in several oxidation states, and a substantial body of research has been conducted on the complexation capacity of iron(II, III) toward catechol-type ligands. The impact of various substituents, including nitro groups, has been investigated with the objective of developing novel effective iron chelators [6,7], antioxidants [8], adsorption surfaces [9], COMT inhibitors [10], reactive species [11], superparamagnetic nanoparticles [12], atmospheric particulate matter [13,14], and complexation agents [15–17].

4-Nitrocatechol (4NC, H_2L) (Figure 1a) occupies a significant position among the catecholic ligands. It is categorized as a non-hazardous substance according to the European Union's Regulation (EC) No. 1272/2008, which classifies substances based on their potential to cause acute or chronic risks to humans and the environment [18]. The presence of a nitro substituent in the aromatic ring of 4NC enhances the acidity of the catechol function, thereby augmenting the complexing power with respect to other ortho-diphenols. This group may increase the ligand field, leading to greater splitting of d-orbitals and its propensity to form low-spin complexes [17]. The employment of 4NC in a variety of applications, including analytical chemistry [19–22], catalysis, and environmental remediation, underscores its versatility and significance. Notably, it serves as a biomarker for breast cancer, a model compound for studying how natural organic matter mobilizes and immobilizes metal ions, and is an important intermediate in the breakdown of nitrogen-containing compounds [22–24]. Despite its classification as one of the most significant organic analytical reagents [19], the application of 4NC in the determination of iron remains unexplored.

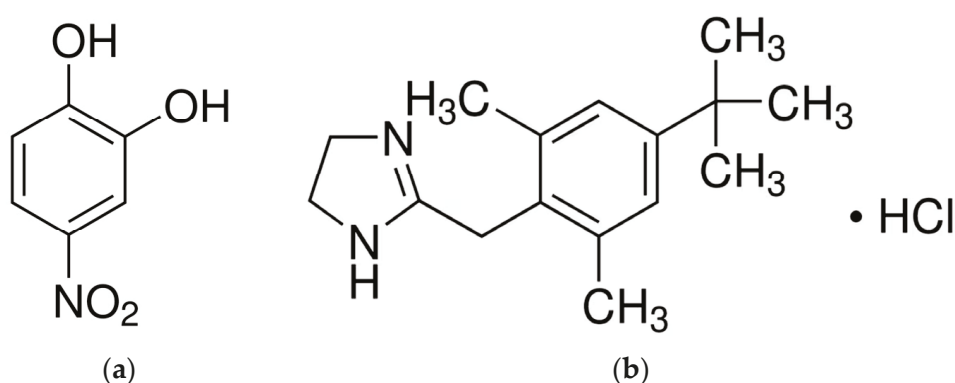


Figure 1. Structural formulas of 4NC (a) and XMH (b).

Nurchi et al. [6] have emphasized the intricate and dynamic nature of the UV-Vis spectra of Fe(III)–4NC species. However, the observed spectral changes have been attributed exclusively to a stepwise complexation process involving the formation of species with formulas FeL^+ , FeL_2^- , and FeL_3^{3-} , where L represents a doubly deprotonated 4NC anion ($L^{2-} = H_2L - 2H^+$) [6,15,25]. Notably, the extant literature contains no reports of protonated Fe(III)–4NC complexes containing HL^- , a phenomenon observed in other ortho-diphenolic ligands [26]. Additionally, the spin state (low spin, high spin, or spin crossover) of the central Fe(III) ion remains undefined, a salient aspect in the context of the findings presented in [8,17,27,28].

The objective of the present study was to examine a liquid-liquid extraction (LLE) system comprising Fe(III), 4NC, xylometazoline hydrochloride (XMH), water, and chloroform. XMH (Figure 1b) has been utilized in a variety of fields and has been incorporated into the WHO Model List of Essential Medicines [29]. Within the domain of analytical chemistry, it has been employed as a colorless cationic ion-association reagent, capable of forming hydrophobic ternary complexes with intensely colored complex anions, as evidenced by several studies [24,30,31]. The present paper encompasses both experimental LLE–spectrophotometric studies and theoretical time-dependent density functional theory (TD DFT) calculations, alongside the simulation of theoretical UV-vis spectra of optimized putative structures and a comparison with experimental spectra. This approach has previously been employed in studies of 4NC complexes of Al(III) [23], V(IV,V) [24], Mo(VI) [20], and W(VI) [21] and has proven fruitful.

2. Results and Discussion

2.1. Extraction-Spectrophotometric Optimization

Figure 2 compares the spectra of the extracted ternary species (1, 2) and the corresponding blanks (1', 2') at two different pH values. It can be concluded that at least two different complexes are formed. One is characterized by the maximum absorption wavelength ($\lambda_{\max}$) = 408 nm. The other one is formed at higher pH values and shows an absorption maximum at 464 nm.

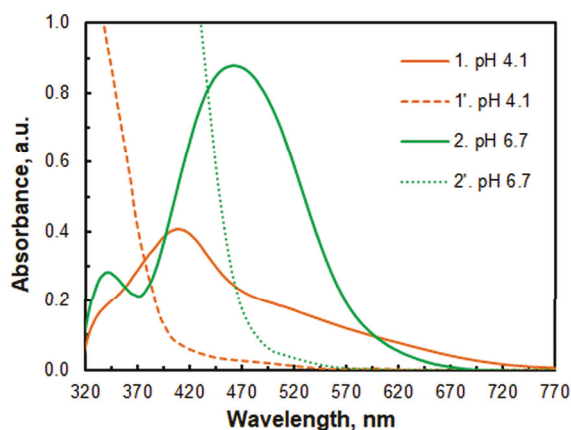


Figure 2. Absorption spectra in chloroform of the ternary species (1, 2) and corresponding blanks (1', 2') at different pH values: $c_{\text{Fe}} = 4.6 \times 10^{-6} \text{ mol dm}^{-3}$, $c_{4\text{NC}} = 2 \times 10^{-4} \text{ mol dm}^{-3}$, $c_{\text{XMH}} = 7.5 \times 10^{-4} \text{ mol dm}^{-3}$, $t_{\text{ex}} = 1 \text{ min}$.

The effect of pH on the absorbance at these two wavelengths is shown in Figure 3. The intersection of the pH curves occurs at a pH close to 4.8, and the maximum absorbance is obtained at a pH of 6.7. A series of buffers prepared from 2 mol dm^{-3} solutions of acetic acid and ammonia were used to maintain pH. The effect of buffer volume (between 0.5 and 3.5 cm^3) was also studied. The results demonstrated that the absorbance remained constant, indicating that the components of the buffer are not involved in complexation. Given the relatively limited buffering capacity at $\text{pH}_{\text{opt}} = 6.7$, the volume of 3 cm^3 was selected for analytical purposes.

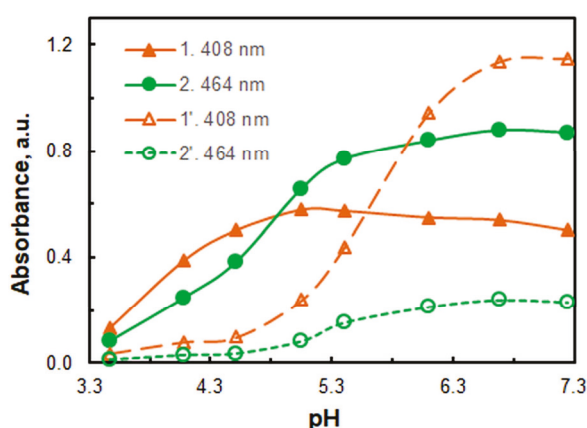


Figure 3. pH curves of the ternary species (1, 2) and corresponding blanks (1', 2') at two different wavelengths. $c_{\text{Fe}} = 4.6 \times 10^{-6} \text{ mol dm}^{-3}$, $c_{4\text{NC}} = 2 \times 10^{-4} \text{ mol dm}^{-3}$, $c_{\text{XMH}} = 7.5 \times 10^{-4} \text{ mol dm}^{-3}$, and $t_{\text{ex}} = 1 \text{ min}$. The pH was maintained using 3 cm^3 of ammonium acetate buffer.

The minimum shaking time required to achieve maximum absorbance was approximately 30–40 s. The resulting absorbance remained constant when the shaking time (t_{ex}) was increased to at least 5 min. The effect of longer extraction times on the process has

not been investigated. To ensure stability of results and maintain operational efficiency, all subsequent experiments were conducted at a t_{ex} of 1 min.

The effect of the concentrations of 4NC and XMH in the aqueous phase on the absorbance is shown in Figure 4. The following concentrations were chosen for further investigation: $c_{4\text{NC}} = 2 \times 10^{-4} \text{ mol dm}^{-3}$ and $c_{\text{XMH}} = 7.5 \times 10^{-4} \text{ mol dm}^{-3}$. The experimental series were conducted with chloroform volumes of 10 cm^3 and 2.5 cm^3 . In alignment with environmental concerns, the well-being of laboratory personnel, sustainability, and the degree of greenness [32], the volume for the chloroform phase was set to 2.5 cm^3 .

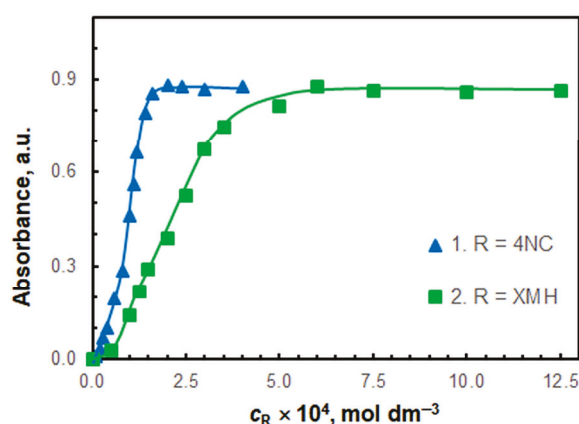


Figure 4. Effect of 4NC (curve 1) and XMH (curve 2) concentrations. $c_{\text{Fe}} = 4.6 \times 10^{-6} \text{ mol dm}^{-3}$, $\text{pH} = 6.7$, $\lambda = 464 \text{ nm}$, $t_{\text{ex}} = 1 \text{ min}$. 1. $c_{\text{XMH}} = 7.5 \times 10^{-4} \text{ mol dm}^{-3}$; and 2. $c_{4\text{NC}} = 2.0 \times 10^{-4} \text{ mol dm}^{-3}$.

Table 1 summarizes all optimized parameters. The optimization intervals and the optimal values found are included.

Table 1. Extraction-spectrophotometric optimization *.

Parameter	Optimization Range	Optimal Value
Wavelength, nm	UV/Vis	464
pH	3.5–7.2	6.7
Volume of the buffer, cm^3	0.5–3.5	3.0
Concentration of 4NC, mol dm^{-3}	$(0.1\text{--}6.0) \times 10^{-4}$	2.0×10^{-4}
Concentration of XMH, mol dm^{-3}	$(0.25\text{--}15) \times 10^{-4}$	7.5×10^{-4}
Shaking time, s	5–300	60
Volume of chloroform, cm^3	2.5 and 10	2.5

* The experiments were conducted at room temperature (22°C).

2.2. Molar Ratios and Suggested Formulas of the Extracted Species

Nurchi et al. [6] and Hakkinen [25] reported that Fe(III) and 4NC form three complexes in aqueous media, which can be represented by the following formulas: $[\text{FeL}]^+$, $[\text{FeL}_2]^-$, and $[\text{FeL}_3]^{3-}$. The 1:3 complex is formed in excess of 4NC at pH levels above 5. This complex is distinguished by a composite band centered at 454 nm in an aqueous medium [6].

The findings of extraction studies in the presence of XMH, processed by the mobile equilibrium method [33] (Figure 5, straight line 1) and the straight-line method of Asmus [34], corroborate the conclusion that the Fe:4NC molar ratio is 1:3. The aforementioned methods yield results indicating that the Fe:XMH molar ratio is also 1:3 when the pH was 6.7 (see Figure 5, straight line 2). Based on these findings, it can be postulated that the formula of the extracted ternary complex at $\text{pH}_{\text{opt}} 6.7$ is $(\text{XMH}^+)_3[\text{Fe}^{\text{III}}\text{L}_3]$. The discrepancy in the absorption maxima of the ternary complex in chloroform (464 nm) and the anionic

chelate in an aqueous medium (454 nm) is negligible. It can be attributed to the influence of both the solvent and the ion-association reagent (XMH).

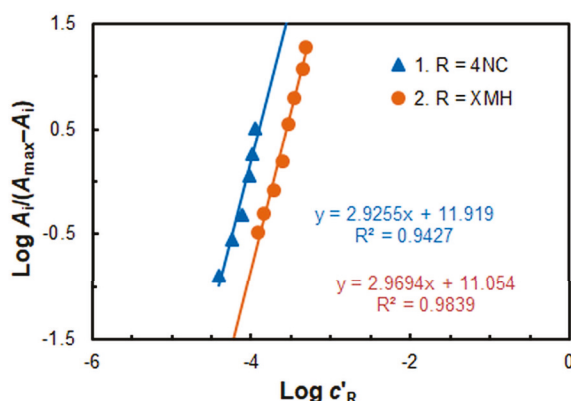


Figure 5. Determination of the 4NC:Fe (1) and XMH:Fe (2) molar ratios by the mobile equilibrium method.

The results obtained at a pH of 5.3 revealed the presence of two distinct ternary complexes, contingent on the concentration of XMH (see Figure 6). At a low XMH concentration, a 1:2 Fe:XMH complex is formed (see Figure 6a), which most likely contains a singly protonated 4NC ligand and can be represented by the formula $(\text{XMH}^+)_2[\text{Fe}(\text{HL})\text{L}_2]$.

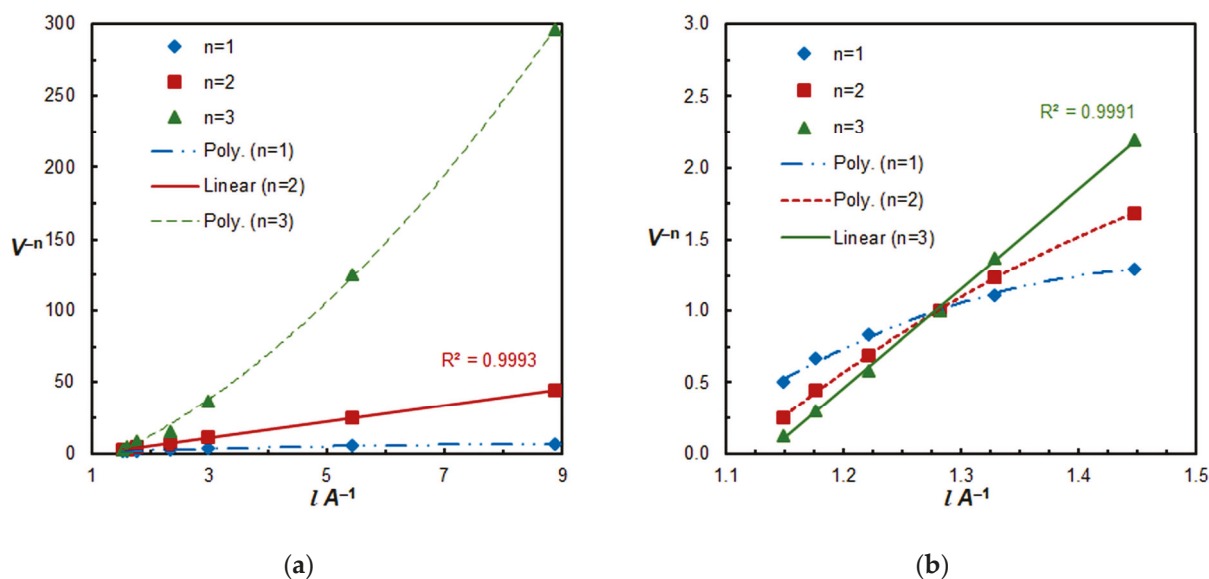


Figure 6. Determination of the XMH:Fe molar ratios by the straight-line method of Asmus. $c_{4\text{NC}} = 4 \times 10^{-4} \text{ mol dm}^{-3}$, pH 5.3 (a) at low XMH concentrations (up to $3.5 \times 10^{-4} \text{ mol dm}^{-3}$), and (b) at higher XMH concentrations.

The Bent–French limited logarithm method [35] (Figure 7) lends further credence to the existence of this complex, as it yields reliable results only at low ligand concentrations.

2.3. Ground-State Equilibrium Geometries of Putative Anionic Structures

The spectral bands observed in the visible region of typical ion-association complexes containing colorless cations (e.g., XMH^+) are attributed to the anionic part of the complex. This peculiarity has been utilized to elucidate the stoichiometry and geometry of 4NC complexes of vanadium [24], molybdenum [20], and tungsten [21] by comparing theoretical spectra of the anionic moiety with experimental spectra of the extracted ternary species.

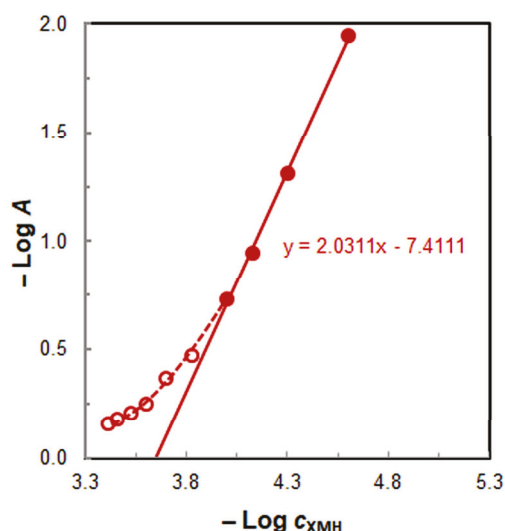


Figure 7. Determination of XMH:Fe molar ratio by the Bent–French limited logarithm method.

In this regard, models of the anionic constituents of some putative structures that are consistent with the experimentally determined mole ratios were constructed and optimized at the theoretical B3LYP/6-311G level (Gaussian 03 software). The calculations were conducted under the assumption that the complex anions, $[\text{Fe}(\text{HL})\text{L}_2]^{2-}$ and $[\text{FeL}_3]^{3-}$, would adopt either a low-spin (multiplicity = 2) or a high-spin (multiplicity = 6) configuration. The optimization results are presented in Figure 8.

As can be observed, the bond lengths in the coordination polyhedra of the low-spin structures (**a** and **c**) are shorter, and the polyhedra themselves (tetragonal pyramid for the protonated complexes and octahedron for the deprotonated complexes) are more regular. For instance, the O–Fe–O angle involving the axial oxygen atoms in the octahedral structures is more closely aligned with 180° in the low-spin configuration (175.50° vs. 170.17°). Furthermore, the displacement of the fourth oxygen atom from the plane of the square base of the pyramid in the protonated anion $[\text{Fe}(\text{HL})\text{L}_2]^{2-}$ is less for the low-spin configuration (dihedral angles close to 20° vs. dihedral angles close to 29°).

It is noteworthy that the benzene nuclei of 4NC maintain their aromatic character in the complexes. This can be determined by the observation that the distances between carbon atoms in these nuclei remain comparable to those reported by Cornard et al. [16] for unbound H_2L and to those in the stable complexes of 4NC with Mo [20] and V [24]. Consequently, the formation of typical quinoidal structures is not observed.

2.4. Simulated Spectra and Spectral Comparison

The optimized structures depicted in Figure 8 were employed to calculate vertical excitation energies with the time-dependent Hamiltonian, with the objective of simulating theoretical absorption spectra. The calculated spectral line locations of the four modeled structures are presented in Table 2. As demonstrated in Figure 9, the application of a scaling coefficient of 0.79 leads to a satisfactory alignment between the experimental spectrum under optimal extraction conditions (Exp.) and the theoretical spectrum of the low-spin structure 3 (Str. 3). Conversely, the spectrum of the high-spin structure 4 (Str. 4) does not align with the experimental spectrum under any scaling conditions. Consequently, it can be deduced that the low-spin electron configuration (single d electron, multiplicity = 2) is realized in the complexation under the optimum conditions.

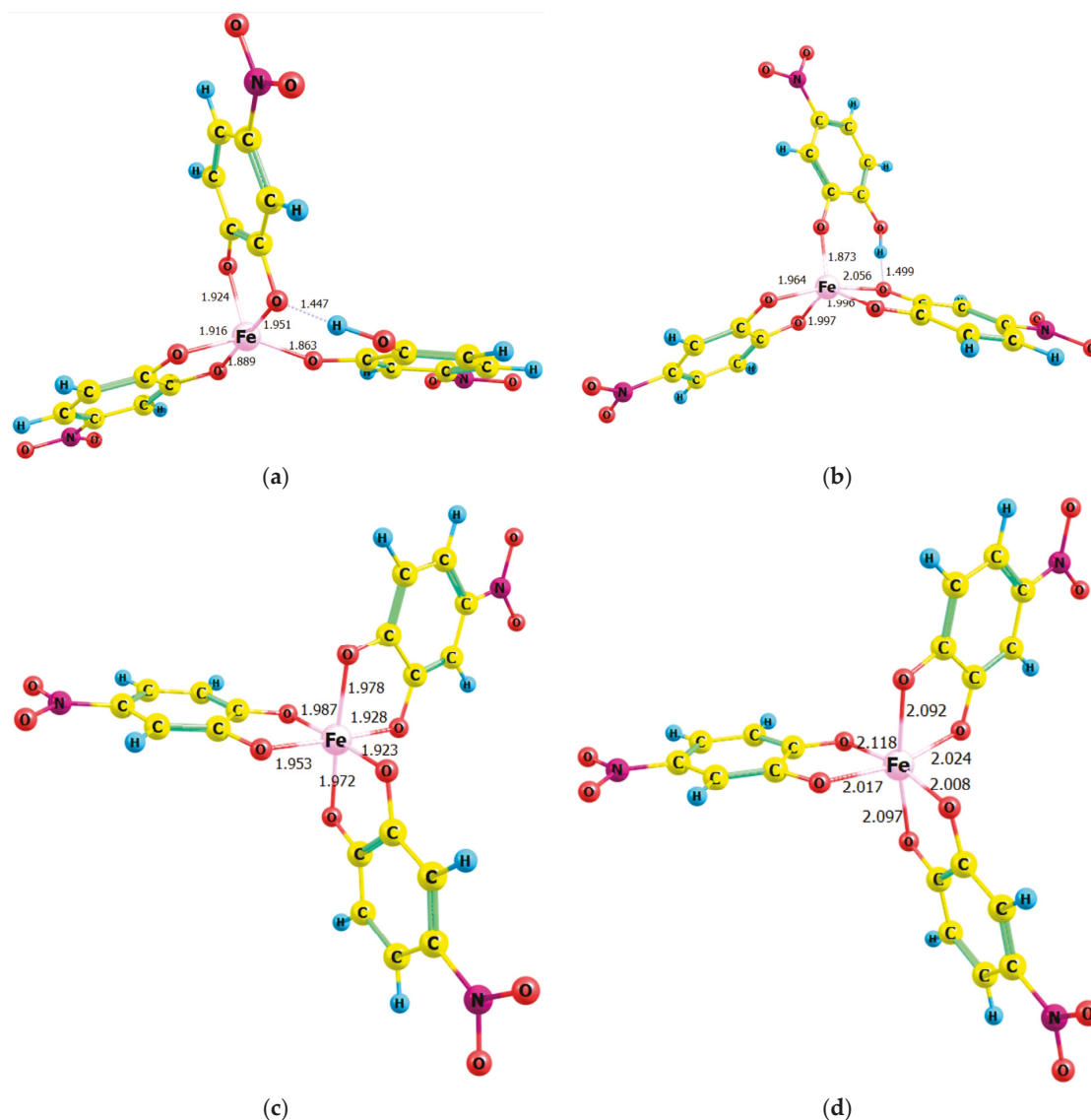


Figure 8. Optimized ground-state equilibrium geometries of some putative structures found at the B3LYP/6-311G level of theory. (a) Structure 1: $[\text{Fe}(\text{HL})\text{L}_2]^{2-}$; charge = -2 , multiplicity = 2. (b) Structure 2: $[\text{Fe}(\text{HL})\text{L}_2]^{2-}$; charge = -2 , multiplicity = 6. (c) Structure 3: $[\text{FeL}_3]^{3-}$; charge = -3 , multiplicity = 2. (d) Structure 4: $[\text{FeL}_3]^{3-}$; charge = -3 , multiplicity = 6.

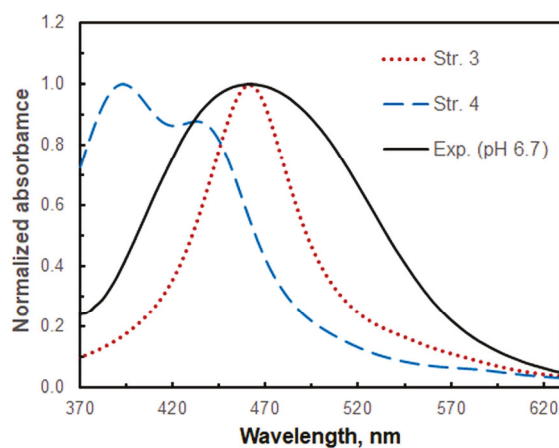


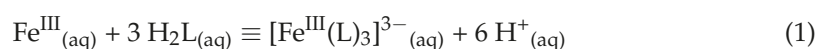
Figure 9. Comparison of the experimental spectrum at the optimum conditions (Exp.) and the theoretical spectra of the low-spin structure 3 and the high-spin structure 4. A scaling coefficient of 0.79 and a Lorentzian broadening were used for the theoretical spectra.

Table 2. Calculated wavelengths of the anionic structures (Str. 1–4). The f denotes the oscillator strength.

Str. 1 (Low-Spin)		Str. 2 (High-Spin)		Str. 3 (Low-Spin)		Str. 4 (High-Spin)	
λ , nm	f	λ , nm	f	λ , nm	f	λ , nm	f
1027	0.0015	740	0.0043	1325	0	738	0.0331
885	0.0003	690	0.0005	1190	0	695	0.0193
875	0.0005	659	0.0001	950	0	672	0.0126
861	0.0001	643	0.0063	771	0	653	0.0219
819	0.0035	615	0.0022	731	0.0076	639	0.0597
810	0.0001	591	0.0015	718	0	630	0.0049
679	0.0007	576	0.0011	695	0.0006	629	0.0007
657	0.0035	567	0.0013	690	0.0014	627	0.0027
644	0.0201	565	0.1092	690	0.0116	626	0.0283
606	0.0028	549	0.0758	672	0.0014	620	0.0232
603	0.0134	543	0.0213	660	0.0006	620	0.0207
596	0.0065	525	0.0008	655	0.0014	618	0.0144
575	0.0494	512	0.0145	655	0.0014	615	0.0023
571	0.0012	510	0.0059	649	0.001	598	0.0372
563	0.0282	508	0.0072	644	0.0015	595	0.0412
555	0.008	504	0.0691	636	0.0013	572	0.0081
548	0.0527	497	0.0192	624	0.0059	569	0.0333
524	0.0515	491	0.0351	590	0.1689	552	0.1013
517	0.0173	489	0.0615	581	0.1269	542	0.0453
514	0.0437	478	0.0473	578	0.0288	536	0.0071
509	0.0017	477	0.0072	564	0.038	522	0.0097
505	0.0184	471	0.0055	509	0.0005	514	0.0209
503	0.0019	464	0.0019	505	0.0028	507	0.0262
481	0.0036	450	0.012	495	0.0009	498	0.0006
		446	0.0004			491	0.0009
		445	0.0018			489	0.0015
		444	0.0036			485	0.0005

2.5. Extraction Characteristics

The findings from both experimental and theoretical studies suggest that under optimal conditions, the complex formation and the subsequent extraction of the ternary complex can be described by the following equations:



The extraction constant was calculated by two different methods based on an XMH saturation curve at the optimal 4NC concentration and pH. The methods employed were (1) the Holme–Langmyhr method [36] and (2) the mobile equilibrium method [33]. As shown in Table 3, the values obtained are statistically indistinguishable. Additionally, Table 3 presents the values of the distribution coefficient (D) and the fraction extracted (% E) determined spectrophotometrically by a known procedure [37].

Table 3. Extraction characteristics.

Characteristic	Value
Extraction constant ($\log K_{ex}$)	11.14 ± 0.07^a ($n = 4$); 11.2 ± 0.4^b ($n = 8$)
Distribution ratio ($\log D$)	1.38 ± 0.23 ($n = 4$)
Fraction extracted (%E)	95.7 ± 1.7 ($n = 4$)

^a Holme–Langmyhr method; ^b Mobile equilibrium method.

2.6. Calibration Graph and Analytical Characteristics

The relationship between the measured absorbance and iron(III) mass concentration was found to be linear up to $0.27 \mu\text{g cm}^{-3}$ Fe(III) ($R^2 = 0.998$). The study design involved a 4-fold enrichment, with the volume of the aqueous phase being 10 cm^3 and that of the organic phase being 2.5 cm^3 . The linear regression equation was $A = 3.40\gamma + 0.005$, where γ is the concentration in $\mu\text{g cm}^{-3}$. The standard deviation of the slope was determined to be 0.005, while that of the intercept was 0.006, indicating that the intercept is statistically indistinguishable from zero. The apparent molar absorptivity coefficient was found to be $1.90 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, and the Sandall's sensitivity was $2.94 \times 10^{-4} \mu\text{g cm}^{-2}$. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated as 3 and 10 times the standard deviation of the blank ($n = 10$) divided by the slope, respectively, yielding values of 5.3 ng cm^{-3} and 18 ng cm^{-3} .

The calculated molar absorptivity coefficient at equal volumes of the two phases (10 cm^3) was approximately four times smaller: $4.8 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$.

2.7. The Effect of Foreign Ions

As demonstrated in Table 4, the presence of substantial quantities of common anions, such as Cl^- , SO_4^{2-} , NO_3^- , and F^- , is permissible without imposing constraints on the method of sample dissolution. Alkali and alkaline earth cations, as well as Zn(II) and Mn(II), also do not induce adverse effects when present in high concentrations. The most substantial interferences are caused by Al(III), which enhances the absorption significantly. Other ions capable of forming stable complexes with 4NC and thereby interfering with the analysis include Mo(VI), Ni(II), V(V), and W(VI).

Table 4. Impact of foreign ions on the determination of $1.3 \mu\text{g Fe(III)}$ ^a.

Foreign Ion (FI)	Formula of the Added Salt	Amount of FI Added/mg	FI:Fe(III) Mass Ratio	Amount of Fe Found/ μg	E%
Al(III)	$\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$	1.3×10^{-3}	1	2.39	184
Ba(II)	BaCl_2	2.6	2000 ^b	1.24	95.2
Ca(II)	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	1.3	1000 ^b	1.29	99.0
Cd(II)	$\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$	0.13	100	1.37	105
Cl^-	NaCl	13	10,000 ^b	1.28	98.2
Co(II)	$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	0.65	500	1.28	98.6
Cr(III)	$\text{Cr}_2(\text{SO}_4)_3$	0.325	250	1.35	104
Cr(VI)	K_2CrO_4	1.3×10^{-2}	10	1.33	102
Cu(II)	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	6.5×10^{-3}	5	1.29	99.5
F^-	NaF	13	10,000 ^b	1.31	101
Hg(I)	$\text{Hg}_2(\text{NO}_3)_2$	0.325	250	1.25	96.4
Hg(II)	$\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$	0.325	250	1.22	94.2
HPO_4^{2-}	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.13	100	1.30	100
I^-	KI	0.13	100	1.22	93.7
Mg(II)	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	13	10,000 ^b	1.28	98.6

Table 4. Cont.

Foreign Ion (FI)	Formula of the Added Salt	Amount of FI Added/mg	FI:Fe(III) Mass Ratio	Amount of Fe Found/ μ g	E%
Mn(II)	MnSO ₄ ·H ₂ O	1.3	1000	1.29	99.4
Mo(VI)	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	1.3×10^{-3}	1	1.42	109
Ni(II)	NiSO ₄ ·7H ₂ O	1.3×10^{-3}	1	1.40	108
NO ₃ [−]	NaNO ₃	13	10,000 ^b	1.24	95.3
Pb(II)	Pb(NO ₃) ₂	0.65	500	1.26	97.0
ReO ₄ [−]	NH ₄ ReO ₄	1.3	1000	1.33	102
SO ₄ ^{2−}	K ₂ SO ₄	13	10,000 ^b	1.31	101
Tartrate	K ₂ NaC ₄ H ₄ O ₆	1.3	1000	1.37	105
V(V)	NH ₄ VO ₃	1.3×10^{-3}	1	1.25	96.5
W(VI)	Na ₂ WO ₄	1.3×10^{-3}	1	1.38	106
Zn(II)	ZnSO ₄ ·7H ₂ O	2.6	2000 ^b	1.25	96.3

^a The volume ratio ($V_{aq}/V_{chloroform}$) was 10/2.5 = 4. ^b Higher FI-to-Fe(III) mass ratios were not studied.

2.8. Analytical Application

The procedure was applied to determine the iron content in Maltofer[®] Fol tablets, which are marketed as containing 100 mg of iron(III) per tablet. The result, 103 ± 4 (mean $\pm$ standard deviation, four replicate analyses), was statistically identical to this value. To assess the inter-day reproducibility, eight replicate analyses were executed on the subsequent two days (four analyses per each day). The results from the three-day period demonstrated adequate reproducibility, with an average value of 102 mg and a relative standard deviation (RSD) of 4.6%. Subsequently, the range of analyses was expanded through the processing of industrial samples (Wälz oxides and calcium carbonate) supplied by a metallurgical plant engaged in the production of zinc and other metals. The results are presented in Table 5.

Table 5. Determination of iron in industrial samples.

#	Sample	Mass Fraction of Iron (w_{Fe}) ^a , %	Other Ingredients ^a	w_{Fe} Determined ^{b,c} , %	RSD, %
1	Wälz oxides	2.55	62.5% Zn, 9.25% Pb, 0.6% Cd, 0.25% Cl, 0.013% F, 0.04% As, 160 μ g g ^{−1} Sb, 16 μ g g ^{−1} Te, 10 μ g g ^{−1} Ge, 10 μ g g ^{−1} Se	2.51 ± 0.11	4.4
2	CaCO ₃	0.095	38.75% Ca, 0.13% Mg, 0.9% SiO ₂	0.098 ± 0.004	4.1
3	CaCO ₃	0.040	39.1% Ca, 0.12% Mg, 0.3% SiO ₂	0.039 ± 0.002	5.1

^a Determined in an alternate laboratory. ^b Mean $\pm$ standard deviation. ^c Four replicate determinations.

2.9. Comparison with Existing Methods

A comparative analysis of the present method with other solvent extraction spectrophotometric methods for the determination of Fe(II,III) ions is provided in Table 6. The present method demonstrates sensitivity, as evidenced by the calculated molar absorptivity (1.9×10^5 dm³ mol^{−1} cm^{−1}) and LOD (5.3 ng cm^{−3}). The method is reliable and robust, as the optimal intervals for the examined parameters are sufficiently broad. The reagents employed are commercially available and cost-effective. Therefore, in contrast to the extant literature [38–43], the synthesis of reagents is not required. The equipment is simple, and all operations can be considered routine, thus eliminating the need for highly trained personnel.

The time required to complete the analysis is shorter than that reported for other methods [39,44–48]. The prolonged duration observed in these methods can be attributed to various factors, including extended equilibration times [44,47], a challenging phase

separation process [44,49], the necessity of drying the organic layer [4,45,46], or the inclusion of labor-intensive steps following extraction, which are essential for the formation of the colored complex [39,50].

Table 6. A comparison of different methods for determining Fe ions using solvent extraction and spectrophotometry.

Reagent(s)	Technique	Organic Solvent	pH	Working Range, $\mu\text{g cm}^{-3}$	λ , nm	$10^{-4}\epsilon$, $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$	Sample	Ref.
DBA	LLE	n-Butanol	8.2	–	430	0.5	Commercial mixtures, pharmaceutical samples and alloys	[51]
DHN	LLE	Ethyl acetate	5–8	0.05–10	485	1.1	Silicate rock samples	[52]
DHN + CTAB	LLE	Ethyl acetate	8–10	0.04–5.0	455	1.2	Geological samples, concentrates, tobacco leaves, cigarettes, and waters	[26]
DM + HAs	LLE	Chloroform	5.3–7.2	0.03–4.2	552–583	3.08–4.33	Pharmaceutical, biological, and food samples	[47]
DPCP	LLE	Chloroform	3.5–4	0.5–10	525	0.1156	Pharmaceutical samples	[38]
DPTP + KSCN	LLE	Chloroform, acetone	6.0	–	480	–	Steel sample	[39]
GA + CTAB	DLLME	C_2Cl_4 + acetone	5.0	0.05–0.65	560	–	Water and food samples	[53]
HHTPs + HAs	LLE	Chloroform	3.5–6.0	0.40–22.5	545–595	3.0–4.5	Oil and oil products	[48]
KSCN + CTAB (TPPC)	LLE	Chloroform	$0.2 \text{ mol dm}^{-3} \text{H}_2\text{SO}_4$	0.056–2.24	473 (506)	3.55	–	[44]
KSCN + CTAB	LLE	Ethyl acetate	3–4	Up to 6.0	474	3.2	Geological and hydrogeochemical samples	[45]
KSCN + DMF	LLE	Benzene	$5 \text{ mol dm}^{-3} \text{H}_2\text{SO}_4$	0.05–12	490	7.2	Model and industrial solutions	[54]
MPDDO	LLE	Dichloromethane	2.0	1–10	235	0.303	Synthetic mixtures	[46]
NH_4SCN + N-OOA	LPME-NDS	Chloroform	Strong acidic solution	0.05–6.0	477	18.0	Food, biological, and environmental samples	[4]
NSTS	LLE	Isoamyl alcohol	4.0–6.0	1–7	435	2.88	–	[55]
PHEN + AA	EIEB	–	4.5	0.050–2.0	510	–	Diesel oil	[49]
TBP + DB18C6, NH_4SCN	LLE	Kerosene	$0.01 \text{ mol dm}^{-3} \text{CH}_3\text{COONa}$	–	475	–	Soap samples	[50]
4NC + XMH	LLE	Chloroform	6.7	0.018–0.27	464	19.0	Pharmaceutical and industrial samples	This work

Abbreviations: 4NC, 4-nitrocatechol; AA, ascorbic acid; CTAB, cetyltrimethylammonium bromide; DB18C6, dibenzo-18-crown-6; DBA, 2, 4-dimethyl-3H-1,5-benzodiazepine; DLLME, dispersive liquid-liquid microextraction; DM, dimercaptophenole; DMF, dimethylformamide; DPCP, 1,3-diphenyl-4-carboethoxy pyrazole-5-one; DPTP, 1,5-dimethyl-2-phenyl-4[(E)-(2,3,4-trihydroxy phenyl)diazonyl]-1,2-dihydro-3H-pyrazole-3-one; DHN, 2,3-dihydroxynaphthalene; EIEB, extraction induced by emulsion breaking; GA, gallic acid; HAs, hydrophobic amines; HHTPs, hydroxyhalogenthiofenols; LLE, liquid-liquid extraction; LPME-NDS, liquid-phase microextraction nanodrop spectrophotometry; MPDDO, 4-methyl-2,3-pentanedione dioxime; N-OOA, N-octylacetamide; NSTS, 5-nitrosalicylaldehyde thiosemicarbazone; PHEN, 1,10-phenanthroline; TBP, tributyl phosphate; TPPC, tetraphenylphosphonium chloride; XMH, xylometazoline hydrochloride.

3. Materials and Methods

3.1. Chemicals and Instruments

An iron(III) stock solution was prepared from ammonium iron(III) sulfate dodecahydrate (Merck, ACS reagent, 99%, Schnellendorf, Germany) in accordance with the procedures

outlined in reference [26,56]. The working solutions were obtained by suitable dilution of the stock solution with water.

A 2×10^{-3} mol dm $^{-3}$ aqueous solution of 4NC (>98%, Fluka AG, Buchs, Switzerland) was prepared by dissolving 0.9695 g of the reagent in a 250 cm 3 calibrated flask.

A solution of XMH at a concentration of 5×10^{-3} mol dm $^{-3}$ (250 cm 3) was prepared by thoroughly dissolving 0.3511 g of the reagent ($\geq 99\%$, Merck, Schnellendorf, Germany) in water. Following preparation, the solution was stored in a dark glass vessel [30].

The acidity of the aqueous medium was established through the use of a buffer solution, which was prepared by combining 2 mol dm $^{-3}$ aqueous solutions of CH $_3$ COOH and NH $_4$ OH. The pH was subsequently verified using an InoLab 720 pH meter (WTW, Weilheim, Germany). Distilled water was used during the experimental process, and chloroform was used repeatedly after pre-distillation. A bottle-top dispenser (Ceramus Classic, Hirschmann, Germany) was used to introduce chloroform into the separating funnel.

Two UV/Vis scanning spectrophotometers were employed to measure the absorbances of the samples: an Ultrospec 3300 pro (Little Chalfont, UK) and a Drawell DU-8800RS (Chongqing, China). Both instruments were equipped with 10 mm macro- and semi-micro cuvettes.

3.2. Samples and Sample Preparation

Maltofer[®] Fol (Vifor Pharma, France) chewable tablets, formulated for the treatment and prevention of iron deficiency, were procured from a local pharmacy. According to the product leaflet, each tablet contains 100 mg of iron(III). The tablets were prepared for analysis by following the procedure outlined in [57,58], which involves heating in a mixture of concentrated nitric acid (10 cm 3) and concentrated sulfuric acid (1 cm 3) on a sand bath.

Industrial samples (Wälz oxides and calcium carbonate) were supplied in powder form by KCM SA, a non-ferrous metallurgical plant located near Plovdiv, Bulgaria. Two samples of calcium carbonate (0.5 g) of different provenance were dissolved in 10 cm 3 sulfuric acid ($w = 10\%$) at gentle heating. The resulting solutions were filtered and transferred to 100 cm 3 flasks. A few drops of 0.02 mol dm $^{-3}$ KMnO $_4$ were added before the solutions were made up to the mark with water, resulting in a stable violet color for a few minutes. Wälz oxides (0.1 g) were dissolved using hydrochloric acid (10 cm 3 , $w = 15\%$) and hydrogen peroxide (1 cm 3 , $w = 30\%$). The latter was added dropwise during the heating process. After filtration, the solution was collected in a 100 cm 3 flask.

3.3. Optimization Procedure

Solutions of Fe(III), 4NC, XMH, and buffer were combined in a separatory funnel, and the total volume was adjusted to 10 cm 3 with water. Then, chloroform (2.5 cm 3) was added, and the mixture was shaken for extraction. A portion of the organic phase was poured into the cuvette, and the absorbance was measured against a similarly prepared blank.

3.4. Determination of the Extraction Characteristics

The constant of extraction (K_{ex}) was calculated by two methods (the Holme–Langmyhr method and the mobile equilibrium method) based on different points from the saturation curve with XMH at the optimal pH and c_{4NC} . The volume of the two phases was set equal to 10 cm 3 . All extraction experiments were conducted at room temperature.

The distribution ratio (D) was determined using the formula $D = A_1 / (A_3 - A_1)$, where A_1 is the measured absorbance after a single extraction and A_3 is the absorbance of the pooled extracts obtained after triple extraction [21,37]. The fraction extracted (E) was then found by the equation $\%E = 100 \times D / (D + 1)$.

3.5. Procedure for the Determination of Iron(III)

An aliquot of the analyzed solution containing 0.2–2.7 µg Fe(III) was placed in a separating funnel, to which 1 cm³ of a 2×10^{-3} mol dm⁻³ 4NC solution, 1 cm³ of a 7.5×10^{-3} mol dm⁻³ XMH solution, and 3 cm³ of a buffer solution (pH 6.7) were added. The volume of the aqueous phase was then adjusted to 10 cm³, 2.5 cm³ of chloroform was dispensed, and the mixture was shaken for one minute. Following phase separation, a portion of the chloroform layer was poured into a semi-micro cuvette (0.7 cm³ volume), and the absorbance was measured at 464 nm against a blank sample. The Fe(III) concentration was calculated using a calibration line.

3.6. Theoretical Calculations

The ground state geometries of the color-bearing anion components of the ternary complexes were optimized at the B3LYP level of theory, utilizing 6-311G basis functions. The spin multiplicities of the two- and three-charge complexes were set to 2 or 6, indicating that 1 or 5 single electrons of Fe(III) were distributed among the split 3d atomic orbitals. To ascertain the absence of imaginary frequencies in the optimized structures, frequency calculations were conducted, thereby confirming their stability as minima. Furthermore, vertical excitation energies were computed to simulate UV-Vis spectra. The calculations were executed using the Gaussian 03 software [59], and the ChemCraft graphical program [60], version 1.8, was employed for visualization of the optimized low- and high-spin structures.

4. Conclusions

An LLE system for Fe(III) was investigated through a combined experimental and theoretical approach, and the optimal conditions for a rapid, simple, sensitive, and robust semi-micro extraction-spectrophotometric determination of Fe(III) were found. The basis of the developed procedure is an intensely colored low-spin complex, which can be represented by the formula (XMH⁺)₃[Fe^{III}(4NC)₃].

Author Contributions: Conceptualization, K.B.G., P.V.R. and G.K.T.; methodology, K.B.G., P.V.R. and V.B.D.; software, V.B.D. and K.B.G.; validation, K.B.G., P.V.R. and A.D.S.; formal analysis, K.B.G., P.V.R., A.D.S., V.V.D. and K.T.S.; investigation, P.V.R., G.K.T., A.D.S., D.D.K., I.P.P., K.T.S., V.V.D., V.B.D. and N.P.M.; resources, K.B.G., V.B.D. and N.P.M.; writing—original draft preparation, K.B.G.; writing—review and editing, K.B.G., D.D.K. and N.P.M.; visualization, A.D.S., I.P.P., P.V.R., N.P.M. and D.D.K.; supervision, K.B.G.; project administration, K.B.G. and V.B.D.; funding acquisition, K.B.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Medical University of Plovdiv, grant number DPDP-4/2024. The theoretical research was conducted using the infrastructure purchased under the National Roadmap for Research and Innovation, which was financially coordinated by the Ministry of Education and Science of the Republic of Bulgaria (grant number D01-325/01.12.2023).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Galy, B.; Conrad, M.; Muckenthaler, M. Mechanisms controlling cellular and systemic iron homeostasis. *Nat. Rev. Mol. Cell Biol.* **2024**, *25*, 133–155. [CrossRef]
2. Anderson, G.J.; Frazer, D.M. Current understanding of iron homeostasis. *Am. J. Clin. Nutr.* **2017**, *106*, 1559S–1566S. [CrossRef]

3. Mobarra, N.; Shanaki, M.; Ehteram, H.; Nasiri, H.; Sahmani, M.; Saeidi, M.; Goudarzi, M.; Pourkarim, H.; Azad, M. A review on iron chelators in treatment of iron overload syndromes. *Int. J. Hematol.-Oncol. Stem Cell Res.* **2016**, *10*, 239–247.
4. Verma, C.; Tapadia, K.; Soni, A.B. Determination of iron(III) in food, biological and environmental samples. *Food Chem.* **2017**, *221*, 1415–1420. [CrossRef] [PubMed]
5. Alian, E.; Semnani, A.; Firooz, A.; Shirani, M.; Azmoon, B. Application of response surface methodology and genetic algorithm for optimization and determination of iron in food samples by dispersive liquid–liquid microextraction coupled UV–visible spectrophotometry. *Arab. J. Sci. Eng.* **2018**, *43*, 229–240. [CrossRef]
6. V Nurchi, V.M.; Pivetta, T.; Lachowicz, J.I.; Crisponi, G. Effect of substituents on complex stability aimed at designing new iron(III) and aluminum(III) chelators. *J. Inorg. Biochem.* **2009**, *103*, 227–236. [CrossRef]
7. Crisponi, G.; Remelli, M. Iron chelating agents for the treatment of iron overload. *Coord. Chem. Rev.* **2008**, *252*, 1225–1240. [CrossRef]
8. Kawabata, T.; Schepkin, V.; Haramaki, N.; Phadke, R.S.; Packer, L. Iron coordination by catechol derivative antioxidants. *Biochem. Pharmacol.* **1996**, *51*, 1569–1577. [CrossRef]
9. Vasudevan, D.; Stone, A.T. Adsorption of 4-nitrocatechol, 4-nitro-2-aminophenol, and 4-nitro-1,2-phenylenediamine at the metal (hydr)oxide/water interface: Effect of metal (hydr)oxide properties. *J. Colloid Interface Sci.* **1998**, *202*, 1–19. [CrossRef]
10. Orama, M.; Tilus, P.; Taskinen, J.; Lotta, T. Iron(III)-chelating properties of the novel catechol O-methyltransferase inhibitor entacapone in aqueous solution. *J. Pharm. Sci.* **1997**, *86*, 827–831. [CrossRef] [PubMed]
11. Lyu, Y.; Wu, H.; Pang, X.; Wang, J.; Zhao, M.; Chen, J.; Qin, K. The complexation of atmospheric Brown carbon surrogates on the generation of hydroxyl radical from transition metals in simulated lung fluid. *Environ. Int.* **2023**, *180*, 108240. [CrossRef] [PubMed]
12. Amstad, E.; Isa, L.; Reimhult, E. Nitrocatechol dispersants to tailor superparamagnetic Fe₃O₄ nanoparticles. *Chimia* **2010**, *64*, 826. [CrossRef] [PubMed]
13. Chin, H.; Hopstock, K.S.; Fleming, L.T.; Nizkorodov, S.A.; Al-Abadleh, H.A. Effect of aromatic ring substituents on the ability of catechol to produce brown carbon in iron(III)-catalyzed reactions. *Environ. Sci. Atmos.* **2021**, *1*, 64–78. [CrossRef]
14. Zhu, Z.; Zhang, J.; Lv, G.; George, C.; Herrmann, H.; Fu, H.; Li, D.; Zhang, L.; Sun, X.; Sun, H.; et al. Complexation of Fe(III)/catechols in atmospheric aqueous phase and the consequent cytotoxicity assessment in human bronchial epithelial cells (BEAS-2B). *Ecotoxicol. Environ. Saf.* **2020**, *202*, 110898. [CrossRef] [PubMed]
15. Luther III, G.W.; Rozan, T.F.; Witter, A.; Lewis, B. Metal–organic complexation in the marine environment. *Geochem. Trans.* **2001**, *2*, 65. [CrossRef]
16. Cornard, J.-P.; Rasmiwetti, Merlin, J.-C. Molecular structure and spectroscopic properties of 4-nitrocatechol at different pH: UV–visible, Raman, DFT and TD-DFT calculations. *Chem. Phys.* **2005**, *309*, 239–249. [CrossRef]
17. Gavazov, K.B.; Delchev, V.B.; Toncheva, G.K.; Georgieva, Z.G. Extraction-spectrophotometric and theoretical (Hartree-Fock) investigations of a ternary complex of iron(II) with 4-nitrocatechol and 2,3,5-triphenyl-2H-tetrazolium. *Russ. J. Gen. Chem.* **2015**, *85*, 1945–1951. [CrossRef]
18. Sigma-Aldrich Safety Data Sheet. 4-Nitrocatechol. Available online: <https://www.sigmaaldrich.com/BG/en/sds/aldrich/n15553?userType=anonymous> (accessed on 22 January 2025).
19. Sommer, L.; Ackermann, G.; Burns, D.T.; Savvin, S.B. Present and future status of organic analytical reagents-Part I: General remarks. *Pure Appl. Chem.* **1990**, *62*, 2147–2166. [CrossRef]
20. Divarova, V.V.; Saravanska, A.D.; Toncheva, G.K.; Milcheva, N.; Delchev, V.B.; Gavazov, K.B. Spectrophotometric determination of molybdenum(VI) as a ternary complex with 4-nitrocatechol and benzalkonium chloride. *Molecules* **2022**, *27*, 1217. [CrossRef]
21. Divarova, V.V.; Stojnova, K.T.; Radkovska, I.D.; Saravanska, A.D.; Toncheva, G.K.; Delchev, V.B.; Gavazov, K.B. Extraction system for the spectrophotometric determination of tungsten(VI) with 4-nitrocatechol and benzalkonium chloride. *Acta Chim. Slov.* **2024**, *71*, 519–527. [CrossRef]
22. Gavazov, K.B. Nitroderivatives of catechol: From synthesis to application. *Acta Chim. Slov.* **2012**, *59*, 1–17.
23. Cornard, J.-P.; Lapouge, C.; Merlin, J.-C. A DFT/TDDFT study of the structural and spectroscopic properties of Al(III) complexes with 4-nitrocatechol in acidic aqueous solution. *Chem. Phys.* **2007**, *340*, 273–282. [CrossRef]
24. Saravanska, A.D.; Racheva, P.V.; Divarova, V.V.; Toncheva, G.K.; Milcheva, N.P.; Delchev, V.B.; Gavazov, K.B. Extraction-spectrophotometric and theoretical studies on a ternary complex obtained from vanadium(V) and 4-nitrocatechol. *Russ. J. Inorg. Chem.* **2021**, *66*, 1880–1886. [CrossRef]
25. Häkkinen, P. Studies on the formation of manganese(II), iron(III), cobalt(II), and nickel(II) complexes of 1, 2 dihydroxy-4-nitrobenzene in aqueous solution. *Finn. Chem. Lett.* **1984**, *3*, 59–62.
26. Tarafder, P.K.; Mondal, R.K. Micelle mediated extraction of iron and its determination in geological, geochemical, hydrogeochemical, biogeochemical and process solutions. *Am. J. Anal. Chem.* **2012**, *3*, 339–346. [CrossRef]
27. Bowman, D.N.; Jakubikova, E. Low-spin versus high-spin ground state in pseudo-octahedral iron complexes. *Inorg. Chem.* **2012**, *51*, 6011–6019. [CrossRef] [PubMed]

28. Tichnell, C.R.; Shultz, D.A.; Popescu, C.V.; Sokirniy, I.; Boyle, P.D. Synthesis, characterization, and photophysical studies of an iron(III) catecholate–nitronylnitroxide spin-crossover complex. *Inorg. Chem.* **2015**, *54*, 4466–4474. [CrossRef] [PubMed]
29. WHO. Model List of Essential Medicines, 23th List. 2023. Available online: <https://iris.who.int/bitstream/handle/10665/371090/WHO-MHP-HPS-EML-2023.02-eng.pdf?sequence=1> (accessed on 22 January 2025).
30. Hristov, D.; Milcheva, N.; Gavazov, K. Extraction-chromogenic systems for vanadium(V) based on azo dyes and xylometazoline hydrochloride. *Acta Chim. Slov.* **2019**, *66*, 987–994. [CrossRef]
31. Racheva, P.V.; Milcheva, N.P.; Saravanska, A.D.; Gavazov, K.B. Extraction of gallium(III) with a new azo dye in the presence or absence of xylometazoline hydrochloride. *Croat. Chem. Acta* **2020**, *93*, 159–165. [CrossRef]
32. Mansour, F.R.; Plotka-Wasyłka, J.; Locatelli, M. Modified GAPI (MoGAPI) tool and software for the assessment of method greenness: Case studies and applications. *Analytica* **2024**, *5*, 451–457. [CrossRef]
33. Zhiming, Z.; Dongsten, M.; Cunxiao, Y. Mobile equilibrium method for determining composition and stability constant of coordination compounds of the form M_mR_n . *J. Rare Earths* **1997**, *15*, 216–219.
34. Asmus, E. Eine neue Methode zur Ermittlung der Zusammensetzung schwacher Komplexe (A New Method for the Determination of Composition of Weak Complexes). *Fresenius' J. Anal. Chem.* **1960**, *178*, 104–116. [CrossRef]
35. Bent, H.E.; French, C.L. The structure of ferric thiocyanate and its dissociation in aqueous solution. *J. Am. Chem. Soc.* **1941**, *63*, 568–572. [CrossRef]
36. Holme, A.; Langmyhr, F.J. A modified and a new straight-line method for determining the composition of weak complexes of the form A_mB_n . *Anal. Chim. Acta* **1966**, *36*, 383–391. [CrossRef]
37. Gavazov, K.B.; Racheva, P.V.; Saravanska, A.D.; Toncheva, G.K.; Delchev, V.B. Extractive spectrophotometric determination and theoretical investigations of two new vanadium(V) complexes. *Molecules* **2023**, *28*, 6723. [CrossRef] [PubMed]
38. Patil, R.; Dhuley, D. Solvent extraction and spectrophotometric determination of Fe(II) with 1,3-diphenyl-4-carboethoxy pyrazole-5-one. *Indian J. Chem.* **2000**, *39A*, 1105–1106.
39. Opara, I.J.; Ukoha, P.O.; Onunze, E.O. Extractive spectrophotometric determination of Fe(III) and Co(II) from steel sample using azo ligand 1,5-dimethyl-2-phenyl-4-[(E)-(2, 3, 4-trihydroxyl phenyl) diazenyl]-1,2-dihydro-3H-pyrazole-3-one. *Int. J. Chem. Mat. Environ. Res.* **2018**, *5*, 148–154.
40. Khoutoul, M.; Djedouani, A.; Lamsayah, M.; Abrigach, F.; Touzani, R. Liquid-liquid extraction of metal ions, DFT and TD-DFT analysis for some pyrane derivatives with high selectivity for Fe(II) and Pb(II). *Sep. Sci. Technol.* **2016**, *51*, 1112–1123. [CrossRef]
41. Lamsayah, M.; Khoutoul, M.; Takfaoui, A.; Touzani, R. High liquid–liquid extraction selectivity of Fe(II) and Pb(II) with TD-DFT theoretical calculations of long chain acid pyrazole- and triazole-based ligands. *Cogent Chem.* **2016**, *2*, 1230359. [CrossRef]
42. Khoutoul, M.; Abrigach, F.; Zarrouk, A.; Benchat, N.-E.; Lamsayah, M.; Touzani, R. New nitrogen-donor pyrazole ligands for excellent liquid–liquid extraction of Fe^{2+} ions from aqueous solution, with theoretical study. *Res. Chem. Intermed.* **2015**, *41*, 3319–3334. [CrossRef]
43. Abou El-Reash, Y.G.; Tantawy, H.A.; Abdel-Latif, E.; Mortada, W.I. Micelle-mediated extraction for speciation and spectrophotometric determination of iron using a newly synthesized salicylic acid derivative. *Microchem. J.* **2020**, *158*, 105280. [CrossRef]
44. Ivšić, A.G.; Tamhina, B. Extraction and formation of iron(III) thiocyanate complexes: Application for spectrophotometric determination of iron. *Croat. Chem. Acta* **2003**, *76*, 323–328.
45. Tarafder, P.K.; Thakur, R. Surfactant-mediated extraction of iron and its spectrophotometric determination in rocks, minerals, soils, stream sediments and water samples. *Microchem. J.* **2005**, *80*, 39–43. [CrossRef]
46. Garole, D.J.; Garole, V.J. Development of an extractive spectrophotometric method for the determination of Fe(III) using 4-methyl 2, 3-pentanedione dioxime. *Der Pharma Chem.* **2012**, *4*, 282–287.
47. Kuliev, K.A.; Verdizade, N.A.; Mamedova, S.A. Development of extractive spectrophotometric methods for the determination of iron(III) with dimercaptophenole and heterocyclic diamines. *J. Chem. Soc. Pak.* **2019**, *41*, 993–1003.
48. Zalov, A.Z.; Rasulov, C.K.; Mammadova, S.A.; Aliyev, S.G. Extraction-photometric determination of iron(III) in oil and oil products of baku using 2-hydroxy-5-halogenothiophenol and hydrophobic amines. *Azerb. Chem. J.* **2023**, *2023*, 126–133. [CrossRef]
49. Pereira, T.V.; Cassella, R.J. Simple spectrophotometric determination of iron in diesel oil after sample preparation employing extraction induced by emulsion breaking. *J. Braz. Chem. Soc.* **2022**, *33*, 1236–1243. [CrossRef]
50. Nikam, G.; Sabale, S.; Tamhankar, B. Synergistic solvent extraction study of Fe(III) from sodium acetate by using DB18C6 and TBP. *Int. J. Res. Biosci. Agric. Techn.* **2015**, *2*, 18–23.
51. Patil, S.S.; Lokhande, R.S.; Dharap, S.B.; Shevede, P.P.; Patil, J. Development of an extractive spectrophotometric method for determination of Fe(III) using 2, 4-dimethyl-3H-1, 5 benzodiazepine. *Int. J. Appl. Chem.* **2013**, *9*, 133–140.
52. Tarafder, P.K.; Sardana, A.K. A rapid method for the spectrophotometric determination of iron in silicate rocks. *Chem. Anal. (Warsaw)* **2000**, *45*, 145–151.
53. Zedan, H.E.; Mortada, W.I.; Khalifa, M.E. Microextraction procedures for preconcentration of Fe(III) in water and food samples prior to colorimetric detection: A comparative study. *J. Iran. Chem. Soc.* **2023**, *20*, 645–653. [CrossRef]

54. Rakhmatullayeva, N.T.; Giyasov, A.S.; Lutfullayeva, N.B.; Niyazova, M.M.; Gapparova, Z.X.; Raxmatov, U.N.; Mirkomilov, S.M.; Rakhmatullayeva, N.T. Selective extraction of iron(III) and its spectrophotometric determination directly in the organic phase. *E3S Web Conf.* **2023**, *371*, 01008. [CrossRef]
55. Lokhande, R.; Khadke, L.; Jain, N.; Jain, K. Liquid-liquid extraction and spectrophotometric determination of Fe(III) using 5-nitrosalicylaldehyde thiosemicarbazone (NSTS) as an analytical reagent. *Asian J. Res. Chem.* **2013**, *6*, 903–905.
56. Marczenko, Z.; Balcerzak, M. *Separation, Preconcentration and Spectrophotometry in Inorganic Analysis*; Elsevier: Amsterdam, The Netherlands, 2000; p. 228.
57. Gavazov, K.B.; Racheva, P.V.; Milcheva, N.P.; Divarova, V.V.; Kiradzhyska, D.D.; Genç, F.; Saravanska, A.D. Use of a hydrophobic azo dye for the centrifuge-less cloud point extraction–spectrophotometric determination of cobalt. *Molecules* **2022**, *27*, 4725. [CrossRef] [PubMed]
58. Gavazov, K.B.; Racheva, P.V.; Saravanska, A.D.; Genc, F.; Delchev, V.B. Mono- and binuclear complexes in a centrifuge-less cloud-point extraction system for the spectrophotometric determination of zinc(II). *Molecules* **2024**, *29*, 4511. [CrossRef] [PubMed]
59. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Montgomery, J.A., Jr.; Vreven, T.; Kudin, K.N.; Burant, J.C.; et al. *Gaussian 03, Revision D.01*; Gaussian, Inc.: Wallingford, CT, USA, 2004.
60. ChemCraft. Available online: <http://www.chemcraftprog.com> (accessed on 22 January 2025).

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

Beer-Derived (Poly)phenol Metabolism in Individuals With and Without Metabolic Syndrome: A Comparative Dietary Intervention

Daniel Hinojosa-Nogueira ^{1,2,*}, Cristina María Díaz-Perdigones ^{1,2,†}, María José García-López ^{1,3,4}, Ascensión Marcos ⁵, María P. Portillo ^{4,6,7}, Rosa María Lamuela-Raventós ^{4,8,9}, Alba Subiri-Verdugo ^{1,2,3}, Esther Nova ⁵, Iñaki Milton-Laskibar ^{4,6,7}, Polina Galkina ^{4,8,9}, Francisco J. Tinahones ^{1,2,3,4,*} and Isabel Moreno-Indias ^{1,2,4}

- ¹ Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina—IBIMA Plataforma, BIONAND, 29590 Málaga, Spain; cristinnemarie@gmail.com (C.M.D.-P.); mari.jose.baza@gmail.com (M.J.G.-L.); alb.sub@gmail.com (A.S.-V.); isabel.moreno@ibima.eu (I.M.-I.)
- ² Unidad de Gestión Clínica de Endocrinología y Nutrición, Hospital Universitario Virgen de la Victoria, 29010 Málaga, Spain
- ³ Department of Medicine and Dermatology, Faculty of Medicine, University of Málaga, 29010 Málaga, Spain
- ⁴ CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBn), Instituto Salud Carlos III, 28029 Madrid, Spain; mariapuy.portillo@ehu.es (M.P.P.); lamuela@ub.edu (R.M.L.-R.); inaki.milton@ehu.es (I.M.-L.); polinagalkina@ub.edu (P.G.)
- ⁵ Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), 28040 Madrid, Spain; amarcos@ictan.csic.es (A.M.); enova@ictan.csic.es (E.N.)
- ⁶ Nutrition and Obesity Group, Department of Nutrition and Food Science, Faculty of Pharmacy and Lucio Lascaray Research Centre, University of the Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain
- ⁷ BIOARABA Health Research Institute, 01006 Vitoria-Gasteiz, Spain
- ⁸ Polyphenol Research Group, Departament de Nutrició, Ciències de l'Alimentació i Gastronomia, Facultat de Farmàcia, Universitat de Barcelona (UB), Av. de Joan XXII, 27-31, 08028 Barcelona, Spain
- ⁹ Institut de Nutrició i Seguretat Alimentària (INSA), Universitat de Barcelona (UB), 08921 Santa Coloma de Gramanet, Spain
- * Correspondence: daniel.hinojosa@ibima.eu (D.H.-N.); fjtinahones@uma.es (F.J.T.)
- † These authors contributed equally to this work.

Abstract: The consumption of low-alcohol fermented beverages has been related to cardiovascular health improvements. Although the underlying mechanism is not completely understood, (poly)phenols have been proposed as one of the mediators. The objective of this study was to evaluate the impact of a controlled intervention with beer on (poly)phenols metabolism in individuals with and without metabolic syndrome (MetS). 20 participants (MetS and control) who consumed a standardized amount of beer during 6 weeks were recruited. Phenolic compounds were assessed in urine. Different changes in phenolic compounds associated with chronic beer consumption were found, particularly related to hesperetin conjugates and to the degradation of phenolic compounds derived from flavonoids and lignans. Noteworthy, MetS and control participants differed in baseline urine phenolic compound profiles and in their metabolization. Significant differences were found in the production and excretion of key (poly)phenols-derived metabolites, such as increased naringenin phase II conjugates in healthy subjects, or increased bacterial flavonoid catabolites. Certain relationships were observed between the phenolic compounds with metabolic and anthropometric variables. These findings suggest that beer-derived (poly)phenols are differentially metabolized according to metabolic-health status, and that they may contribute to certain metabolic health benefits through the modulation of specific metabolic pathways.

Keywords: beer; (poly)phenols; dietary intervention; metabolism; metabolic syndrome

1. Introduction

Metabolic syndrome is defined as a cluster of cardiometabolic risk factors that includes elevated blood pressure, hyperglycemia, increased waist circumference or abdominal obesity, elevated plasma triglycerides, and low levels of high-density lipoprotein (HDL) cholesterol. The condition is diagnosed when at least three of the following criteria are met: waist circumference > 102 cm in men or > 88 cm in women; triglyceride levels ≥ 150 mg/dL (1.7 mmol/L) or specific treatment for this lipid abnormality; HDL cholesterol < 40 mg/dL (1.0 mmol/L) in men or < 50 mg/dL (1.3 mmol/L) in women, or a specific treatment for reduced HDL; blood pressure $\geq 130/85$ mmHg or the use of antihypertensive medication; and fasting plasma glucose ≥ 100 mg/dL (5.6 mmol/L) or treatment for elevated blood glucose [1,2]. Furthermore, the presence of these factors increases by 5-fold the probability of developing type 2 diabetes, by 2-fold the risk of cardiovascular disease and by 1.5-fold the risk of mortality [2]. The global prevalence of metabolic syndrome is estimated to range between 20% and 40%, with significant variations across different countries. Moreover, an unhealthy lifestyle, including factors such as smoking, low or no physical activity, a poor-quality diet and other elements, is under the main factors that may potentially increase the metabolic syndrome prevalence [1].

Although the pathophysiology of metabolic syndrome is multifactorial, oxidative stress, resulting from an imbalance between oxidants and antioxidants, has been demonstrated to be a pivotal factor in the pathogenesis of metabolic disorders [1,2].

It is important to note the role of dietary compounds in the maintenance of oxidative stress and its associated effects. The evidence indicates that dietary patterns, particularly those comprising elevated contents of fat or added simple carbohydrates, are linked to oxidative damage through the elevation of protein carbonylation and lipid peroxidation products while concurrently reducing the antioxidant defense status [3]. However, these effects can be mitigated by the intake of specific nutrients, including some minerals and vitamins, as well as non-nutrient bioactive phytochemicals such as (poly)phenols [3,4]. Dietary (poly)phenols are molecules with high antioxidant capacity, also being capable of modulating gene expression and modifying epigenetic alterations, which is essential for the prevention of the pathological conditions associated with metabolic dysfunction [4]. Polyphenol-rich foods include a variety of fermented foods, which are notable for their nutraceutical potency [5]. These foods include certain beverages such as wine, beer, or new beverages [5–7]. Although these beverages are classified as alcoholic, and thus may have adverse effects when consumed in excess or over an extended period, numerous studies have demonstrated an association between their moderate consumption and an improvement in metabolic syndrome parameters, which is attributed to the presence of (poly)phenols [6]. In this sense, beer is one of the most consumed alcoholic beverages and has a polyphenolic composition comprising compounds such as phenolic acids and tannins, which have high bioactive potential [8].

Therefore, the aim of this study was to evaluate the effect of a controlled dietary intervention, including beer, on (poly)phenols metabolism in individuals with and without metabolic syndrome.

2. Results

2.1. Characteristics of the Subjects

This study was a randomized open-label crossover trial (ClinicalTrials.gov Identifier NCT05300165) conducted at Virgen de la Victoria University Hospital (Málaga, Spain). Although the main outcomes were published elsewhere [9], data relative to the aim of the current study, to decipher how chronic beer consumption (six weeks, one beer per day) alters the urine (poly)phenols profile in volunteers with and without metabolic syndrome, is currently presented. The composition of the study population was 65% females and 35% males. The mean age of the participants was 45.6 ± 8 years. The analysis of anthropometric parameters revealed statistically significant differences ($p < 0.05$) between the control (C) vs. metabolic syndrome (MetS) groups. It is important to highlight that the study population was constituted by an equal number of individuals in each group. The mean body weight was higher in MetS (93 ± 15 kg) than in C (66 ± 7.6 kg). Similarly, the mean body mass index (BMI) was higher in MetS (34 ± 4.8 kg/m²) compared to C (23 ± 2.8 kg/m²). The results showed statistically significant differences ($p < 0.05$) between the C and MetS groups for most biochemical parameters irrespective of the time, including blood pressure, fasting glucose levels, and the lipid profile, which were higher in MetS than in C (Table 1). In these parameters, no statistically significant differences were found between the beginning and the end of the intervention. It is noteworthy that no statistically significant differences in nutritional parameters were neither identified during the study, in which a standardized Mediterranean diet was followed. Furthermore, the principal values obtained for dietary intake with regard to energy and macronutrient consumption are presented in Table 1.

Table 1. Mean and standard deviation of selected anthropometric, biochemical and nutritional parameters.

Parameters (Control n = 10/ MetS n = 10)	Control—Baseline	Control—End Time	MetS—Baseline	MetS—End Time
Weight (kg) ^{a,b}	65.6 ± 7.8	65.4 ± 7.4	93.7 ± 14.4	93.1 ± 15.1
BMI (kg/m ²) ^{a,b}	22.9 ± 2.5	22.8 ± 2.4	34.6 ± 4.8	34.3 ± 4.9
Waist (cm) ^{a,b}	80.3 ± 8.9	79.6 ± 7.5	109.1 ± 10.2	104.9 ± 8.5
Hip (cm) ^{a,b}	97.0 ± 6.4	97.0 ± 6.6	114.9 ± 12.8	112.6 ± 12.4
Systolic blood pressure (mmHg) ^{a,b}	114.0 ± 14.5	112.1 ± 12.4	137.5 ± 13.2	129.4 ± 12.1
Diastolic blood pressure (mmHg) ^{a,b}	69.4 ± 8.3	71.1 ± 10.1	88.8 ± 10.3	86.7 ± 6.7
Glucose (mg/dL) ^{a,b}	82.6 ± 6.8	88.5 ± 5.8	114.7 ± 36.2	119.0 ± 24.6
Creatinine (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.2
Uric Acid (mg/dL) ^b	4.2 ± 0.8	4.5 ± 1.1	5.3 ± 1.4	5.9 ± 1.3
Na (mEq/L)	140.0 ± 1.3	140.5 ± 2.2	140.3 ± 2.2	139.0 ± 3.7
Fe (µg/dL)	77.5 ± 20.4	75.6 ± 24.2	71.1 ± 34.4	62.1 ± 27.6
Cholesterol (mg/dL)	192.5 ± 31.0	190.1 ± 30.3	208.6 ± 43.1	214.4 ± 45.4
HDL (mg/dL) ^{a,b}	64.0 ± 12.6	65.9 ± 12.4	48.7 ± 11.7	47.9 ± 12.1
LDL (mg/dL)	113.6 ± 28.6	109.6 ± 27.8	129.6 ± 31.2	127.9 ± 39.3
Triglycerides (mg/dL) ^{a,b}	74.6 ± 38.6	73.1 ± 33.5	151.7 ± 89.2	206.4 ± 168.2
Energy (kcal)	1610.1 ± 291.6	1704.3 ± 203.3	1617.2 ± 549.3	1697.6 ± 297.8
Total Protein (g)	65.7 ± 15.2	68.0 ± 13.0	71.0 ± 34.0	74.1 ± 14.0
Total Fat (g)	81.7 ± 24.4	79.6 ± 16.8	82.4 ± 38.7	89.9 ± 22.0
Total Carbohydrates (g)	155.3 ± 26.8	178.4 ± 27.6	147.8 ± 44.8	147.3 ± 29.3
Dietary Fiber (g)	16.6 ± 6.4	16.1 ± 12.2	14.6 ± 5.0	14.3 ± 5.7

Values are presented as mean ± SD (standard deviation). *p* values were calculated for differences between groups using a Mann–Whitney U test. Statistically significant differences ($p < 0.05$) at both baseline (^a) and end time (^b).

2.2. Phenolic Profiles of the Whole Population During the Intervention

Urine samples were subjected to high-performance liquid chromatography coupled to high-resolution mass spectrometry for (poly)phenols screening, particularly those related with microbial metabolism. In order to know the variation followed by the phenolic compounds, principal component analysis (PCA) and other variable reduction analyses were performed (Figure 1). Multivariate analysis techniques were employed to reveal a partial yet consistent clustering of samples according to intervention time (baseline vs. end time) and treatment group (C vs. MetS). While the overlap between the time points suggests a moderate change in phenolic compound profiles over a six-week period of daily consumption of a bottle of beer, Procrustes analysis supported this observation with a high disparity value (0.817), indicating a substantial, though not complete, reorganization of the metabolic space. In contrast, a clearer separation between the intervention groups was observed, particularly in the PCA and partial least squares (PLS) projections. A similar trend is observed with t-SNE (T-distributed stochastic neighbor embedding) and isomap analyses, although it is much less pronounced. This finding was further confirmed by a higher disparity (0.923).

In order to know the particular metabolites that significantly changed in the whole population after the beer intervention, different approximations were performed. Firstly, a fold change analysis was assessed. In this manner, we found that a total of 20 phenolic metabolites, 8 down and 12 up, significantly changed (Figure 2). In particular, hesperetin glucuronide, hesperetin sulfate_2, hesperetin sulfate_1, naringenin glucuronide, naringenin, 3-hydroxyphenylacetic glucuronide_3, dihydroresveratrol sulfate_1, sinapic acid glucuronide_1, coumaric acid sulfate_2, hippuric acid glucuronide_1, hydroxybenzoic acid glucuronide_1, and sinapic acid sulfate_1 suffered an increment in their concentrations, while 3,4-dihydroxyphenylacetic acid sulfate_2, chlorogenic acid, hydroxytyrosol sulfate_2, vanillic acid sulfate_2, 3,4-dihydroxyphenylacetic acid glucuronide_1, 3,4-dihydroxyphenylacetic acid sulfate_3, tyrosol sulfate_1, and sinapic acid suffered a reduction.

In order to gain a deeper insight into these data, we performed a paired *t*-test analysis which revealed that six phenolic metabolites changed between times: hesperetin sulfate_1 (*p*-value = 0.002, FDR-*p*-value = 0.121), hippuric acid glucuronide_1 (*p*-value = 0.002, FDR-*p*-value = 0.121), hesperetin glucuronide (*p*-value = 0.003, FDR-*p*-value = 0.121), 3,4-dihydroxyphenylacetic acid sulfate_2 (*p*-value = 0.004, FDR-*p*-value = 0.121), protocatechuic acid glucuronide (*p*-value = 0.008, FDR-*p*-value = 0.199), and hesperetin sulfate_2 (*p*-value = 0.009, FDR-*p*-value = 0.199), where all the metabolites but 3,4-dihydroxyphenylacetic acid sulfate_2 increased their amounts.

Finally, a volcano plot analysis that mixed both statistical analyses used before (fold change >2 and *fdr-p*-value-corrected ≤ 0.25) (Figure 3) found that five phenolic metabolites are the most important: hesperetin glucuronide, hesperetin sulfate_1, 3,4-dihydroxyphenylacetic acid sulfate_2, hippuric acid glucuronide_1 and hesperetin sulfate_2, with 3,4-dihydroxyphenylacetic acid sulfate_2 being the only one that decreased.

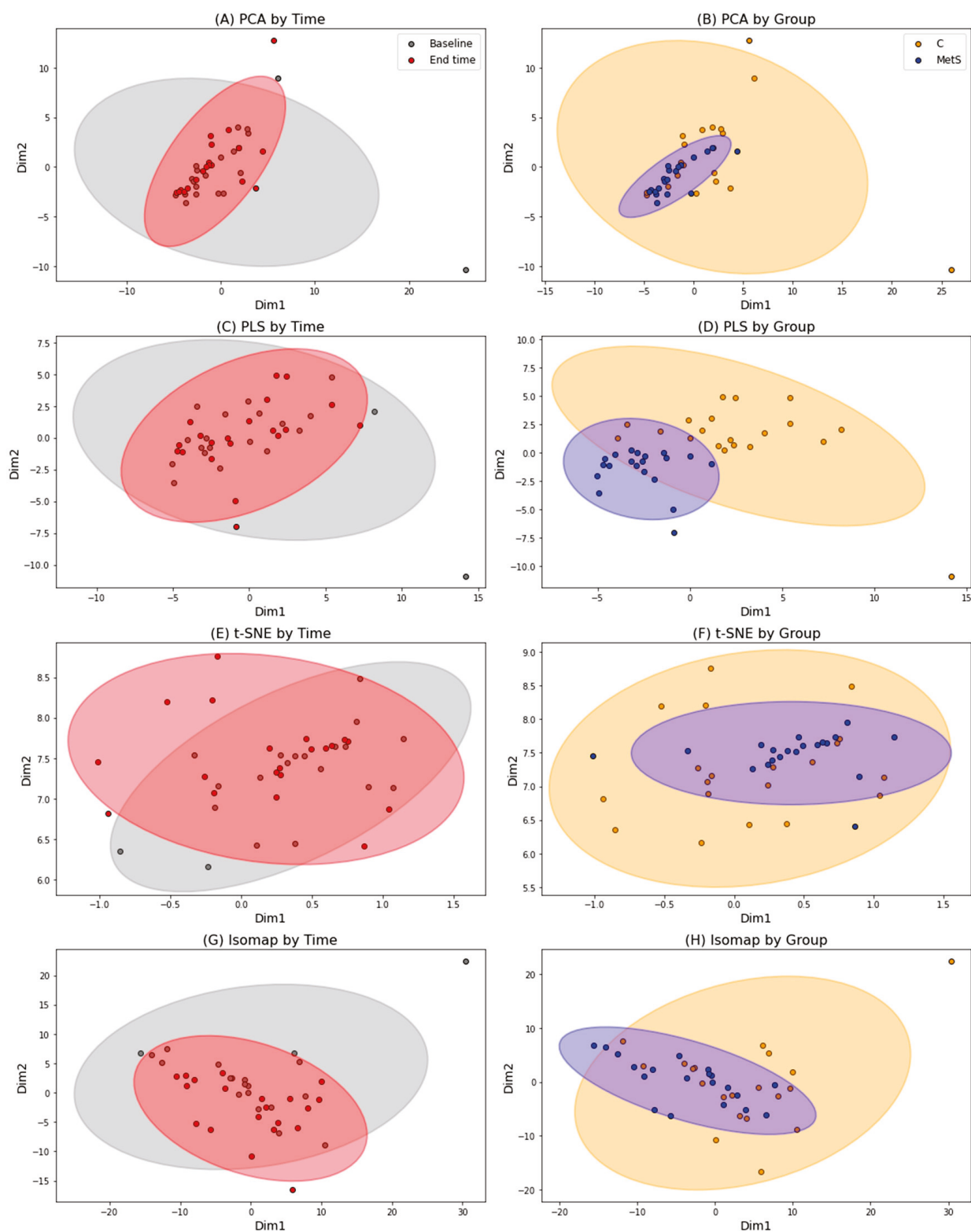


Figure 1. Comparative visualization of PCA, PLS, t-SNE, and isomap by group and time. Color legend: grey = baseline; red = end time; orange: MetS group; blue: Control group.

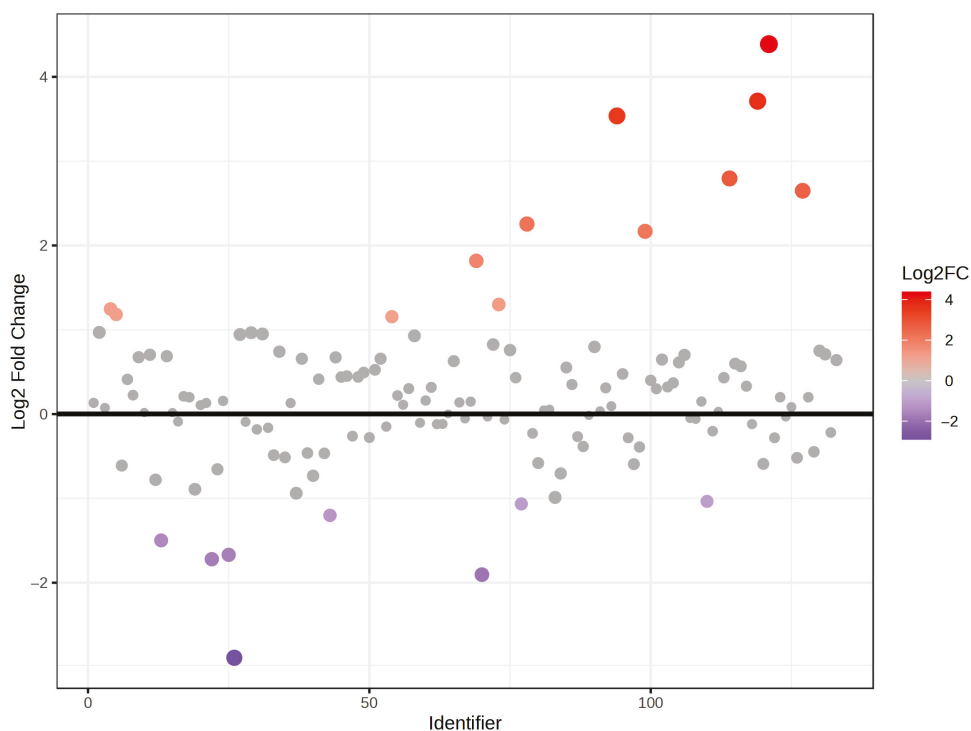


Figure 2. Fold change analysis of phenolic metabolites after the beer intervention in the whole population of study.



Figure 3. Volcano plot of the phenolic compounds that changed with the beer intervention in the whole population.

2.3. Phenolic Profiles According to the Metabolic Status of the Participants

Volunteer participants were divided according to their metabolic status between C and MetS participants. Considering the total amount of phenolic compounds between groups at baseline, healthy participants showed a higher content of total phenolic compounds (Figure 4).

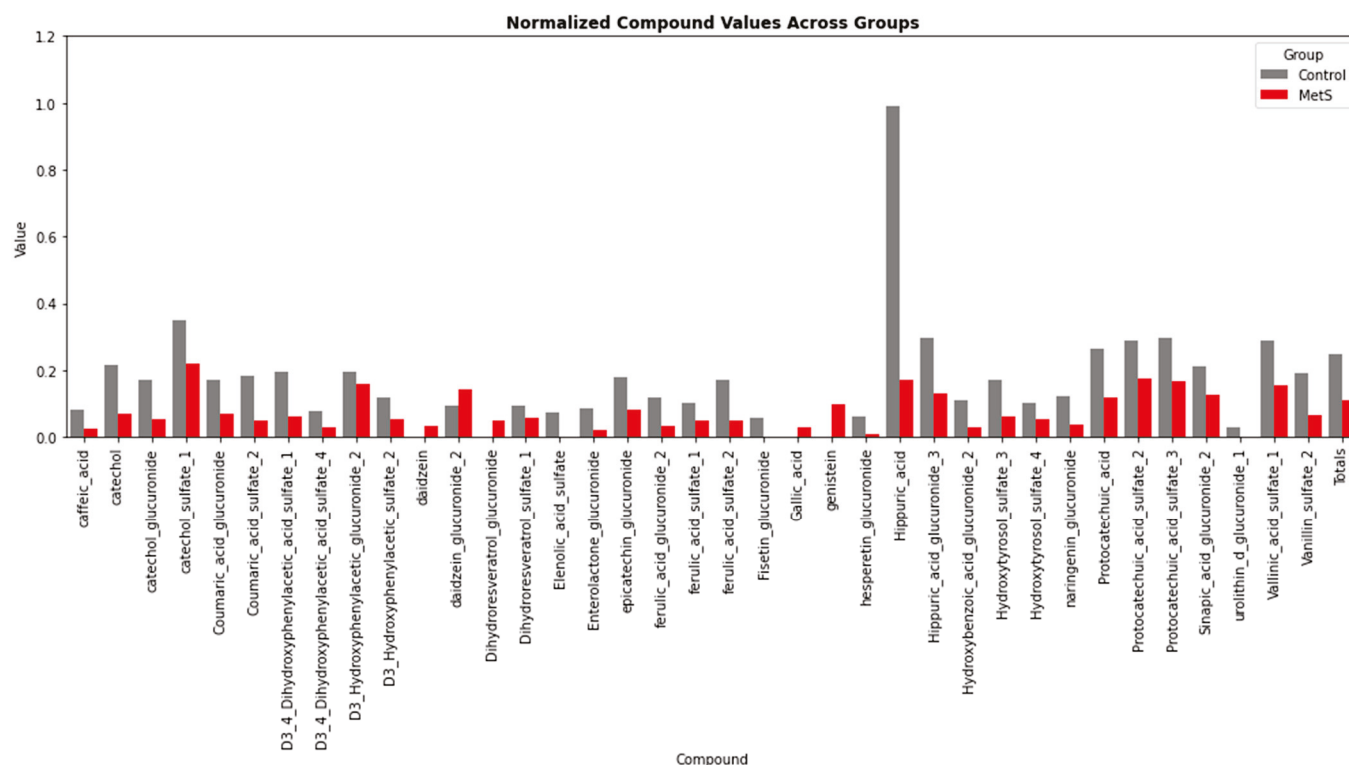


Figure 4. Difference in the concentration of phenolic compounds according to subject groups at baseline.

Interestingly, a PCA analysis did not show statistical differences between the metabolic groups ($p > 0.05$), although according to a posterior fold-change analysis, 15 phenolic compounds were diminished and 46 increased in the MetS group with respect to the control group. This trend continued after the chronic beer consumption under a Mediterranean diet, as the PCA analysis did not show differences between the two groups at the final stage ($p > 0.05$), although the fold change analysis indicated that, at this level, 18 metabolites diminished in the MetS group and 36 increased with respect to the Control group (Figure 5).

Following these initial prospective analyses, we performed a linear model with covariate adjustment to find those phenolic metabolites that differently changed in both groups after the beer intervention, finding that six phenolic metabolites changed differently across the different phenotypes, healthy or MetS. Thus, while hesperetin glucuronide, hesperetin sulfate_1, hesperetin sulfate_2, protocatechuic acid glucuronide and naringenin increased in the C group, they decreased or remained almost unchanged in the MetS group. On the other hand, the 3,4-dihydroxyphenylacetic acid sulfate_2 diminished in the C group, while increasing in the MetS group (Figure 6).

2.4. Relationships Between Biochemical Phenolic Profiles According to the Metabolic Status of the Participants

The relationship among the most relevant (poly)phenols seen previously, and their impact on certain biochemical values related to metabolic syndrome, was also evaluated. Significant correlations were found between most of them with the different variables. Furthermore, consideration was given to the (poly)phenols associated with metabolic changes. Those (poly)phenols exhibiting significant opposite or similar correlations for both groups were selected (Figure 7).

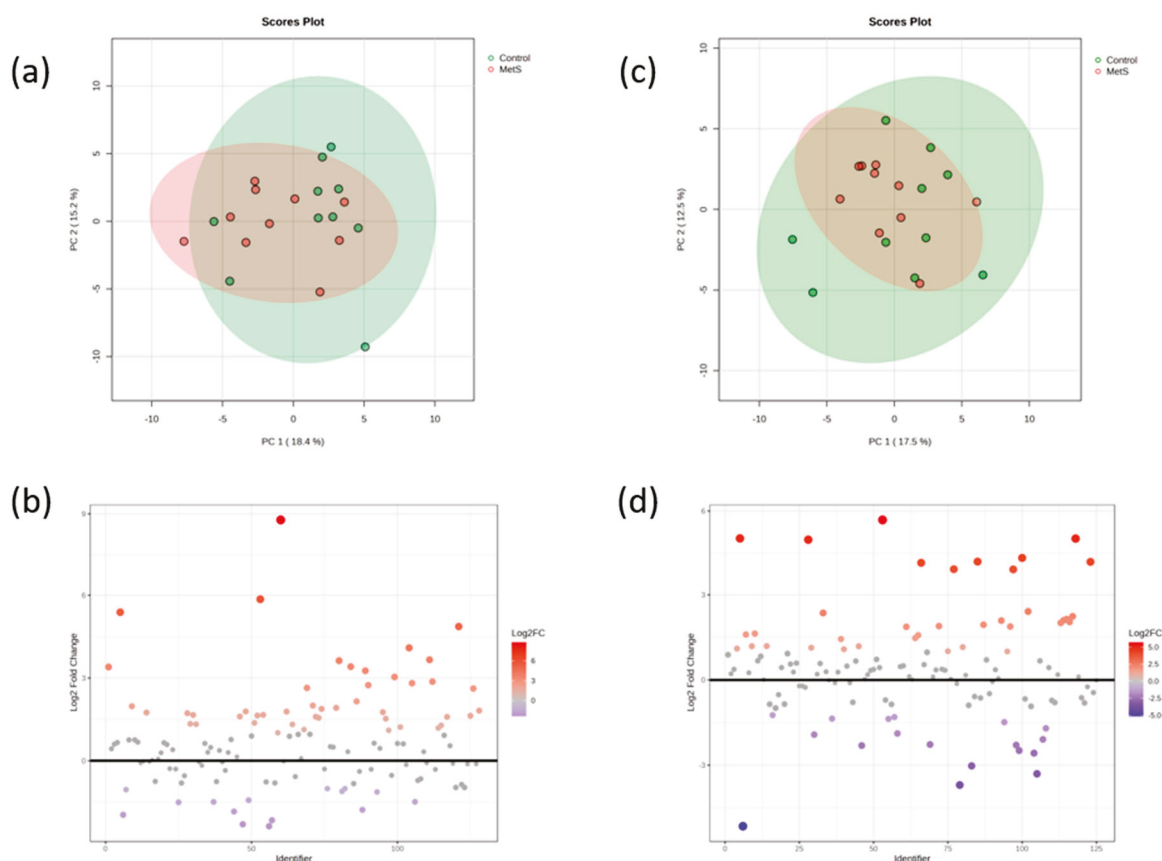


Figure 5. Basal and final patterns of phenolic compounds between the control group and metabolic syndrome patients (MetS). **(a)** Representation of the PCA analysis of phenolic compounds at baseline between MetS and Control groups; **(b)** Fold change analysis of phenolic compounds at baseline between MetS and Control groups; **(c)** Representation of the PCA analysis of phenolic compounds at a final point between the MetS and Control groups; **(d)** Fold change analysis of phenolic compounds at a final point between the MetS and Control groups.

The figure presents a bubble plot of the Spearman correlations between different (poly)phenols and metabolic parameters. The initial segment of the figure presents the (poly)phenols highlighted in the previous analysis and various metabolic parameters. As demonstrated in the figure, the (poly)phenols protocatechuic acid glucuronide and 3,4-dihydroxyphenylacetic acid sulfate 2 predominantly exhibit a negative correlation with parameters associated with the anthropometric profile. In addition, naringenin, hesperetin sulfate 1 and 2, and hesperetin glucuronide correlate negatively with other parameters such as insulin, C-peptide, triglycerides and correlate positively with HDL cholesterol and protein parameters. The following section of the figure shows the correlations between another group of (poly)phenols and metabolic parameters, differentiated by group. With the exception of certain (poly)phenols which demonstrate contradictory and substantial correlations according to the metabolic state of the individual, mainly positive correlations are observed between several (poly)phenols such as tyrosol glucuronide, urolithin B glucuronide, vanillic acid and D3,4-dihydroxyphenylacetic acid with metabolic parameters such as Homeostasis Model Assessment of Insulin Resistance (HOMA) index, glucose, BMI, blood pressure, and insulin. In contrast, compounds such as enterodiol glucuronide show negative correlations with anthropometric parameters regardless of the subject's metabolic status.

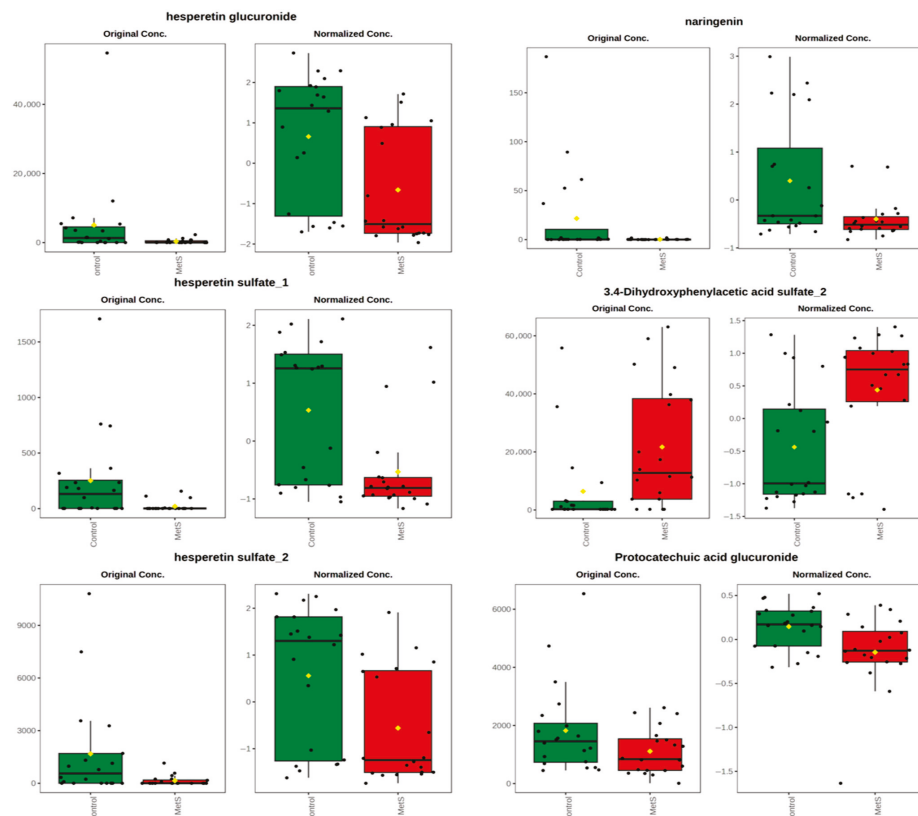


Figure 6. Phenolic compounds that changed differently among study groups according to the linear models results. The figure shows changes in concentration between times and groups. Control group: green; metabolic syndrome (MetS) patients: red. The yellow diamond represents the group mean value.

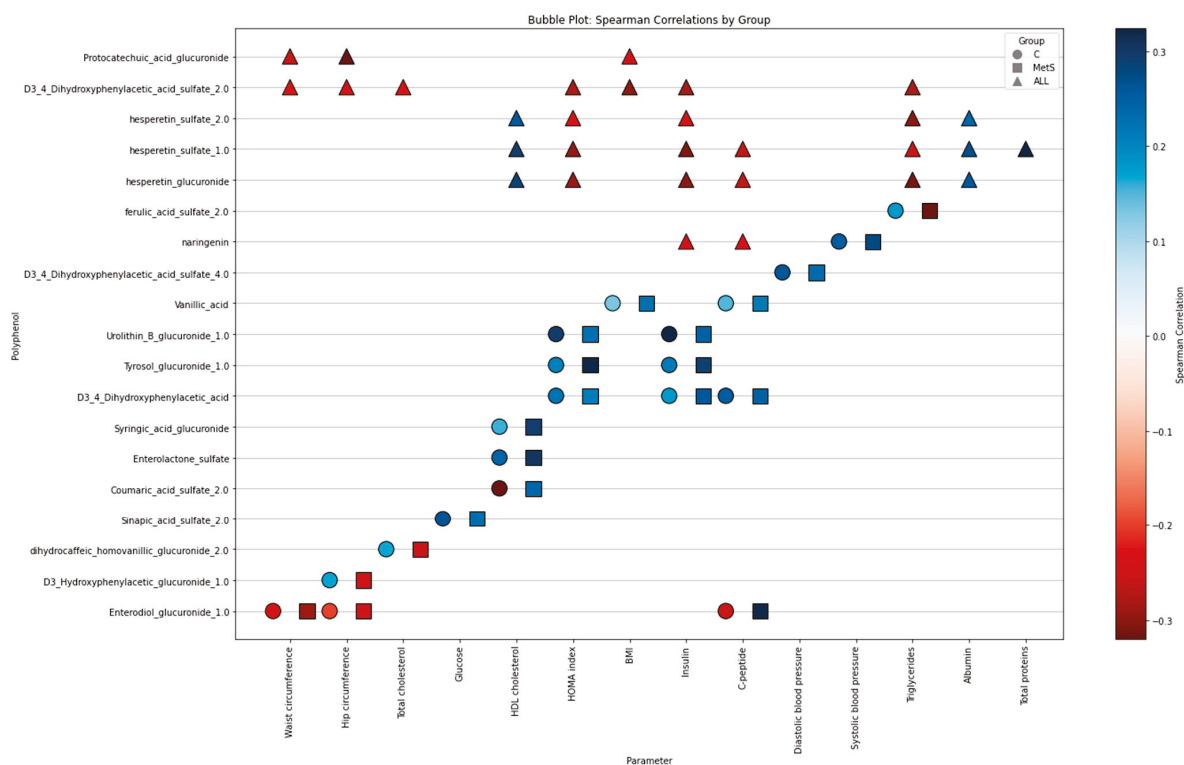


Figure 7. Relationship between (poly)phenols and metabolic parameters: Spearman's correlations by group and totals of relevant (poly)phenols. Subject groups: circle = Control, square = MetS, triangle = All subjects. Blue for positive correlations and red for negative correlations.

3. Discussion

Fermented food, and particularly, fermented beverages, have been the focus of extensive research because of their relationships with healthy outcomes. Most of these improvements have been related to their polyphenolic compounds. In this study, we have observed that the chronic consumption of the fermented beverage beer, within a context of a Mediterranean diet, is able to change the urine phenolic compound profile. Moreover, the presence or absence of metabolic syndrome among participants influenced the changes observed in phenolic compound profiles.

Our data clearly indicates that a chronic consumption of beer, equivalent to one bottle per day over a period of six weeks, results in changes to the phenolic compounds present in urine, particularly, suggesting a shift towards a more homogeneous profile. Indeed, (poly)phenol intake has been related to improvements in the metabolic profile [10], so the continued (poly)phenol intake through beer consumption could lead to metabolic changes that affect (poly)phenol levels. Furthermore, our results are similar to those described in the literature, where the circulating forms of the different (poly)phenols are glucuronides and sulfoglucuronides [11]. Thus, this may support a greater homogeneity in the phenolic profile after the intervention, suggesting an overall metabolomic response to regular beer consumption [12].

When we focused on the abundance changes of different phenolic compounds through a fold change analysis, we observed a notable increase, in the whole population, in metabolites primarily derived from citrus flavanones, such as hesperetin glucuronide and hesperetin sulfates, as well as naringenin and its conjugates. These compounds, abundant in beer due to its hop and cereal content [13], could reflect both intestinal absorption and hepatic and microbial metabolism of flavones [14]. The observed increases in dihydroresveratrol sulfate and synaptic acid glucuronide also suggest an effect of gut microbiota on the transformation of stilbenes and phenolic acids [15]. On the other hand, reductions were observed in compounds such as 3,4-dihydroxyphenylacetic acid sulfate, chlorogenic acid, and hydroxytyrosol sulfate and vanillic acid. These decreases could be due to the competition in hepatic conjugation pathways that modulates the catabolism of certain phenols [16].

These results were reinforced by posterior analysis. Indeed, our volcano plot results indicated a selective increase in 3,4-dihydroxyphenylacetic acid sulfate₂, which means an activation of the microbial or host catabolic pathways associated with the degradation of phenolic compounds derived from diverse (poly)phenols and dopamine. These results are relevant since there are preclinical studies conducted in animals that have associated this compound with the prevention of obesity [17]. Concurrently, a consistent decrease in hesperetin conjugates (glucuronide and sulfates) was detected in MetS subjects, which could be due to an increased clearance rate mediated by hepato-renal transporters [18]. Furthermore, the reduction in hippuric acid glucuronide suggests a potential alteration in the gut microbiota responsible for its production [19]. In a clinical context, this profile could be indicative of intestinal dysbiosis and oxidative stress, which is consistent with the metabolic characteristics of the study population [19].

Our study included two specific types of participants, healthy volunteers and MetS patients. In fact, the two groups were clearly differentiated. In particular, MetS patients showed higher body weight and BMI compared to the C group, indicative of a state of overweight or obesity that is characteristic of metabolic syndrome. Furthermore, the subjects with MetS had higher blood pressure, fasting glucose, and lipid profile alterations, which are common in people with metabolic syndrome [20].

Regarding the phenolic compounds, higher concentrations were found in healthy volunteers than in MetS patients. This outcome could be due to several factors, such

as better dietary habits or metabolic regulation, which may affect the absorption and metabolism of phenolic compounds [10]. Phenolic compounds are also recognized for their antioxidant and anti-inflammatory properties, and therefore their levels may vary in response to inflammation and oxidative stress as the body tries to protect against these changes by using them as a response [9,10,21]. Thus, taking into account that low-grade inflammation and oxidative stress are present in metabolic syndrome, lower amounts of these compounds are to be expected.

Moreover, differences in the magnitude and direction of phenolic changes were also identified in the two types of participants: healthy subjects showed greater increases, whereas individuals with MetS had attenuated or even decreased responses in the compounds. This finding suggests the potential for alterations in the absorption, conjugation, or excretion pathways of (poly)phenols in individuals with MetS [22]. For example, studies suggest that increases in some of these organic acids may indicate good metabolic function and good gut function, as in the case of hippuric acid [23,24].

Even though PCA revealed no statistically significant differences between the metabolic groups according to their phenolic profiles ($p > 0.05$), a thorough examination revealed significant alterations. Specifically, a decrease in 15 phenolic compounds and an increase in 46 phenolic compounds were observed in the MetS group compared to the C group. The remaining analyses corroborated this finding. This trend was sustained in the post-intervention phase, with no discernible alterations in the overall structure of the PCA ($p > 0.05$). These findings suggest that, although the differences are not reflected in the overall patterns of variance, there are specific modifications at the metabolomic level that could be relevant in the context of metabolic syndrome.

Looking at the six (poly)phenols with the greatest observed differences suggests that a beer intervention supports a higher (poly)phenols intake, but it is the metabolic state of the subject what mainly determines the metabolic pathways. In healthy subjects, the phase II conjugates of hesperetin and naringenin were increased, indicating efficient intestinal absorption and preserved hepatic metabolism. Conversely, within MetS patients, these conjugates remained stable or decreased, suggesting alterations in hepatic detoxification (typical of insulin resistance) and/or increased passage from the aglycone to the colon, where they are degraded by the microbiota before being conjugated. This pattern is consistent with the findings of in vitro studies, which demonstrate that hesperetin-7-O-glucoside elevates levels of *Bifidobacterium* and enhances the process of β -deglycosylation. The availability of conjugates is found to be contingent on intestinal integrity [25].

Concurrently, the MetS patients had increased levels of 3,4-dihydroxyphenylacetic sulfate, which is a bacterial phenolic catabolite. This finding indicates that the microbiota present in patients may have the capacity to synthesize simple phenolic acids. However, the loss of conjugates has the potential to compromise vasculoprotective effects [26]. Together, dysbiosis and liver dysfunction in metabolic syndrome appear to shift the balance from active conjugated metabolites to bacterial catabolites.

While most (poly)phenols can improve lipid and glycemic profiles, in many cases, it is the combination of several related (poly)phenols that provide synergistic effects and generate more significant health effects [27]. These synergistic effects may underlie specific metabolic associations observed in clinical studies. Considering the relationship of (poly)phenols with metabolic parameters, the clinical relevance of these changes was reinforced: protocatechuate glucuronide and 3,4-dihydroxyphenylacetic sulfate showed negative correlations with anthropometric markers, while hesperetin and naringenin were positively associated with HDL-cholesterol and negatively with insulin and triglycerides. These findings provide support for the role of beer-derived phenolics as modulators of

antioxidant and inflammatory pathways, with potential benefits in improving lipid profiles and insulin sensitivity [8,10,28–30].

Moreover, it has been observed that the alterations produced by metabolic syndrome can result in changes in the metabolism of certain (poly)phenols. These alterations can have a positive or negative effect on these parameters, depending on the subject's condition [31].

Although the current study presents some strengths such as the fact that it has been performed under a registered clinical trial, we also acknowledge some limitations in our study, such as the reduced number of participants or the fact that some of the metabolized (poly)phenols could have originated from dietary sources beyond the beer consumption. However, mitigation activities were introduced, such as different statistical approaches for the first one and a standardization of the diet and follow-up throughout the trial for the second.

4. Materials and Methods

4.1. Intervention Design and Sample Processing

The present study was conducted at the Hospital Universitario Virgen de la Victoria in Málaga, Spain. A total of 20 adult volunteers, aged between 30 and 60 years, were recruited to participate in a randomized, crossover study with an open-label design (ClinicalTrials.gov identifier NCT05300165). The main outcome was previously published elsewhere [9]. Briefly, in order to be eligible for participation in the trial, individuals were required to have a BMI of $<40 \text{ kg/m}^2$ and to abstain from the consumption of more than 20 g and 30 g of alcohol per day for women and men, respectively. The subjects were divided into two groups: a control group (C) ($n = 10$) comprising subjects with a BMI of $<30 \text{ kg/m}^2$, and a group of subjects with metabolic syndrome (MetS) ($n = 10$), defined according to the National Cholesterol Education Program Adult Treatment Panel III criteria [32].

The participants were randomly assigned to a crossover design, after a two-week period of washout, in which the participants were trained to follow a Mediterranean diet, which was maintained during the whole study. Particularly, the participants were instructed to consume olive oil as the primary source of fat, as well as a diet rich in fruits, vegetables, legumes, nuts, whole grains, as well as a moderate consumption of fish, seafood, poultry, eggs, and a low consumption of red and processed meats. These patterns were distributed in 3 main meals (breakfast, lunch, and dinner), and an additional 2 snacks periods.

Participants were instructed to consume one bottle (330 mL) of beer per day for a total period of six weeks. The quantity supplied guaranteed that the maximum daily alcohol intake did not exceed 12 g. During the intervention, participants were permitted to consume only the beers listed in the study protocol, and no other alcoholic beverage. Information relative to the kind of beer consumed can be found in our previous manuscript [9], but briefly: all participants consumed three types of beer for a period of two weeks each one: an alcohol-free beer (low (poly)phenols content— 12.2 mg/100 mL); a lager beer (intermediate (poly)phenols content— 27.83 mg/100 mL ; 4.2% alcohol by volume); and a dark beer (high (poly)phenols content— 41.6 mg/100 mL ; 4.5% alcohol by volume). (Poly)phenols content was estimated using the public database Phenol-Explorer [33]. Beer consumption was allocated into a randomized model mitigating the possible interference due to the different sequences performed. Anthropometric, biochemical, and dietary assessments were conducted at baseline and final time-points (after 6 weeks of a daily beer bottle consumption). Subjects were evaluated at the beginning of the day; which was the time-point when all measurements were taken. Furthermore, a urine sample was collected and immediately frozen at -80°C until subsequent analysis. In addition, a dietary survey was completed by each subject to facilitate the monitoring of their dietary habits. Blood samples

were collected after 10 h of fasting. Weight, height, and waist and hip circumferences were measured according to standardized procedures and BMI was calculated as weight (kg) divided by height² (m²). Biochemical parameters including total cholesterol (mg/dL), HDL cholesterol (mg/dL), triglycerides (mmol/L), and serum glucose were measured using a standard enzymatic method (Randox Laboratories Ltd., Crumlin, UK). Low-density lipoprotein (LDL) cholesterol (mg/dL) was calculated by using the Friedewald formula.

The informed consent of all subjects participating in the study was obtained. The study was conducted in accordance with the ethical guidelines established in the Declaration of Helsinki and received approval from the Ethics Committee of the Hospital Universitario Virgen de la Victoria (reference ID BEEROTA18).

4.2. Phenolic Compound Analysis

The analysis of profiles of phenolic compounds was performed using urine samples obtained from the subjects participating in the study. Urine was selected as the matrix for the analyses due to its common employment in the study of (poly)phenols metabolism, as it reflects the absorbed and metabolized forms of these compounds. The excretion of these compounds primarily occurs via the urine, and the cumulative excretion of these substances provides a more accurate evaluation of absorption than plasma levels, given that urinary concentrations are typically higher [34].

Sample preparation and analysis were conducted following a previously described protocol [35], with minor modifications to expand the method for the comprehensive analysis of urinary phenolic metabolites. The samples were subjected to a dilution process involving the addition of 10 µL of urine to 80 µL of water (with the presence of 0.05% formic acid), along with 10 µL of internal standard. The final concentration was of 100 µg/L. The internal standards utilized included hippuric-d5, abscisic acid-d6, hydroxytyrosol-d4, vanillic-d3 acid, and enterodiol-d6, with a final concentration of 100 µg/L. A standard curve was constructed in water at concentrations ranging from 1 to 7500 ppb (µg/L) for the performance of a quantitative analysis. The following compounds were used: apigenin; caffeic acid; caffeic glucuronide 3-hydroxyphenylacetic acid; 3-hydroxybenzoic acid; 3,4-dihydroxyphenylacetic acid; 4-hydroxybenzoic acid; ferulic acid; fisetin; genistein; hesperetin; hesperidin; catechin; epicatechin; epicatechin gallate; gallic acid; chlorogenic acid; daidzein; dihydroresveratrol; elenolic acid; enterodiol, and enterolactone. The samples were subsequently analyzed by high-performance liquid chromatography coupled to high-performance mass spectrometry (HPLC-ESI-QTOF-HRMS), at the Separation Techniques Unit of the Scientific and Technological Centers (CCiTUB). Chromatographic separation was performed on an Agilent 1290 Infinity II (Agilent Technologies, Santa Clara, CA, USA) using a Kinetex F5 column coupled with a SecurityGuardTM UHPLC F5 precolumn (Phenomenex, Torrance, CA, USA) were utilized, with water and acetonitrile as the mobile phases, both containing 0.05% acetic acid. The mass spectrometry was conducted using an Agilent 6560 QTOF-HRMS instrument, with the polarity set to negative. The identification and quantification of phenolic metabolites were carried out using Agilent MassHunter Workstation Software (Qualitative Analysis Version 10.0 and Quantitative Analysis Version 10.0) (Agilent Technologies, Santa Clara, CA, USA). Compounds were identified by comparing retention times and MS/MS spectra with those of commercial standards, when available. If not, identification was based on their fragmentation patterns obtained through product ion scans. Quantification was performed using calibration curves prepared in synthetic urine. Standard curves were constructed at concentrations ranging from 1 to 7500 ppb (µg/L). Each compound was quantified in relation to its pre-established standard. In the absence of a standard, an analogous alternative was selected based on chemical

similarity to its most relative compound within the standards, i.e., hydroxytyrosol sulfate was quantified with the hydroxytyrosol curve.

4.3. Statistical Analysis

The statistical analysis of the results was conducted using SPSS 22.0 (SPSS Inc., Chicago, IL, USA), R 4.4.2 (www.r-project.org), and Python 3.12.1 (www.python.org). The more complex analyses of the phenolic compounds were conducted using MetaboAnalyst 6.0, which offers a comprehensive range of methods for the analysis and visualization of metabolomics data [36]. In particular, data was primarily analyzed through paired samples after normalization of the concentrations, particularly the PCA, Fold change, and Volcano plot utilities from the statistical analysis (one factor), and after that, the linear model analysis from the statistical analysis (metadata table) between groups and times.

Moreover, in selected cases, outliers were excluded prior to conducting the statistical analyses. In the majority of cases, non-parametric methods were utilized. The Mann–Whitney U test was employed to evaluate the independent variables and identify any significant differences between the participant groups (C and MetS). Furthermore, PCA, t-SNE, Isomap and PLS regression were employed as analytical techniques. In addition, the procrustes analysis was performed to evaluate the degree of dissimilarity between sample configurations. Statistically significant differences were identified ($p < 0.05$).

5. Conclusions

The present findings show that beer-derived (poly)phenols are metabolized in a differential way depending on the metabolic health status of the subjects. This outcome suggests that the variability in metabolic profile may be attributed to differences in enzyme activity, gut microbial composition, or genetic factors that modulate the absorption, metabolism or biotransformation of (poly)phenols.

Furthermore, these bioactive compounds may exert regulatory effects on specific metabolic pathways associated with oxidative stress, systemic inflammation, and lipid metabolism. These results provide support for the hypothesis that beer-derived (poly)phenols may contribute to certain metabolic health benefits by acting as the modulators of specific biochemical processes, thereby conferring them a certain degree of therapeutic potential. Nevertheless, it is imperative to exercise caution and avoid making recommendations regarding consumption levels, due to the damaged effects of certain compounds, such as an excess of alcohol.

Author Contributions: Conceptualization, I.M.-I. and F.J.T.; methodology, D.H.-N., C.M.D.-P., M.J.G.-L., R.M.L.-R., A.S.-V. and P.G.; software, D.H.-N. and I.M.-I.; formal analysis, D.H.-N., R.M.L.-R., P.G. and I.M.-I.; investigation, C.M.D.-P., M.J.G.-L., A.S.-V., R.M.L.-R. and P.G.; resources, I.M.-I. and F.J.T.; data curation, D.H.-N. and I.M.-I.; writing—original draft preparation, D.H.-N. and I.M.-I.; writing—review and editing, all authors; visualization, D.H.-N. and I.M.-I.; supervision, F.J.T. and I.M.-I.; project administration, I.M.-I. and F.J.T.; funding acquisition, A.M., M.P.P., E.N., I.M.-L., F.J.T. and I.M.-I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly funded by a grant from “Foro para la Investigación de la Cerveza y Estilos de Vida (FICYE)” as well as by the “Network of Centers for Biomedical Research” (CIBER) of the Carlos III Health Institute (ISCIII) (CB06/03/0018), and research grants from the ISCIII (PI18/01160, PI21/01677) and co-financed by the Regional Development Fund (ERDF). Moreover, D.H.-N. was supported by a Sara Borrell postdoctoral contract (CD23/00111) from ISCIII-Madrid (Spain). C.M.D.-P. is supported by a postdoctoral Río Hortega (CM23/00128) from ISCIII-Madrid (Spain). M.J.G.-L. was funded by the Agencia Estatal de Investigación (PTA2022-021854-I). A.S.-V. was supported by a predoctoral PFIS (FI22/00193) from ISCIII-Madrid (Spain). I.M.-I. is supported by the “Miguel Servet Type II” program (CPII21/00013) under ISCIII-Madrid (Spain).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by Ethics Committee of Virgen de la Vitoria University Hospital (protocol code Beerota18, approved at 27 September 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available under request from the corresponding author due to ethical reasons.

Acknowledgments: Foro para la Investigación de la Cerveza y Estilos de Vida (FICYE) donated the beers in kind used in this study as part of a Manuel de Oya Research fellowship. The authors thank the volunteers for their patience and interest shown during the study. Also thanks to the Government of the Basque Country (IT1482-22).

Conflicts of Interest: The authors declare no conflicts of interest. None of the funding bodies had direct influence or input into the research design, execution, data analysis, or interpretation of the results.

References

1. Åberg, F.; Byrne, C.D.; Pirola, C.J.; Männistö, V.; Sookoian, S. Alcohol Consumption and Metabolic Syndrome: Clinical and Epidemiological Impact on Liver Disease. *J. Hepatol.* **2023**, *78*, 191–206. [CrossRef] [PubMed]
2. Masenga, S.K.; Kabwe, L.S.; Chakulya, M.; Kirabo, A. Mechanisms of Oxidative Stress in Metabolic Syndrome. *Int. J. Mol. Sci.* **2023**, *24*, 7898. [CrossRef]
3. Jiang, S.; Liu, H.; Li, C. Dietary Regulation of Oxidative Stress in Chronic Metabolic Diseases. *Foods* **2021**, *10*, 1854. [CrossRef] [PubMed]
4. Borsoi, F.T.; Neri-Numa, I.A.; De Oliveira, W.Q.; De Araújo, F.F.; Pastore, G.M. Dietary Polyphenols and Their Relationship to the Modulation of Non-Communicable Chronic Diseases and Epigenetic Mechanisms: A Mini-Review. *Food Chem. Mol. Sci.* **2023**, *6*, 100155. [CrossRef] [PubMed]
5. Sahoo, M.; Aradwad, P.; Sanwal, N.; Sahu, J.K.; Kumar, V.; Naik, S.N. Fermented Foods in Health and Disease Prevention. In *Microbes in the Food Industry*; Chhikara, N., Panghal, A., Chaudhary, G., Eds.; Wiley: Hoboken, NJ, USA, 2023; pp. 39–85. ISBN 978-1-119-77558-4.
6. Kang, Q.; Sun, J.; Wang, B.; Sun, B. Wine, Beer and Chinese Baijiu in Relation to Cardiovascular Health: The Impact of Moderate Drinking. *Food Sci. Hum. Wellness* **2023**, *12*, 1–13. [CrossRef]
7. Lopes, A.C.A.; Eda, S.H.; Andrade, R.P.; Amorim, J.C.; Duarte, W.F. New Alcoholic Fermented Beverages—Potentials and Challenges. In *Fermented Beverages*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 577–603. ISBN 978-0-12-815271-3.
8. Ambra, R.; Pastore, G.; Lucchetti, S. The Role of Bioactive Phenolic Compounds on the Impact of Beer on Health. *Molecules* **2021**, *26*, 486. [CrossRef]
9. Martínez-Montoro, J.I.; Quesada-Molina, M.; Gutiérrez-Repiso, C.; Ruiz-Limón, P.; Subiri-Verdugo, A.; Tinahones, F.J.; Moreno-Indias, I. Effect of Moderate Consumption of Different Phenolic-Content Beers on the Human Gut Microbiota Composition: A Randomized Crossover Trial. *Antioxidants* **2022**, *11*, 696. [CrossRef]
10. Chiva-Blanch, G.; Badimon, L. Effects of Polyphenol Intake on Metabolic Syndrome: Current Evidences from Human Trials. *Oxidative Med. Cell. Longev.* **2017**, *2017*, e5812401. [CrossRef]
11. Manach, C.; Morand, C.; Gil-Izquierdo, A.; Bouteloup-Demange, C.; Rémésy, C. Bioavailability in Humans of the Flavanones Hesperidin and Narirutin after the Ingestion of Two Doses of Orange Juice. *Eur. J. Clin. Nutr.* **2003**, *57*, 235–242. [CrossRef]
12. Grao-Cruces, E.M.; Montserrat-de La Paz, S.; Martin, M.E. Moderate Beer Consumption and Metabolic Health: A Comprehensive Review from the Lipoprotein Perspective. *J. Funct. Foods* **2022**, *95*, 105188. [CrossRef]
13. Kováčová, J.; Lehotay, J.; Ůrgeová, E.; Mocák, J.; Čižmárik, J. Determination of Selected Flavonoids in Hop Extract by HPLC. *J. Liq. Chromatogr. Relat. Technol.* **2011**, *34*, 329–340. [CrossRef]
14. Espín, J.C.; González-Sarriás, A.; Tomás-Barberán, F.A. The Gut Microbiota: A Key Factor in the Therapeutic Effects of (Poly)Phenols. *Biochem. Pharmacol.* **2017**, *139*, 82–93. [CrossRef]
15. Corrêa, T.A.F.; Rogero, M.M.; Hassimotto, N.M.A.; Lajolo, F.M. The Two-Way Polyphenols-Microbiota Interactions and Their Effects on Obesity and Related Metabolic Diseases. *Front. Nutr.* **2019**, *6*, 188. [CrossRef] [PubMed]
16. Clifford, M.N.; Kerimi, A.; Williamson, G. Bioavailability and Metabolism of Chlorogenic Acids (Acyl-quinic Acids) in Humans. *Comp. Rev. Food Sci. Food Safe* **2020**, *19*, 1299–1352. [CrossRef]

17. Chen, W.; Liu, R.; Zhu, X.; Lu, Q.; Yang, H.; Liu, R. Microbial Phenolic Metabolites 3-(3',4'-Dihydroxyphenyl)Propanoic Acid and 3',4'-Dihydroxyphenylacetic Acid Prevent Obesity in Mice Fed High-Fat Diet. *Food Sci. Hum. Wellness* **2024**, *13*, 327–338. [CrossRef]
18. Jakobek, L.; Blesso, C. Beneficial Effects of Phenolic Compounds: Native Phenolic Compounds vs. Metabolites and Catabolites. *Crit. Rev. Food Sci. Nutr.* **2024**, *64*, 9113–9131. [CrossRef]
19. Pallister, T.; Jackson, M.A.; Martin, T.C.; Zierer, J.; Jennings, A.; Mohney, R.P.; MacGregor, A.; Steves, C.J.; Cassidy, A.; Spector, T.D.; et al. Hippurate as a Metabolomic Marker of Gut Microbiome Diversity: Modulation by Diet and Relationship to Metabolic Syndrome. *Sci. Rep.* **2017**, *7*, 13670. [CrossRef]
20. Neeland, I.J.; Lim, S.; Tchernof, A.; Gastaldelli, A.; Rangaswami, J.; Ndumele, C.E.; Powell-Wiley, T.M.; Després, J.-P. Metabolic Syndrome. *Nat. Rev. Dis. Primers* **2024**, *10*, 77. [CrossRef]
21. Zugravu, C.-A.; Medar, C.; Manolescu, L.S.C.; Constantin, C. Beer and Microbiota: Pathways for a Positive and Healthy Interaction. *Nutrients* **2023**, *15*, 844. [CrossRef]
22. García-Conesa, M.-T. Dietary Polyphenols against Metabolic Disorders: How Far Have We Progressed in the Understanding of the Molecular Mechanisms of Action of These Compounds? *Crit. Rev. Food Sci. Nutr.* **2015**, *57*, 1769–1786. [CrossRef]
23. De Simone, G.; Balducci, C.; Forloni, G.; Pastorelli, R.; Brunelli, L. Hippuric Acid: Could Become a Barometer for Frailty and Geriatric Syndromes? *Ageing Res. Rev.* **2021**, *72*, 101466. [CrossRef] [PubMed]
24. Lord, R.S.; Bralley, J.A. Clinical Applications of Urinary Organic Acids. Part 2. Dysbiosis Markers. *Altern. Med. Rev.* **2008**, *13*, 292–306.
25. Pan, L.; Ye, H.; Pi, X.; Liu, W.; Wang, Z.; Zhang, Y.; Zheng, J. Effects of Several Flavonoids on Human Gut Microbiota and Its Metabolism by in Vitro Simulated Fermentation. *Front. Microbiol.* **2023**, *14*, 1092729. [CrossRef]
26. Giménez-Bastida, J.A.; González-Sarriás, A.; Vallejo, F.; Espín, J.C.; Tomás-Barberán, F.A. Hesperetin and Its Sulfate and Glucuronide Metabolites Inhibit TNF- α Induced Human Aortic Endothelial Cell Migration and Decrease Plasminogen Activator Inhibitor-1 (PAI-1) Levels. *Food Funct.* **2016**, *7*, 118–126. [CrossRef] [PubMed]
27. Gasmi, A.; Mujawdiya, P.K.; Noor, S.; Lysiuk, R.; Darmohray, R.; Piscopo, S.; Lenchyk, L.; Antonyak, H.; Dehtiarova, K.; Shanaida, M.; et al. Polyphenols in Metabolic Diseases. *Molecules* **2022**, *27*, 6280. [CrossRef]
28. Sluik, D.; Atkinson, F.S.; Brand-Miller, J.C.; Fogelholm, M.; Raben, A.; Feskens, E.J.M. Contributors to Dietary Glycaemic Index and Glycaemic Load in the Netherlands: The Role of Beer. *Br. J. Nutr.* **2016**, *115*, 1218–1225. [CrossRef]
29. Chiva-Blanch, G.; Magraner, E.; Condines, X.; Valderas-Martínez, P.; Roth, I.; Arranz, S.; Casas, R.; Navarro, M.; Hervás, A.; Sisó, A.; et al. Effects of Alcohol and Polyphenols from Beer on Atherosclerotic Biomarkers in High Cardiovascular Risk Men: A Randomized Feeding Trial. *Nutr. Metab. Cardiovasc. Dis.* **2015**, *25*, 36–45. [CrossRef]
30. López-Yerena, A.; Muñoz-García, N.; De Santisteban Villaplana, V.; Padro, T.; Badimon, L. Effect of Moderate Beer Intake on the Lipid Composition of Human Red Blood Cell Membranes. *Nutrients* **2024**, *16*, 3541. [CrossRef]
31. Laveriano-Santos, E.P.; Quifer-Rada, P.; Marhuenda-Muñoz, M.; Arancibia-Riveros, C.; Vallverdú-Queralt, A.; Tresserra-Rimbau, A.; Ruiz-León, A.M.; Casas, R.; Estruch, R.; Bodega, P.; et al. Microbial Phenolic Metabolites in Urine Are Inversely Linked to Certain Features of Metabolic Syndrome in Spanish Adolescents. *Antioxidants* **2022**, *11*, 2191. [CrossRef]
32. National Cholesterol Education Program (US). Expert Panel on Detection; Treatment of High Blood Cholesterol in Adults. In *Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)*; The Program; National Heart, Lung, and Blood Institute: Bethesda, MD, USA, 2002; ISBN 1-58808-003-X.
33. Showing Report on Alcoholic Beverages—Phenol-Explorer. Available online: <http://phenol-explorer.eu/reports/38#beertext> (accessed on 7 July 2025).
34. Marhuenda-Muñoz, M.; Laveriano-Santos, E.P.; Tresserra-Rimbau, A.; Lamuela-Raventós, R.M.; Martínez-Huélamo, M.; Vallverdú-Queralt, A. Microbial Phenolic Metabolites: Which Molecules Actually Have an Effect on Human Health? *Nutrients* **2019**, *11*, 2725. [CrossRef]
35. Campins-Machado, F.M.; Casas, R.; Lamuela-Raventós, R.M.; Galkina, P.; Martínez-González, M.Á.; Fitó, M.; Ros, E.; Estruch, R.; Domínguez-López, I.; Pérez, M. Microbiota-Derived Resveratrol Metabolites: New Biomarkers of Red Wine Consumption Are Inversely Associated with Inflammation in a Longitudinal Study of a Mediterranean Population. *J. Nutr. Health Aging* **2025**, *29*, 100542. [CrossRef] [PubMed]
36. Pang, Z.; Lu, Y.; Zhou, G.; Hui, F.; Xu, L.; Viau, C.; Spigelman, A.F.; MacDonald, P.E.; Wishart, D.S.; Li, S.; et al. MetaboAnalyst 6.0: Towards a Unified Platform for Metabolomics Data Processing, Analysis and Interpretation. *Nucleic Acids Res.* **2024**, *52*, W398–W406. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

Anti-*Helicobacter pylori* Activity and Gastroprotective Effects of Diacetylcurcumin and Four Metal Derivatives

Almanelly Agabo-Martínez ¹, Erika Gomez-Chang ¹, Erick Hernández-Hipólito ¹, Elizabet Estrada-Muñoz ², Carolina Escobedo-Martínez ³, Marco A. Obregón-Mendoza ⁴, Raúl G. Enríquez ⁴, Libia Vega ² and Irma Romero ^{1,*}

¹ Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria, Ciudad de México 04510, Mexico; amagcmtz94@gmail.com (A.A.-M.); ergoc@bq.unam.mx (E.G.-C.); erick.hipolito27@gmail.com (E.H.-H.)

² Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Zacatenco, Ciudad de México 07360, Mexico; eestrada@cinvestav.mx (E.E.-M.); lvega@cinvestav.mx (L.V.)

³ Departamento de Farmacia, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Campus Guanajuato, Guanajuato 36050, Mexico; c.escobedo@ugto.mx

⁴ Instituto de Química, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria, Ciudad de México 04510, Mexico; obregonmendoza@yahoo.com.mx (M.A.O.-M.); enriquezhabib@gmail.com (R.G.E.)

* Correspondence: irma@bq.unam.mx; Tel.: +52-555-623-2533

Abstract: *Helicobacter pylori* is the main etiological factor of gastritis, peptic ulcers, and gastric cancer. This bacterium's antibiotic resistance has led to a lower eradication rate; therefore, new drugs with anti-*H. pylori* activity are needed. Curcumin exhibits multiple biological activities, but it has low stability and poor bioavailability. To overcome these disadvantages, different metal complexes have been synthesized. The objective of this study was to determine the in vitro anti-*H. pylori* activity of diacetylcurcumin (DAC), DAC₂-Cu, DAC₂-Zn, DAC₂-Mn, and DAC₂-Mg by obtaining the minimum inhibitory concentration of bacterial growth, and to investigate some mechanisms by which they could affect the bacteria (urease and DNA gyrase activities). Moreover, their gastroprotective potential was assayed in an ethanol-induced gastric ulcer model in mice. The results showed that DAC₂-Cu and DAC₂-Zn have good anti-*H. pylori* activity, exhibit specific activity against this bacterium, inhibit the urease activity, and provide 70% gastroprotection at a dose of 200 mg/kg of body weight. In a subacute toxicity study in mice, DAC₂-Cu and DAC₂-Zn did not cause death or any deleterious symptoms, nor did they have a significant effect on serum and urine biochemical parameters compared to control mice. These compounds are promising candidates for use in *H. pylori* eradication schemes.

Keywords: *Helicobacter pylori*; diacetylcurcumin complexes; gastroprotection; MIC; urease; subacute toxicity

1. Introduction

Helicobacter pylori is a gastric human pathogen that infects 44.3% of the world's population [1]. Eighty percent of infected people are asymptomatic; however, all of them have gastritis, 10–20% develop duodenal or gastric ulcer, 1–2% progress toward gastric cancer, and less than 1% evolve into MALT-type lymphoma [2]. The outcome of the infection depends on three key elements: the bacterial genotype, the host's genetic predisposition, and environmental factors such as diet, among others.

This bacterium has developed several pathogenic factors that allow it to survive, cause damage to the gastric epithelium, and persist in the stomach, despite the hostile acidic environment. The eradication of *H. pylori* is crucial for reducing gastric mucosal inflammation, promoting ulcer healing, lowering the risk of gastric cancer, and decreasing mortality associated with these conditions. Currently, there is no way to prevent *H. pylori* infection except for basic hygiene rules.

Standard triple therapy is the most common treatment for *H. pylori* eradication, which is based on the administration of a proton pump inhibitor (PPI) in conjunction with two antibiotics, usually clarithromycin and amoxicillin or metronidazole or tetracycline for 14 days. However, this therapy often fails, so quadruple therapy is increasingly used as a first-line treatment. In addition to PPI and antibiotics, this regimen includes bismuth salts. Other antibiotics used may comprise quinolones, such as levofloxacin [3]. The main reasons for treatment failure are escalating antibiotic resistance and adverse effects that make patients uncomfortable and lead them to abandon therapy [4]. Therefore, it is important to explore novel, more efficacious, and less aggressive treatments for *H. pylori* eradication.

Curcumin ($C_{21}H_{20}O_6$) is a compound obtained from the rhizome of *Curcuma longa*, which is used in Asia as a food flavoring, coloring, food preservative, and even in other countries as a dietary supplement [5]. This compound has three chemical entities in its structure: two aromatic rings with o-methoxylated phenolic groups connected through a 7-carbon bond with a β -diketone α , β -unsaturated center (Figure 1A), which are responsible for its multiple biological activities [6]. Many in vitro and in vivo studies have demonstrated its antioxidant, antiproliferative, anti-inflammatory, anticancer, and antimicrobial activities [7].

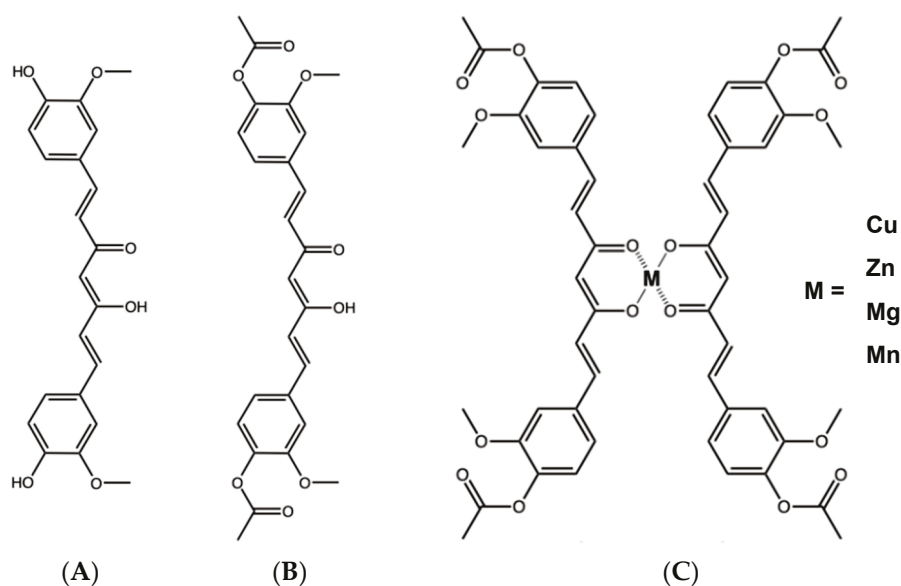


Figure 1. Chemical structure of (A) curcumin, (B) diacetylcurcumin, and (C) its metallic complexes.

The activity of curcumin against *H. pylori* has been assessed in vitro, revealing a Minimum Inhibitory Concentration (MIC) ranging from 5 to 50 $\mu\text{g/mL}$ against clinical isolates of the bacterium [8,9]. An in vivo study demonstrated the therapeutic potential of curcumin in a murine model of *H. pylori* infection, where it effectively eradicated the bacteria and ameliorated gastric damage induced by the infection [9]. Additional studies found that curcumin reduced the activation of proinflammatory mediators [10]. Notably, this compound has demonstrated non-toxicity in humans at doses up to 12 g per day [11].

In contrast to the promising activity demonstrated by curcumin in preclinical in vitro and in vivo studies, clinical trials have revealed that curcumin shows a poor eradication effect against *H. pylori* [12]. This limitation can be attributed to the compound's low bioavailability in the body. To address this challenge and enhance the pharmacokinetic profile of curcumin, nanoparticles, micelles, and synthetic derivatives have been developed [13]. A notable advancement in this area involves leveraging the chelating properties of curcumin's β -diketone center to synthesize stable metal complexes [6]. Diacetylcurcumin (DAC) (Figure 1B) is a derivative of curcumin in which the phenolic OH groups have been acetylated. Dr. Enriquez's group successfully synthesized four metal complexes from DAC with a 2:1 DAC/metal ratio (Figure 1C): DAC₂-Cu, DAC₂-Zn, DAC₂-Mn, and DAC₂-Mg [14]. These compounds exhibited antioxidant activity in a lipid peroxidation model and cytotoxic effects against three cancer cell lines: colorectal adenocarcinoma (HCT-15), breast adenocarcinoma (MCF-7), and lung adenocarcinoma (SKLU-1), and no acute toxicity in a murine model (LD > 3 g/kg) [14].

Based on the above-described effects of curcumin on *H. pylori* and its associated diseases, it would be desirable to find out whether DAC and its metal derivatives retain these properties. Therefore, the present work aimed to determine the effect of DAC, DAC₂-Cu, DAC₂-Zn, DAC₂-Mn and DAC₂-Mg on the growth of *H. pylori* and their impact on two important enzymes of bacterial metabolism such as urease, an enzyme essential for colonization and survival in the acid environment of the stomach [15], and DNA gyrase, a key enzyme in DNA metabolism [16]. We also assessed the anti-ulcerogenic effect of the compounds. This information provides an essential basis for the use of these curcumin derivatives in anti-*H. pylori* therapies.

2. Results

2.1. Anti-Helicobacter pylori Activity

Initially, we evaluated the anti-*H. pylori* effect of curcumin, DAC, and their metallic complexes. All the tested compounds showed inhibitory activity against *H. pylori* growth, considering the MIC values obtained (Table 1). This activity was higher than the inhibition of metronidazole, but none surpassed the MIC of clarithromycin. DAC₂-Cu and DAC₂-Mn had the lowest MIC, followed by DAC₂-Mg and DAC₂-Zn.

Table 1. Anti-*H. pylori* activity of DAC and its metallic derivatives.

Compound	MIC ¹	
	$\mu\text{g/mL}$	μM
Curcumin	1.95	5.29
DAC	62.50	138.12
DAC ₂ -Cu	15.62	15.00
DAC ₂ -Zn	31.25	29.86
DAC ₂ -Mn	15.62	14.90
DAC ₂ -Mg	31.25	28.84
Metronidazole ²	300.00	1752.84
Clarithromycin ²	0.05	0.07

¹ MIC: Minimum inhibitory concentration. ² Reference antibiotics for anti-*H. pylori* test.

Activity Versus Other Bacteria

To investigate if the inhibitory activity of the compounds was specific to *H. pylori*, their effect on other bacteria was tested [*Escherichia coli* (EPEC), *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*]. Experiments were performed up to a concentration of 62.5 $\mu\text{g/mL}$, equivalent to the MIC for DAC. At this concentration, none of

the compounds completely inhibited the growth of any of the bacteria tested (Table 2); DAC and its metal derivatives inhibited less than 25%. Curcumin, which was used as a control, inhibited in a higher percentage; however, these values are lower than the effect of curcumin against *H. pylori* (100% inhibition at 1.95 µg/mL).

Table 2. Growth inhibition of *E. coli*, *S. enterica* and *S. aureus* by DAC and its metallic derivatives.

Compound (62.5 µg/mL)	<i>E. coli</i> EPEC	<i>S. enterica</i>	<i>S. aureus</i>
Inhibition (%)			
Curcumin (170 µM)	25.7 ± 3.3	47.5 ± 10.1	67.2 ± 4.0
DAC (138 µM)	21.3 ± 3.0	15.2 ± 2.4	16.7 ± 6.9
DAC ₂ -Cu (60 µM)	10.7 ± 2.8	3.9 ± 1.2	10.9 ± 1.8
DAC ₂ -Zn (60 µM)	19.3 ± 4.5	10.0 ± 3.1	23.7 ± 6.3
DAC ₂ -Mn (56 µM)	8.6 ± 3.4	6.5 ± 4.1	10.9 ± 2.0
DAC ₂ -Mg (58 µM)	14.2 ± 2.3	11.0 ± 8.7	17.4 ± 1.7
Ampicillin (40 µM) (14 µg/mL)	100%		

2.2. *Helicobacter pylori* Urease Inhibition

H. pylori urease is an enzyme that is crucial for the colonization, survival, and pathogenicity of the bacterium. Figure 2 shows that DAC₂-Zn inhibits 86.3% of urease activity at 62.5 µg/mL, while DAC₂-Cu inhibits 75% (there was no statistically significant difference between both at this concentration ($p > 0.05$)). The respective IC₅₀ values calculated were 13.0 and 21.2 µg/mL (12.4 and 20.4 µM). These compounds had better activity than the positive control, acetohydroxamic acid (AHA), which has an IC₅₀ value of 35.7 µg/mL (488.4 µM). Curcumin and DAC inhibited 30% of urease activity at 125.0 µg/mL (339.3 and 276.2 µM, respectively).

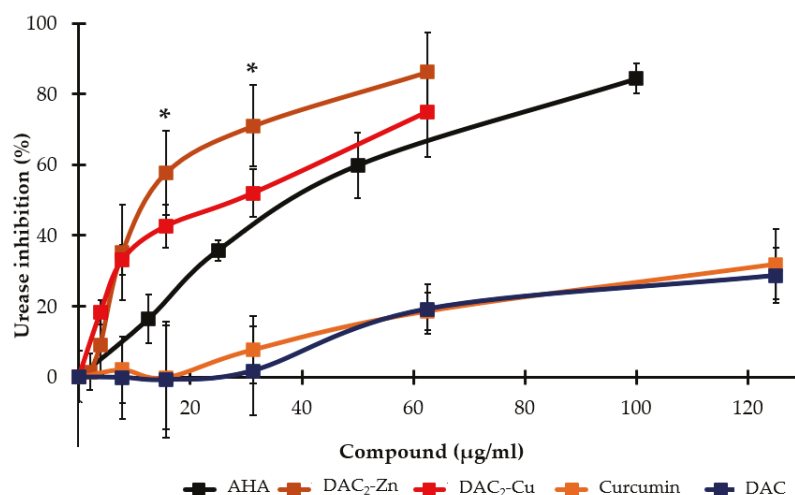


Figure 2. Effect of DAC and its metal derivatives on *H. pylori* urease activity. AHA: Acetohydroxamic acid, DAC: diacetylcurcumin. Data presented as mean ± SD. $n = 3$ per value. * Statistically significant difference between DAC₂-Cu and DAC₂-Zn ($p < 0.05$).

In contrast to the inhibition of urease activity, DAC₂-Mn exhibited a dose-dependent activation, reaching a maximum of 76.3% at 62.5 µg/mL (56.1 µM). Meanwhile, DAC₂-Mg did not affect the enzyme.

2.3. Effect on DNA Gyrase Activity

DNA gyrase is another essential enzyme for *H. pylori*. Its function is critical for DNA replication, recombination, and transcription. Except for DAC₂-Mn, none of the compounds had a significant effect on *E. coli* DNA gyrase supercoiling activity. DAC₂-Mn inhibited gyrase activity by 40% at 55.7 µg/mL (50 µM) (Table S1).

2.4. Gastroprotective Effect In Vivo

After demonstrating the anti-*H. pylori* activity of the studied compounds, their gastroprotective activity was evaluated using an acute ethanol-induced gastric ulcer model in mice. It was observed that the DAC₂-Cu and DAC₂-Zn were the only two compounds that significantly reduced the ulcer index in a dose-dependent manner (Figure 3). At a concentration of 200 mg/kg body weight (BW), both compounds exhibited approximately 70% gastroprotective activity (Table 3), which was statistically equivalent to the activity of curcumin at the same concentration and carbenoxolone at 50 mg/kg BW. DAC, DAC₂-Mn, and DAC₂-Mg did not have statistically significant gastroprotection (Table 3).

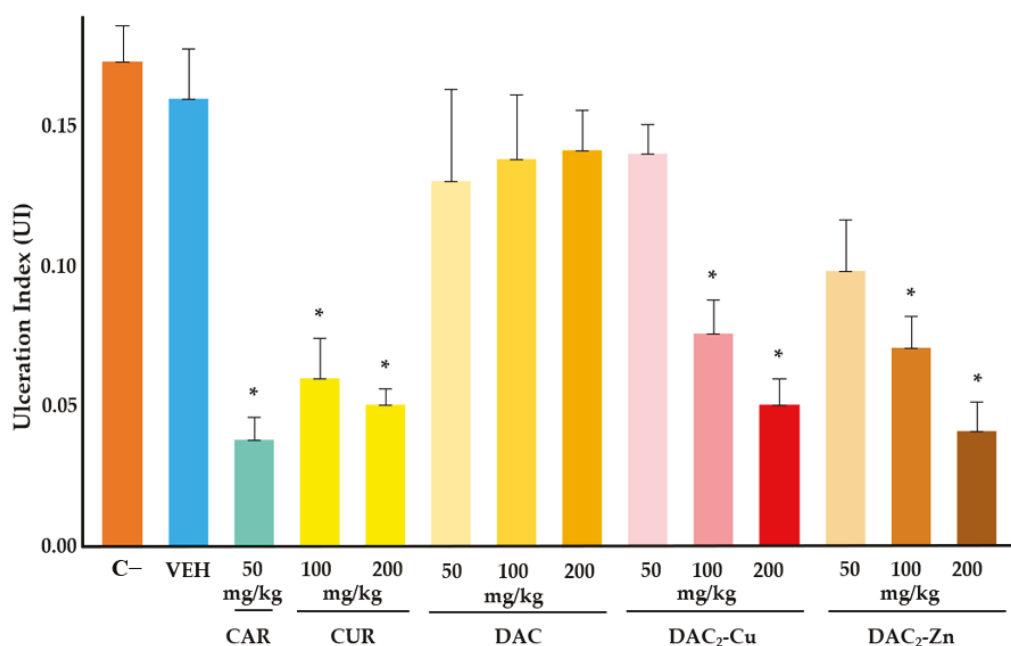


Figure 3. The anti-ulcerogenic effects of DAC, DAC₂-Cu, and DAC₂-Zn in an acute ethanol-induced gastric ulcer model. C−: Negative control [Isotonic sodium saline solution (NSS)], VEH: Vehicle [sodium carboxymethylcellulose (CMC) 0.5%], CAR: Carbenoxolone. CUR: Curcumin. DAC: Diacetylcurcumin. *n* = 6 per treatment. Data presented as mean ± SEM. * Statistically significant difference with respect to negative control (*p* < 0.05).

Macroscopic images of representative stomachs are shown in Figure 4. In contrast to a normal stomach (Figure 4A), the negative control and the vehicle stomachs (Figures 4C and 4D) presented severe hemorrhagic ulcer areas in the glandular epithelium (black arrows), while those treated with carbenoxolone, positive control (50 mg/kg BW) (Figure 4B), curcumin, DAC₂-Cu, and DAC₂-Zn (Figures 4E, 4F and 4G, respectively) with 200 mg/kg BW, exhibit few visible lesions on the stomach surface.

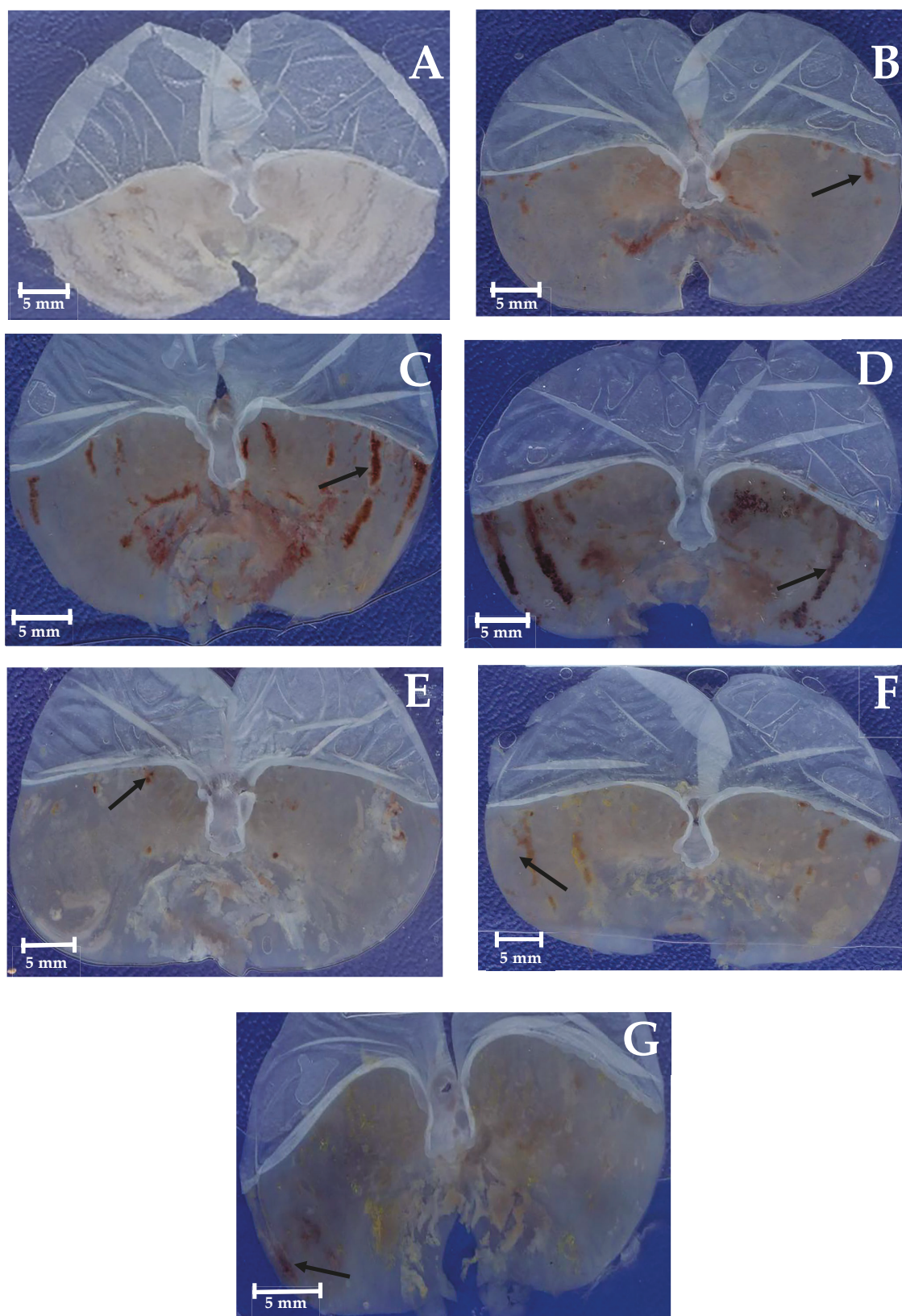


Figure 4. Representative stomachs. (A) Normal stomach. (B) Carbenoxolone (50 mg/kg BW). (C) Negative control, isotonic sodium saline solution (NSS). (D) Vehicle, 0.5% CMC. (E) Curcumin (200 mg/kg BW). (F) DAC₂-Cu (200 mg/kg BW). (G) DAC₂-Zn (200 mg/kg BW). Hemorrhagic ulcers (black arrows).

The histological examination of the stomachs is shown in Figure 5. The lining of the normal stomach shows a healthy epithelium, with normal glandular structures, and virtually no inflammatory cells (Figure 5A). This structure is similar to that observed in the stomach treated with 50 mg/kg BW of carbenoxolone. Although this compound is a known gastroprotective agent that favors the gastric glands' preservation, certain damage, such as mild edema in the submucosa, as well as some inflammatory and erythrocyte infiltrates, can be observed (Figure 5B). In the negative control and vehicle representative stomachs, the presence of abundant erythrocytes and inflammatory cell infiltrates in the mucosa are evident. Also, there is loss of the glandular architecture, exhibited by destruction of the epithelial cells, as well as edema in the submucosa (Figures 5C and 5D). With the highest tested dose of DAC₂-Cu and DAC₂-Zn (200 mg/kg body weight), maximum gastroprotection was achieved, as evidenced by the preservation of the glandular mucosa structure and the reduction in inflammatory and erythrocytic infiltrates (Figures 5F and 5G). These results were also obtained when using curcumin as treatment (200 mg/kg BW); however, low mucosa damage persisted, which could be seen by some red cell blood infiltration (Figure 5E).

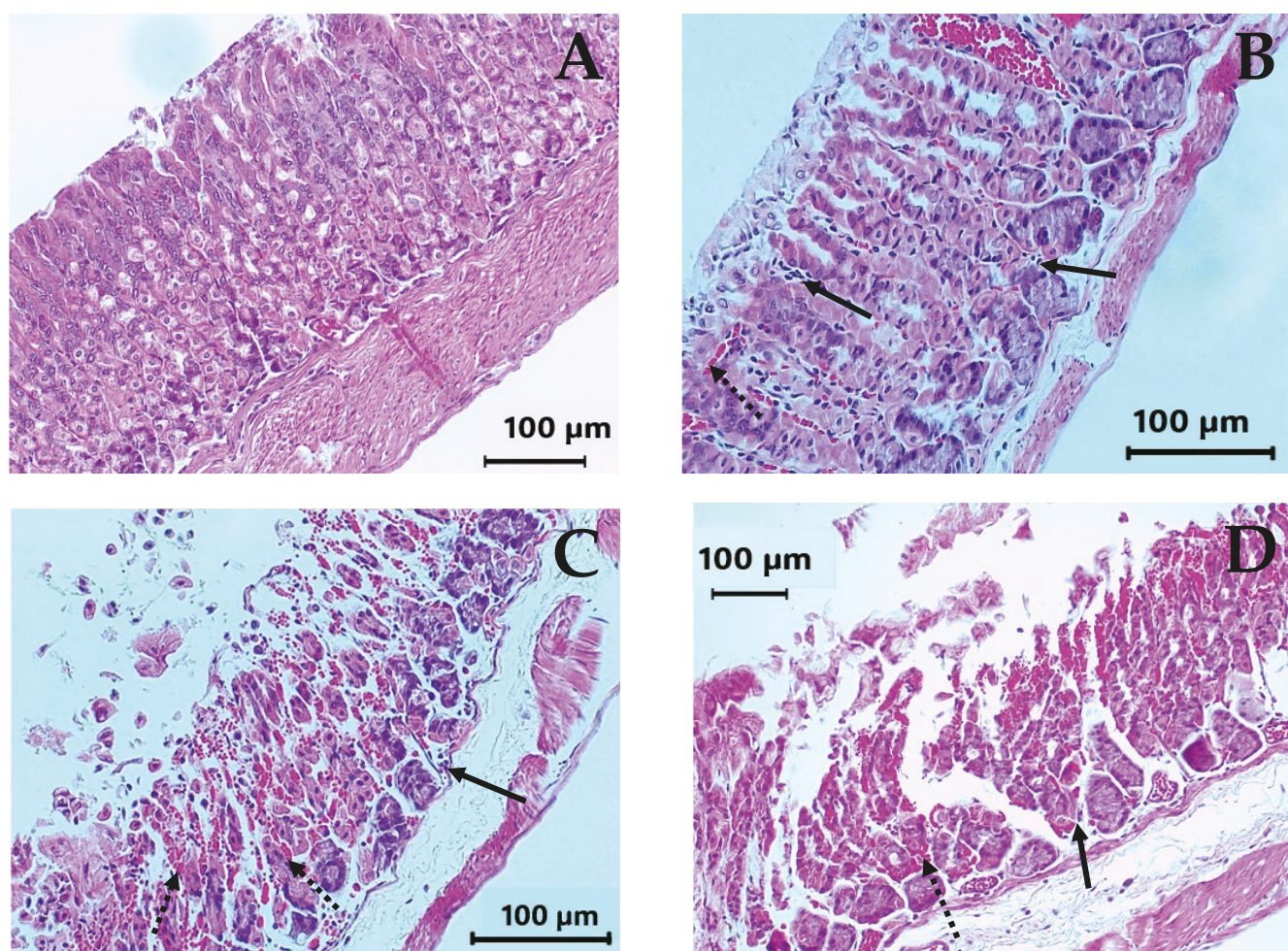


Figure 5. *Cont.*

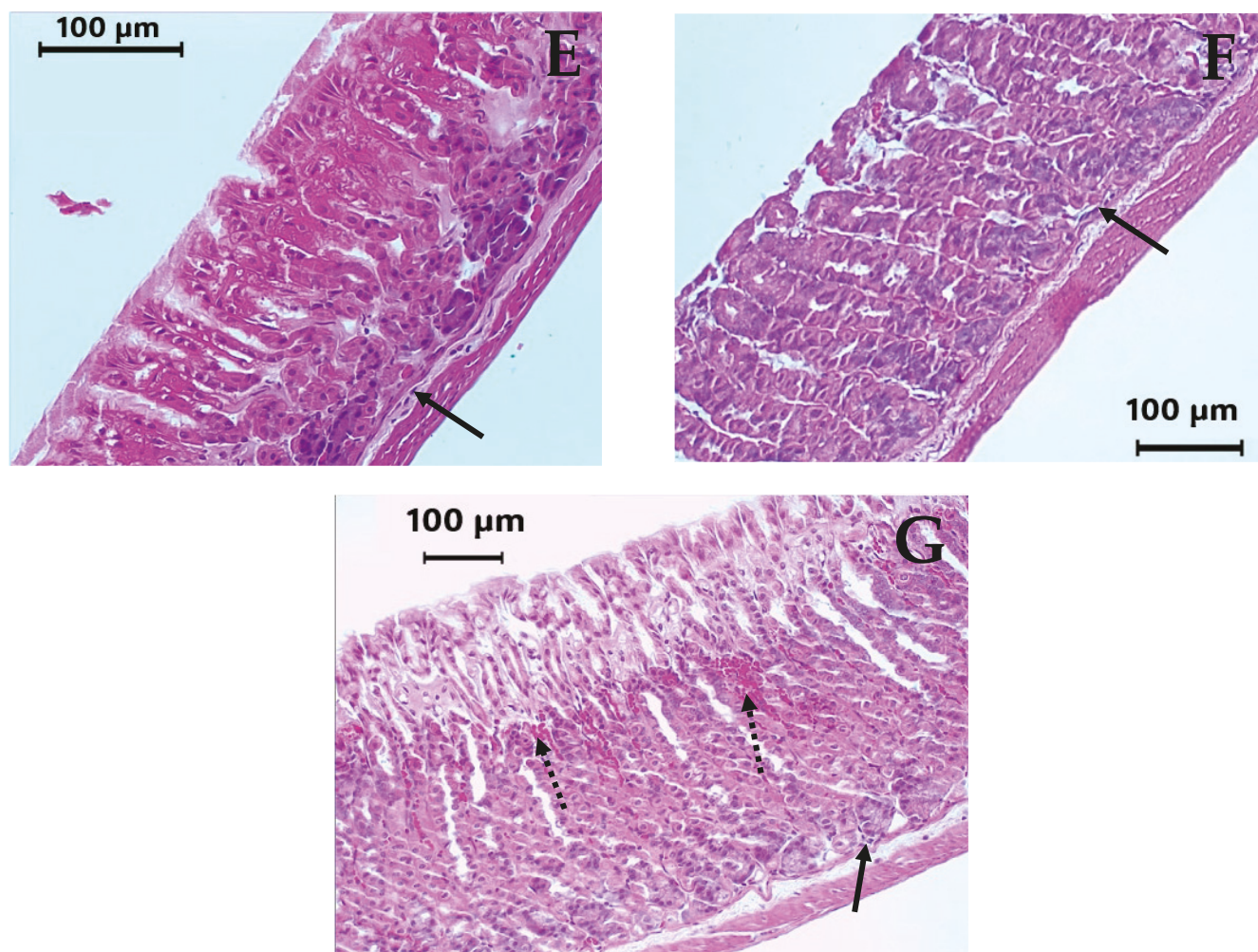


Figure 5. Representative microphotographs of the glandular portion of the mouse stomach after administration of different treatments. (H&E, 200×). (A) Normal stomach. (B) Carbenoxolone (50 mg/kg BW). (C) Negative control, NSS. (D) Vehicle, 0.5% CMC (E) Curcumin (200 mg/kg BW). (F) DAC₂-Cu (200 mg/kg BW). (G) DAC₂-Zn (200 mg/kg BW). Dotted black arrows: erythrocyte infiltrate. Black arrows: inflammatory infiltrate.

Table 3. Percentage of gastroprotection.

Treatment	50 mg/kg	100 mg/kg	200 mg/kg
Carbenoxolone	78.3 ± 4.8		
Curcumin	-	65.5 ± 8.4	71.1 ± 3.4
DAC	24.8 ± 19.0	20.3 ± 13.4	18.6 ± 8.6
DAC ₂ -Cu	19.0 ± 6.1	56.5 ± 7.0 *	71.2 ± 5.5 *
DAC ₂ -Zn	43.6 ± 10.8	59.3 ± 6.5	76.6 ± 6.3
DAC ₂ -Mn	20.8 ± 11.6	21.1 ± 16.5	24.0 ± 15.6
DAC ₂ -Mg	0 ± 10.0	0.2 ± 19.5	11.4 ± 14.3

* Statistically significant differences with respect to the dose of 50 mg/kg of the compound ($p < 0.05$).

2.5. Subacute Toxicity Assay of DAC₂-Cu and DAC₂-Zn

Subacute toxicity was evaluated by administering a single daily oral dose of 200 mg/kg BW of either DAC₂-Cu or DAC₂-Zn to groups of CD1 mice for 28 days, in addition to a control group that received the vehicle (0.5% carboxymethyl cellulose solution, CMC). Recovery groups were also included for the same variables, which were observed for 28 days after the treatment was suspended.

No deaths were recorded in any of the groups, nor were any changes in the general behavior of the animals, except mild fur changes in all groups during the 56 days of the experiment, not attributed to the treatments. There were no significant differences ($p > 0.05$) in body weight between the treatment groups and the control group throughout the experiment (Figure S1). In general, the organ-weight ratio was also similar between the control and treatment groups for both the experimental and recovery phases (Figure 6). It was found that the stomachs of animals treated with DAC₂-Zn, and after the recovery stage, were significantly smaller ($p = 0.031$) than their corresponding experimental group. Similarly, the spleens of control mice (VEH) in the recovery phase are smaller ($p = 0.040$) than their experimental counterparts.

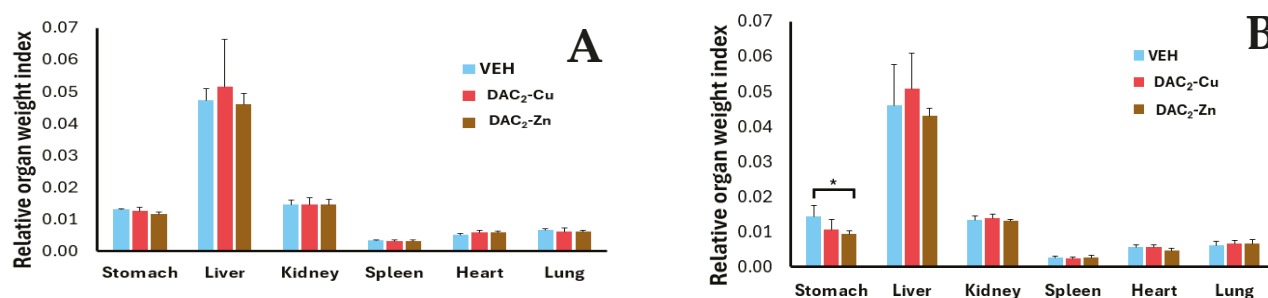


Figure 6. Relative organ weight index of mice. (A) experimental phase, and (B) recovery phase. There were no statistically significant differences ($p < 0.05$) in the relative weight of the organs between the control (VEH) and the DAC₂-Cu and DAC₂-Zn treatments, except in the case of the stomachs of the mice in the recovery group that received DAC₂-Zn, which were significantly smaller than their controls ($p < 0.05$) (*). Data presented as mean $\pm$ SD.

Macroscopic examination of the organs revealed no treatment-related findings in the general appearance and the architecture of the vital organs, except for a darkening in the upper right lobe of the lung in 3/5 animals of the DAC₂-Zn experimental group and 2/4 animals of the DAC₂-Cu recovery group (Table S2 and Figure S2).

In the histopathological examination, the livers of VEH groups (experimental and recovery phases) stained with H&E exhibited typical histological characteristics (Figures 7A and 7B). They had a normal structure with central veins and portal triads. The hepatocytes were arranged in cords that radiated outward from the central veins (plates), and there was a network of sinusoids between the hepatocyte cords. The hepatocytes showed a typical feature in the shape and size, with eosinophilic cytoplasm containing small vacuoles and visible acidophilic nuclei. In contrast, the liver of DAC₂-Cu and DAC₂-Zn treated groups exhibited a disorganized cellular structure, with loss of the radial liver plate and sinusoids, and extensive central veins congestion was observed. The hepatocytes showed slightly acidic cytoplasm and increased vacuolization, compared to the control group (Figures 7C and 7E). Some cells in degeneration/necrosis can also be seen (Figure S3). However, the histological images of the recovery phase for both treatments showed a clear improvement of damaged liver tissue (Figure 7C vs. Figures 7D and 7E vs. Figure 7F), and the radial organization of the hepatocyte cords and sinusoids is restored. Likewise, congestion of the central veins is reduced. The acidophilic cytoplasm of the hepatocytes disappears completely, although vacuolization persisted.

Regarding the kidney, the control VEH group of mice showed normal histological structure of the renal parenchyma, with intact tubular lining cells and glomeruli as evidenced by H&E staining (Figure 8A). The groups treated with DAC₂-Cu and DAC₂-Zn showed no histopathological alterations (Figures 8C and 8E). Similarly, all recovery groups demonstrated normal structural organization (Figures 8B, 8D and 8F).

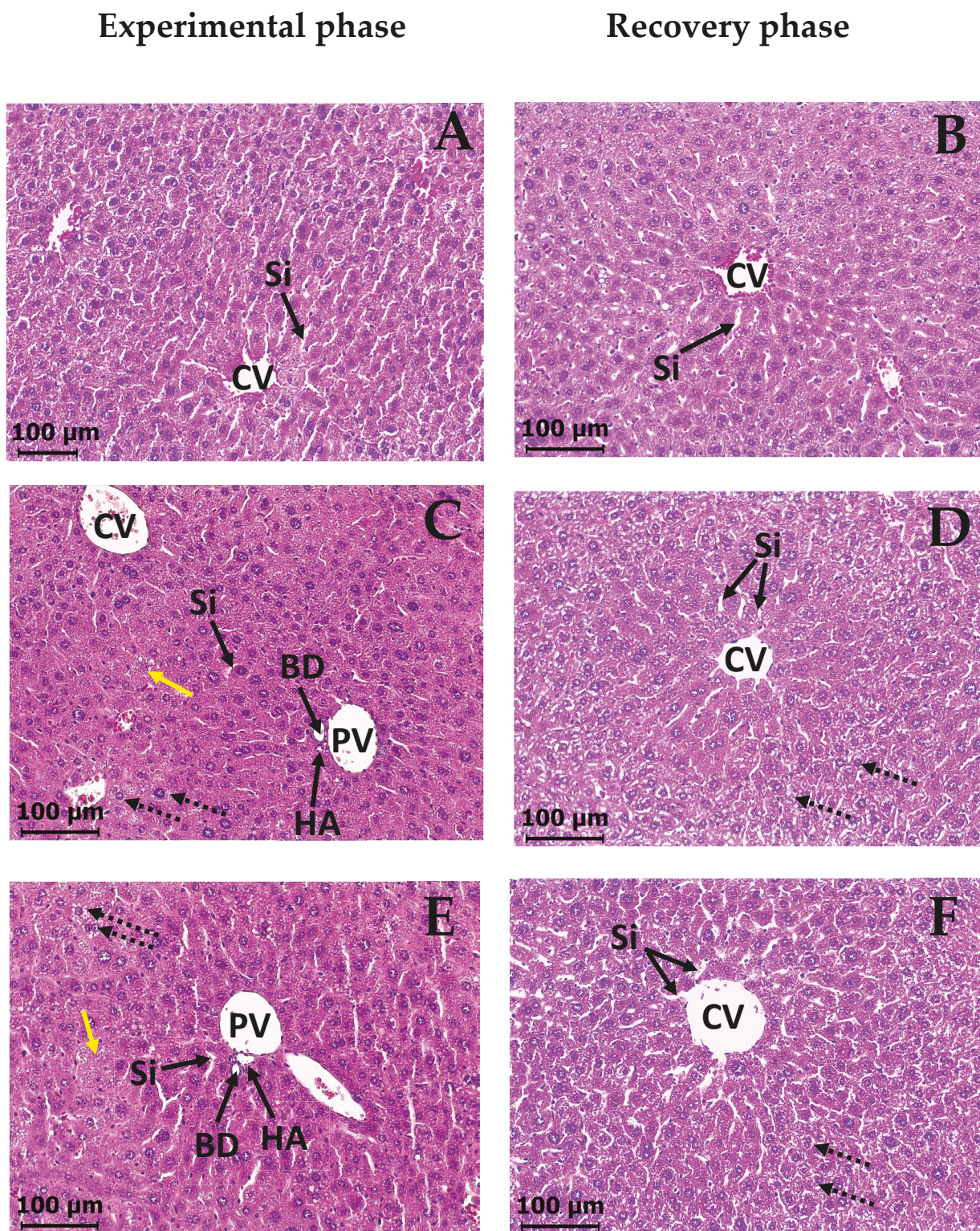


Figure 7. Representative microphotographs of a mouse liver section after DAC₂-Cu and DAC₂-Zn administration. (H&E, 200). (A) Control, vehicle 0.5% CMC, (C) DAC₂-Cu, 200 mg/kg BW, and (E) DAC₂-Zn, 200 mg/kg BW of the experimental phase. (B) Control. (D) DAC₂-Cu, and (F) DAC₂-Zn, corresponding images from the recovery phase. (PV) portal vein. (Si) sinusoids. (HA) hepatic artery. (BD) bile duct. (CV) central vein. Vacuolar degeneration (dotted black arrows). Apoptotic cells (yellow arrows).

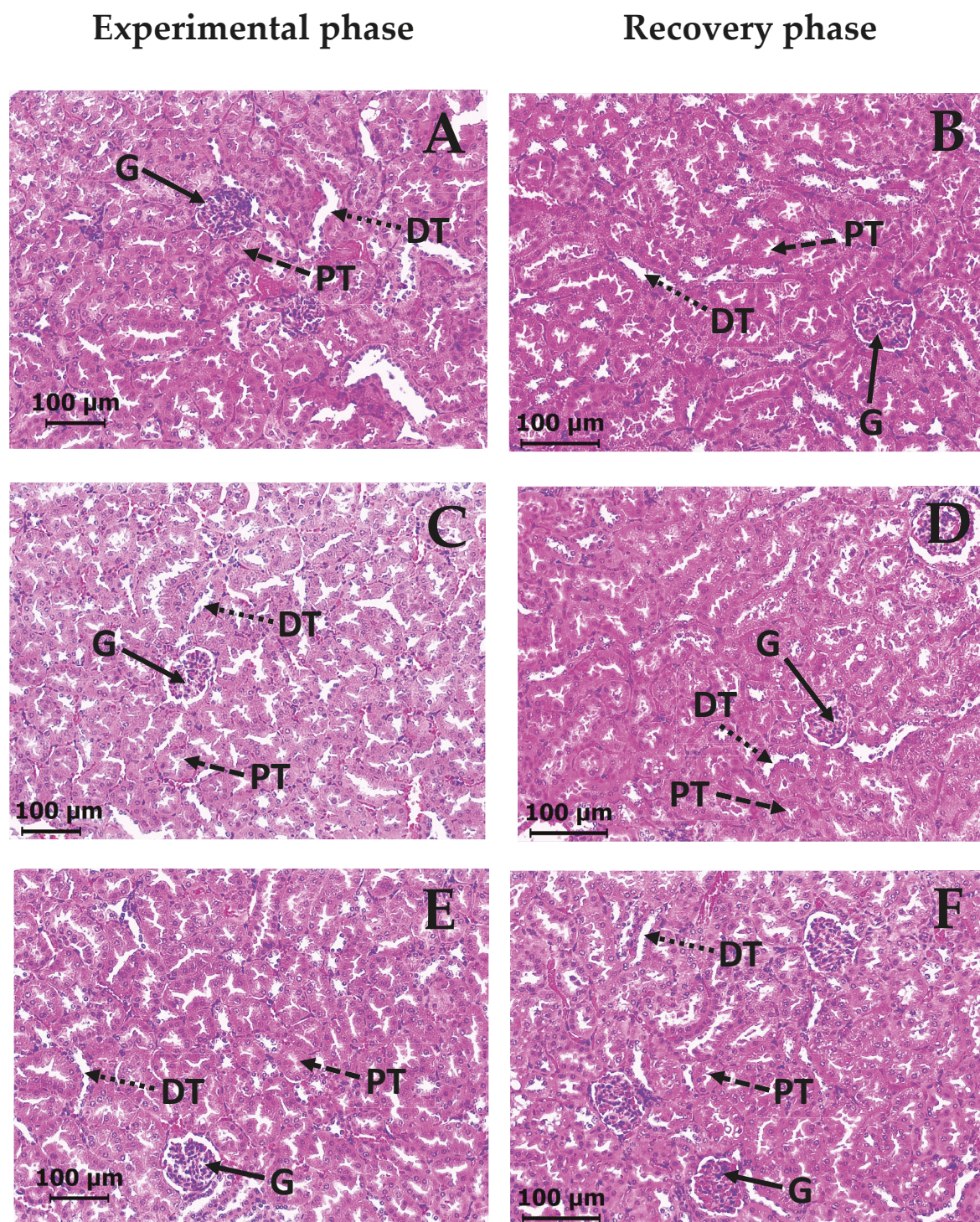


Figure 8. Representative microphotographs of a mouse kidney section after DAC₂-Cu and DAC₂-Zn administration. (H&E, 200×). (A) Control, vehicle 0.5% CMC, (C) DAC₂-Cu, 200 mg/kg BW, and (E) DAC₂-Zn, 200 mg/kg BW of the experimental phase. (B) Control. (D) DAC₂-Cu, and (F) DAC₂-Zn, corresponding images from the recovery phase. (G) glomerulus. (PT) proximal tubule. (DT) distal tubule.

The clinical biochemistry results are presented in Table 4. No statistically significant changes were observed in DAC₂-Cu and DAC₂-Zn treated animal groups compared to the controls in any of the serum parameters tested: total protein, albumin, creatinine, total bilirubin, glucose, and alanine aminotransferase and lactate dehydrogenase enzymes in

either the experimental or recovery phases. The same can be observed for total protein, creatinine, and glucose in urine samples.

Table 4. Clinical biochemistry parameters on CD1 mice administered orally with 0.5% CMC (vehicle), DAC₂-Cu (200 mg/kg BW), and DAC₂-Zn (200 mg/kg BW) for 28 days.

Serum	Experimental Phase			Recovery Phase		
	Vehicle	DAC ₂ -Cu	DAC ₂ -Zn	Vehicle	DAC ₂ -Cu	DAC ₂ -Zn
Total protein	5.3 ± 0.3	5.5 ± 0.4	5.4 ± 0.4	5.2 ± 0.5	5.8 ± 1.0	5.6 ± 0.4
Albumin	3.5 ± 0.5	3.5 ± 0.4	3.5 ± 0.4	3.1 ± 1.01	3.4 ± 0.3	3.5 ± 0.7
Creatinine	0.37 ± 0.08	0.30 ± 0.06	0.34 ± 0.04	0.44 ± 0.18	0.37 ± 0.10	0.31 ± 0.14
Total bilirubin	0.17 ± 0.05	0.19 ± 0.062	0.16 ± 0.03	0.25 ± 0.04	0.34 ± 0.04	0.29 ± 0.05
Glucose	164.9 ± 52.8	178.7 ± 41.1	147.3 ± 14.0	200.6 ± 25.6	232.5 ± 29.2	203.6 ± 56.9
ALT	25.0 ± 14.8	12.3 ± 9.0	24.0 ± 8.2	21.5 ± 8.7	23.4 ± 12.0	20.0 ± 5.2
LDH	891.8 ± 395.8	1465.1 ± 636.7	1051.1 ± 220.2	1945.1 ± 1399.5	1789.7 ± 916.3	1205.8 ± 283.5
Urine						
Total protein	3.9 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	5.1 ± 0.3	5.6 ± 0.06	5.4 ± 0.3
Creatinine	25.56 ± 1.9	22.17 ± 5.3	28.16 ± 6.9	16.33 ± 8.0	22.36 ± 4.9	25.89 ± 10.7
Glucose	0.7 ± 1.0	0.1 ± 0.5	1.5 ± 0.7	2.2 ± 2.2	3.4 ± 3.1	2.0 ± 1.6

ALT: Alanine aminotransferase. LDH: Lactate dehydrogenase. According to the nonparametric Kruskal–Wallis test, there are no statistical differences between the vehicle and the treatments (DAC₂-Cu and DAC₂-Zn) for any of the parameters ($p < 0.05$).

3. Discussion

Several studies have recently focused on the identification of novel alternatives for *H. pylori* eradication that exhibit superior efficacy in comparison with conventional therapeutic regimens, reduced adverse effects, and greater accessibility for patients. Curcumin, extracted from the rhizome of *Curcuma longa*, has attracted significant attention due to its numerous reported pharmacological effects [17]. These effects include anti-*H. pylori* activity, antiulcerogenic properties, and anticancer activity [9,18–20]. However, the limited bioavailability of curcumin due to high decomposition, low absorption, and rapid metabolism and excretion has limited its therapeutic use [17]. Intending to enhance the pharmacokinetic profile of the compound, Dr. Enriquez’s group used DAC to synthesize DAC₂-Mg, DAC₂-Mn, DAC₂-Cu, and DAC₂-Zn [14]. In this context, we decided to explore whether the chemically modified compounds could be exploited for use as new alternatives for *H. pylori* eradication or even for the treatment of its associated diseases, such as gastric ulcers.

DAC and its derivatives successfully inhibited the growth of *H. pylori* (Table 1). The activity of all compounds was higher than metronidazole’s effect, though not compared to clarithromycin. Since the *H. pylori* strain used here is metronidazole-resistant, having compounds that improve the antimicrobial activity could contribute to eradication therapies. DAC₂-Cu and DAC₂-Mn have the best anti-*H. pylori* activity (MIC = 15.6 µg/mL) among DAC and its metal derivatives.

The anti-*H. pylori* activity of natural plant-derived products has previously been reported. These products include flavonoids, quinones, coumarins, terpenoids, alkaloids, and phenolic compounds, such as curcumin, whose MICs range from 0.156 to 25 µg/mL, or greater than 100 µg/mL for some coumarins [21].

Curcumin was included in the assay as a control, exhibiting an MIC of 1.95 µg/mL. This value is lower than those previously reported [8,9]; the difference could be due to the method used for the evaluation, which in this work was by broth dilution, and in the other cases were by agar dilution and agar well diffusion.

Interestingly, neither DAC nor the derivatives improved the MIC of curcumin. This finding suggests that acetylation of the molecule is deleterious to anti-*H. pylori* activity;

however, the formation of metal complexes, particularly with copper and manganese, enhanced the DAC effect (Table 1).

To explore the specificity of the compounds, their effect against other bacteria was investigated. At the highest concentration used (62.5 µg/mL), at which DAC and all its metal complexes inhibited *H. pylori* at 100%, none of them significantly inhibited the growth of *E. coli* (EPEC), *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* (Table 2). These compounds do not appear to act based on the type of bacterial cell wall, as *H. pylori*, EPEC, and *S. enterica* are Gram-negative, whereas *S. aureus* is Gram-positive. This indicates a specificity toward *H. pylori*, which could be advantageous in eradication therapies by providing a more precise and effective antibiotic with a lower risk of antimicrobial resistance development. However, this should be explored with a larger number of pathogenic bacteria.

This assay also included curcumin as a control, this compound is known to have a broad in vitro bactericidal spectrum against Gram-positive and Gram-negative bacteria, including members of the species tested in this work [22,23]. At the concentrations used in this study, curcumin inhibits, but not completely, the growth of EPEC, *S. enterica*, or *S. aureus*. However, compared to the effects of DAC and its metal derivatives, curcumin is more efficient. Although there are no data on the antibacterial activity of DAC metal complexes, there are few reports on the effect of DAC on the growth of certain bacteria. Tajbakhsh et al. [24] found that the compound did not affect *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, or *E. coli* at concentrations up to 373 µg/mL, which is consistent with our results. On the other hand, a mild effect against *Mycobacterium tuberculosis* was observed (MIC = 200 µg/mL) [25]. Also, a significant DAC activity was reported against *Enterococcus faecalis* [26] and *Streptococcus mutans* [27], with an MIC value of 15.6 µg/mL for both.

In an effort to elucidate the mechanism by which DAC and the metal complexes inhibit *H. pylori*, their effect on the activity of two essential enzymes, urease and bacterial gyrase, was evaluated.

The urease of *H. pylori* is vital for colonization in the human stomach. This cytosolic enzyme, which represents 6–10% of the total protein, uses nickel as a cofactor and is primarily responsible for the bacterium's acid acclimation. The hydrolysis of urea into ammonia and carbon dioxide contributes to buffering the environment of the bacteria by protecting them from damage caused by gastric acid and allows maintenance of proton motive force for bacterial growth in the gastric environment [28].

Other functions attributed to *H. pylori* urease include reducing mucus viscosity, altering epithelial integrity, inducing proinflammatory cytokines, and protecting against reactive oxygen species. Urease activity is also associated with an increased risk of histopathological alterations and gastric carcinogenesis [15].

It has been reported that *H. pylori* strains that do not express urease are not able to colonize the human stomach [29–31]. Considering the role of this enzyme in the colonization and virulence of the bacterium, it has become an important therapeutic target.

Figure 2 shows that DAC₂-Zn and DAC₂-Cu were the curcuminoids that most effectively inhibited *H. pylori* urease activity in a concentration-dependent manner. They reached 86.3% and 75% inhibition, respectively, at 62.5 µg/mL. Higher concentrations could not be used due to solubility issues. Analyzing the IC₅₀ values obtained for these complexes (12.4 µM for DAC₂-Zn and 20.4 µM for DAC₂-Cu) and the value calculated for AHA (a competitive and irreversible inhibitor of the enzyme with an IC₅₀ value of 488.4 µM) reveals the higher efficiency of the metal complexes. Curcumin and DAC, on the other hand, inhibited urease by less than 30% at 125 µg/mL (339.3 and 276.2 µM, respectively). Considering that these two compounds retain a β-diketonic center in their structure, which has transition

metal chelating capacity (e.g., nickel), one might expect these two compounds to inhibit urease; however, this was not observed. Unexpectedly, DAC₂-Mn increased urease activity by 76.3% at 62.5 µg/mL. This is not particularly relevant to this study, but urease activation could be useful in other applications, such as in the bioremediation industry [32]. One possible explanation for the activating effect of DAC₂-Mn could be its interaction with the protein in such a way that it modifies its structure, making catalysis at the active site more efficient; further studies, such as molecular docking, are required to establish whether this possibility is feasible.

Comparing the *H. pylori* growth inhibitory effects of DAC₂-Zn and DAC₂-Cu (MIC = 31.2 and 15.6 µg/mL, respectively) with their corresponding urease enzyme inhibition (~80% at 62.5 µg/mL, in both cases), more compound is required to inhibit the enzyme than to stop bacterial growth. DAC₂-Mg did not affect urease but had anti-*H. pylori* activity (MIC = 31.2 µg/mL), while DAC₂-Mn activated the enzyme, which could be advantageous for *H. pylori*, but in contrast, it was one of the compounds with higher activity against the bacteria (MIC = 15.6 µg/mL). These data lead us to conclude that the anti-*H. pylori* mechanism of action of DAC and its metal derivatives is not through urease inhibition, although it could be a secondary target for DAC₂-Zn and DAC₂-Cu.

Another essential enzyme of *H. pylori* is DNA gyrase, a type II topoisomerase found in bacteria but not in mammals. This enzyme is unique in introducing ATP-dependent negative supercoils during DNA replication and transcription, preventing strand stress [16]. Quinolones are the only antibiotics used in *H. pylori* eradication therapies that inhibit DNA gyrase activity, leading to DNA fragmentation and bacterial death [16,33]. However, the emergence of *H. pylori* strains resistant to levofloxacin, the quinolone most used in traditional therapies, has made DNA gyrase an important pharmacological target that has been studied in recent years.

In this work, the *E. coli* DNA Gyrase Drug Screening kit from TopoGEN was used to determine the effect of DAC and its metal derivatives on the supercoiling activity of the enzyme. DNA gyrases are highly conserved enzymes in function and structure across bacteria [34,35]; therefore, the results obtained are likely to reflect the effects that the compounds could have on the *H. pylori* enzyme.

Under the experimental conditions and within the concentration range of 0.1–50 µM, none of the compounds, including curcumin, inhibited DNA gyrase activity. On the contrary, the positive control ciprofloxacin inhibited 100% the supercoiling activity with 3 µM. This suggests that bacterial DNA gyrase is unlikely to be the target of DAC or its metal derivatives in *H. pylori*. However, this result on *H. pylori* DNA gyrase remains to be corroborated.

To further explore the pharmacological properties of the compounds, the gastroprotective effect of DAC and its metal derivatives was investigated in a model of acute ulceration induced with absolute ethanol in CD1 mice. This model is widely used to study the efficacy of compounds that are believed to have gastroprotective effects.

Ulcers are lesions that originate in the gastric mucosa due to an imbalance between protective and harmful mucosal factors. Ethanol causes acute hemorrhages, vascular congestion, edema, and necrosis of the mucosa by solubilization of the mucus layer, which leaves the superficial mucous cells in contact with stomach acid and proteolytic enzymes that damage the cell membrane. Exposure to ethanol also increases HCl secretion, decreases blood flow, increases permeability and free radical production, and alters bicarbonate secretion, all these factors together alter the structure of gastric mucosa [36].

Among the compounds tested, only DAC₂-Cu and DAC₂-Zn showed gastroprotective activity, with maximum values of 71.2% and 76.6%, respectively, at 200 mg/kg BW (Table 3). Macroscopic and microscopic histological analyses of the stomachs confirmed the anti-ulcer

effect of the compounds. Their gastroprotective activity was comparable to the positive control, carbenoxolone, which showed 78.3% protection at 50 mg/kg BW.

Similarly, the gastroprotective effect of the compounds was comparable to the effect obtained with 200 mg/kg of curcumin (71.1%). Curcumin's antiulcer activity has been widely demonstrated in many ulceration models [37–40]. Percentages vary depending on the agent used to generate the ulcer, but differences have also been reported in the same model. For instance, using an ethanol-induced ulcer model in rats similar to the one in this study, Chattopadhyay et al. [37] reported 95% gastroprotection at a dose of 25 mg/kg, whereas Czekaj et al. [39] obtained 89% at 100 mg/kg.

The differences in effects observed in in vitro compound susceptibility experiments and in vivo gastroprotection experiments are due to the different metabolic conditions to which the complexes are exposed. In vitro experiments involve direct contact between the compounds and the bacteria, whereas gastroprotection experiments may involve a local effect in the stomach or exposure to the animal's own biotransformation.

The present work is the first report on the antiulcer activity of DAC and its metal complexes. To our knowledge and regarding complexes with curcumin, the gastroprotective activity of a Zn(II)–curcumin complex in solid dispersions (SDs) has been reported in pylorus-ligature [41], cold-restraint stress [42], and ethanol [43] induction of gastric ulcer models. The results showed gastroprotective percentages of 73.7, 81.1, and 75.0%, respectively, at a dose of 48 mg/kg of BW. The authors also report that the percentage achieved with the complex is slightly higher than the gastroprotective effect of curcumin SDs. The complex was characterized using mass spectrometry, thermal gravimetry, and differential thermal analysis. These analyses indicated that the curcumin-Zn(II) complex contains a zinc atom that is coordinated through the keto-enol group of curcumin, as well as an acetate group and a water molecule [41].

Although our results confirm the anti-ulcerogenic effect of DAC₂-Cu and DAC₂-Zn, further studies are needed to elucidate their precise gastroprotective mechanism.

The in vitro and in vivo results obtained in this study lead us to identify two of the compounds studied, DAC₂-Cu and DAC₂-Zn, as candidates for use in *H. pylori* eradication therapies and in the treatment of diseases associated with it. Both compounds have very good inhibitory activity against *H. pylori* growth (MIC = 15.2 and 31.2 µg/mL, respectively), which appears to be species-specific since they did not inhibit other bacteria; they inhibit the urease of the bacterium (IC₅₀ = 13 and 21.2 µg/mL, respectively), which may be one of their mechanisms of action; and they also have a good gastroprotective effect (71% and 76%, respectively, with 200 mg/kg). Therefore, in order to be implemented in *H. pylori* eradication therapies and in the treatment of associated diseases, such as peptic ulcers, it is necessary to determine their pharmacological biosafety. Toxicity studies provide essential data on a compound's safety profile, identifying risks before human clinical trials.

In this regard, the only study conducted to evaluate the in vivo toxicity of DAC and its four metal derivatives was an acute toxicity study in a murine model, conducted by Meza-Morales et al. [14]. The authors observed no clinical signs of toxicity with any of the compounds administered orally at doses up to 3000 mg/kg BW. Slight hair frizzing was only observed with DAC₂-Cu and DAC₂-Mn treatments. However, these results only provide an initial indication of the gross signs of toxicity of the compounds and do not reflect long-term continuous exposure.

Eradication therapy for *H. pylori* can vary from 10 to 14 days, depending on the type, to achieve the best effectiveness rates [3]. Therefore, in accordance with OECD Guidelines for the Testing of Chemicals 407 [44], we consider that a 28-day study at a fixed dose of 200 mg/kg BW would provide the basic necessary information on the effects of repeated

oral exposure to DAC₂-Cu and DAC₂-Zn. The dosage was chosen based on our previous in vitro and in vivo results.

The subacute toxicity study was divided into two phases. In the first experimental phase, one group was treated with the vehicle, and two others were treated with DAC₂-Cu and DAC₂-Zn at a dose of 200 mg/kg BW for 28 days. In the second phase, the recovery phase, three other groups were treated the same as in the first phase, but after finishing administration, these groups were observed for an additional 28 days to detect any toxic effects or reversibility of the effects produced by the compounds.

During our study, no mortality was observed in any of the groups during the 56-day evaluation period (28 days of experimental phase and 28 days of recovery phase). The administration of DAC₂-Cu and DAC₂-Zn did not cause abnormal alterations in the behavior of mice. No significant modifications in body weight between treated and untreated groups of animals were found. In general, no macroscopic changes in the organs or considerable alterations in the relative organ weight were observed. These findings suggest that the experimental compounds do not alter the normal growth and development of the animals. Furthermore, the biochemical parameters that were determined did not show any significant alterations compared to the control group.

The major sites of metabolism and excretion of many chemicals and drugs are the liver and the kidneys. Histopathological analysis demonstrates no renal toxicity. Tissue samples show no evidence of cellular damage, inflammation, or other abnormalities, suggesting that DAC₂-Cu and DAC₂-Zn are not nephrotoxic. On the other hand, the analysis of the liver indicates that these compounds induce some changes in the liver structure (Figures 7C and 7E), such as loss of overall cellular structure, venous congestion, and increased vacuolization of hepatocytes. However, these abnormalities are almost completely reversed 28 days after the administration of the compounds has ended (recovery phase, Figures 7D and 7F), which demonstrates that the injury caused by the compounds is reversible and that the tissue is expected to recover completely. The observed changes in the liver's histological architecture 28 days after administration of the compounds probably represent the onset of a low-grade, temporary inflammatory process, as there is no massive infiltration of immune cells. In these circumstances, there is no alteration to liver-specific biochemical parameters, suggesting that the organ's physiology has not yet been affected.

As these compounds are intended for use within a sufficient timeframe for treating *H. pylori*-related diseases (10–14 days), they do not pose a health risk. However, based on the results obtained in this study, prolonged administration is not recommended until any potential long-term effects on liver function have been confirmed or ruled out.

Considering that the lability of the complexes within the organism has not yet been demonstrated, it would be advisable to quantify the concentrations of free ions and ascertain their potential impact on the experiments conducted in this study.

The findings of this study are promising because they provide concrete data on the toxicological safety of the curcuminoid complexes DAC₂-Cu and DAC₂-Zn when administered daily for 28 days in an in vivo model. However, to evaluate the safety and therapeutic potential of these compounds in humans, further research is necessary at a preclinical level to understand their mechanism of action, to know more about their toxicological profile, and their therapeutic efficacy. Finally, their pharmacokinetic and pharmacodynamic properties must be assessed.

4. Materials and Methods

4.1. Curcumin, DAC, and Its Metal Derivatives

Diacetylcurcumin and its metal derivatives (DAC₂-Cu, DAC₂-Zn, DAC₂-Mn, and DAC₂-Mg) were synthesized by Dr. Raúl G. Enríquez's group from the Chemistry Institute

of UNAM according to reference [14]. Curcumin was extracted from a natural source through conventional procedures and subsequently purified by crystallization.

4.2. Strains and Growing Conditions

Helicobacter pylori ATCC 43504 was cultured on Casman Agar (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood, 10 mg/L vancomycin, 5 mg/L trimethoprim, 2 mg/L amphotericin B, and 2.5 mg/L polymyxin B at 37 °C for 24 h under microaerophilic conditions (10% CO₂, 5% O₂, 85% N₂). Cultures were stored at −70 °C in Brucella broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies Corporation, Grand Island, NY, USA) and 10% glycerol. The strains were identified by Gram staining and biochemical tests.

Escherichia coli O127:H6 E2348/69 (EPEC), *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 25923 were grown on Luria–Bertani (LB) agar at 37 °C for 24 h under aerobic conditions. Cultures were stored at −70 °C in Brucella broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies Corporation, Grand Island, NY, USA) and 50% glycerol. The strains were identified by Gram staining and biochemical tests.

4.3. Antibacterial Activity

All strains were evaluated for antimicrobial susceptibility according to Clinical and Laboratory Standards Institute [45] using the broth dilution method. Bacteria were grown in Mueller-Hinton broth (DIFCO, Becton Dickinson and Company, Sparks, MD, USA). For *H. pylori*, it was supplemented with 0.2% β-cyclodextrin, vancomycin (10 mg/L), trimethoprim (5 mg/L), amphotericin B (2 mg/L), and polymyxin B (2.5 mg/L). Different concentrations of the compounds (3.9–62.5 µg/mL), dissolved in dimethyl sulfoxide (DMSO), were added to the bacterial cultures at the beginning of the log growth phase (1×10^8 CFU/mL). ΔA₆₀₀ was measured after 24 h of incubation under microaerophilic conditions, 37 °C, and shaking at 150 rpm for *H. pylori*, and after 3.5 h of incubation at 37 °C, aerobic conditions, and 180 rpm for *E. coli*, *S. aureus*, and *S. enterica*; in all cases, the time corresponded to the end of the log phase of growth. Percentage inhibition was calculated relative to a culture growing only in the presence of solvent DMSO (100% growth). The final solvent concentration in the assay did not affect the experiments. The Minimum Inhibitory Growth Concentration (MIC) was defined as the lowest concentration of the compound at which there is no growth. Metronidazole and clarithromycin were used as positive controls for *H. pylori*, and ampicillin for the other bacteria.

4.4. Urease Assay

4.4.1. Partial Purification of Urease

Urease was obtained from an *H. pylori* broth culture in the logarithmic phase of growth. Bacteria were washed twice with PBS pH 7.4 and resuspended in PBS/protease inhibitor cocktail (cOmplete Roche TM, Roche Applied Science, Mannheim, Germany). Subsequently, the sample was subjected to sonication at 40 W in a Branson 250 Sonicator (Branson Ultrasonics Co., Danbury, CT, USA) at 4 °C. This process was repeated for three intervals of 30 s, with a 30 s rest period between each cycle. The lysate was centrifuged at 13,500 rpm (Centronix Microcentrifuge, Spintron Inc. Metuchen, NJ, USA) for 10 min at 4 °C. The supernatant was used as the urease source and was stored at 4 °C until use. Protein content was quantified by Bradford's method using bovine serum albumin as a standard [46].

4.4.2. Urease Inhibition Assay

The urease activity was evaluated by quantifying the NH_4^+ liberated after the hydrolysis of urea using the colorimetric Berthelot method with some modifications [47]. The reaction included in a final volume of 130 μL , 3 μg protein of urease which were preincubated for 5 min at 37 °C with 5 μL of the compounds dissolved in DMSO at different concentrations (3.9–125 $\mu\text{g}/\text{mL}$), to start the reaction, 20 μL of 37.5 mM urea (5 mM final concentration) were added and incubated for 10 min at 37 °C. For the colorimetric reaction, 50 μL of 354 mM phenol and 100 μL of 345 mM NaOH/176 mM NaClO were added. After 5 min, the absorbance was recorded at 600 nm using a Bio-Rad 2550 EIA reader (Bio-Rad Laboratories, Inc., Pleasanton, CA, USA). Ammonium sulfate was used as a standard for the calibration curve. The percentage of enzymatic activity was calculated with respect to the activity without inhibitor. Acetohydroxamic acid (AHA) was used as a positive control.

4.5. DNA Gyrase Supercoiling Assay

The *E. coli* DNA Gyrase Drug Screening kit from TopoGEN (TopoGEN, Inc. Port Orange, FL, USA) was used. The kit is based on gyrase supercoiling activity on a relaxed plasmid DNA substrate. The reaction medium contained 4 μL of 5 $\times$ buffer (35 mM Tris-Cl, pH 7.5, 24 mM KCl, 4 mM MgCl_2 , 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, and 0.1 mg BSA/mL), 0.3 μL of relaxed plasmid pHOT1 (0.3 μg), and 0.15 μL of DNA gyrase (1 U/ μL) in a final volume of 20 μL . To evaluate the effect of the compounds on DNA gyrase supercoiling activity, 1 μL of different concentrations (0.1–50 μM) of the compounds dissolved in DMSO was added before DNA gyrase. The reactions were incubated at 37 °C for 1.5 h. The products of the reactions were run on a 1% agarose gel at 50 V, stained with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide, and photodocumented on a UV transilluminator. The supercoiled plasmid was quantified with ImageJ[®] free software, version 1.54d. 100% of the activity corresponded to a reaction in which only the solvent (DMSO) was added. Ciprofloxacin (3 μM , final concentration) was used as a positive control.

4.6. In Vivo Gastroprotective Activity

4.6.1. Animals

Adult male CD1 mice weighing 35–40 g provided by the vivarium of the Faculty of Medicine of UNAM were used. The animals were maintained at a controlled temperature of 21 ± 2 °C in 12/12 h light/dark cycles with free access to water and standard rodent food. The experimental protocol was approved by the Ethics Committee for the Care and Use of Laboratory Animals, Faculty of Medicine, UNAM, Mexico City, Mexico (CICUAL 076/2021) and was carried out in accordance with the Official Mexican Standard of Care and Animal Handling and in compliance with international guidelines for ethical standards for animal research.

4.6.2. Anti-Ulcerogenic Activity

Acute gastric ulceration with ethanol was performed according to the method described by Bucciarelli [36]. The mice were divided into groups, $n = 6$. The negative control groups received either isotonic sodium saline solution (NSS) or the vehicle, 0.5% sodium carboxymethylcellulose (CMC), both at 7 mL/kg. Carbenoxolone, 50 mg/kg body weight (BW) in NSS, in the case of the positive control. The experimental groups received the compounds suspended in the vehicle at doses of 50, 100, and 200 mg/kg BW. All the samples were prepared freshly and administered by gastric gavage. After an hour of administration, all groups received orally absolute ethanol (7 mL/kg) to induce ulceration, and 1.5 h later, the mice were sacrificed in a CO_2 chamber. Each stomach was dissected out, insufflated

with 10% formalin, and fixed in the same solution for 24 h. The stomachs were opened along the greater curvature, pressed between two glass plates, and scanned. Areas of mucosal damage of the glandular stomach estimated in square millimeters were quantified with ImageJ[®] free software. The ulcer index was calculated by the following expression:

$$UI = \frac{\text{Ulcerated area}}{\text{Total stomach area}} \quad (1)$$

And the percentage of gastroprotection by:

$$\%G = \frac{UI \text{ control} - UI \text{ test}}{UI \text{ control}} \times 100 \quad (2)$$

For histological analysis, a 10% formalin fixed glandular portion of the stomach was embedded in paraffin wax, and sagittal sections of 5 µm of thickness were obtained. Tissue sections were stained with hematoxylin-eosin (H&E) and were examined under the light microscope for either morphological or cellular changes.

4.7. Subacute Toxicity Assay

4.7.1. Animals

CD1 male mice aged 6 to 7 weeks, obtained from the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav) animal facility, were used. Animals were randomly grouped in autoclaved bedding in microisolator cages (Super Mouse, model 750, Lab Products LLC, Aberdeen, MD, USA) within an OC 2100 mouse rack (Lab Products Inc., Seaford, DE, USA). Throughout the trial, they were maintained in pathogen-free conditions with 12/12 h light/dark cycles, at 20 and 24 °C, relative humidity 40–70%, and a noise level below 70 dB. The mice had free access to ultrafiltered tap water and sterilized chow (PicoLab Rodent Diet, 5058, Richmond, IN, USA). Animals were acclimatized for one week before experimentation. The experimental procedure was approved by Cinvestav's Internal Committee for the Care and Use of Laboratory Animals (CICUAL, Protocol No. 0413-25) and complied with the Mexican Guideline Regulations of Animal Care and Maintenance and with international guidelines for ethical standards for animal research.

4.7.2. Study Design

The subacute toxicity of DAC₂-Cu and DAC₂-Zn was assessed in CD1 mice according to Organisation for Economic Co-operation and Development (OECD) Test Guidelines 407 [44], with modifications. The animals were separated in three different groups ($n = 5$); the first was the control group (vehicle; 0.5% sodium carboxymethylcellulose (CMC) solution), the second and third groups received orally 200 mg/kg BW of DAC₂-Cu or DAC₂-Zn for a period of 28 days once a day. Three other groups ($n = 4$), corresponding to a recovery phase of the study, were included, and treated exactly as the previous ones, to observe the reversibility, persistence, or delayed onset of possible toxic effects. These groups were kept for 28 days without treatment after the last administration.

The animals were examined daily for signs of toxicity, such as changes in behavioral responses (locomotion, aggression), spontaneous activity (response to tail nibbling and noises), fecal appearance, tremors, bristling, and mortality. Their weight was monitored every third day. At the end of the experimental protocol, the mice were fasted for 12 h. Urine samples were collected during this period. The mice were then euthanized using isoflurane and dissected. Blood was drawn by cardiac puncture for the analysis of biochemical parameters, specifically for the determination of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), as well as for the determination of glucose, total protein, albumin,

creatinine, and bilirubin. Biochemical parameters were determined using commercial Randox kits following the manufacturer's protocols (Randox Laboratories Ltd., London, UK). All organs, mucous membranes, body cavities, etc., were examined for macroscopic pathological alterations. Major organs (stomach, liver, kidney, heart, lung, and spleen) were weighed. Absolute values were recorded, and relative values (organ weight/body weight ratio) were calculated. The organs were preserved in 10% neutral buffered formalin. Histopathological examination of the liver and kidney was performed. Tissues were dehydrated, embedded in paraffin, and sectioned at 5 μ m thickness. Sagittal sections were stained with H&E and examined under the microscope.

4.8. Statistical Analysis

The Kolmogorov–Smirnov test was used to determine the normal distribution of the data, comparisons by dose or concentration were analyzed with the nonparametric Kruskal–Wallis test, and Dunn's post hoc test was used to determine significant differences between groups. For all tests, $p < 0.05$ was considered statistically significant. All data were reported as mean $\pm$ standard deviation (SD), except for gastroprotection results and body weight change analysis, which were reported as mean $\pm$ standard error of the mean (SEM). At least three independent experiments were performed in triplicate for each of the conditions. In animal tests, at least three animals were used per treatment. All statistical tests were performed using the R program, 4.2.1 version [48].

5. Conclusions

The current study highlights the anti-*H. pylori* properties of DAC and its four metal derivatives (DAC₂-Cu, DAC₂-Zn, DAC₂-Mn, and DAC₂-Mg). Interestingly, none of these compounds affected the growth of *E. coli*, *S. enterica*, and *S. aureus*, suggesting bacteriospecific activity against *H. pylori*. Moreover, DAC₂-Zn and DAC₂-Cu also inhibit *H. pylori* urease, with an IC₅₀ lower than the positive control, AHA. Furthermore, these two compounds exhibit gastroprotective effects in mice, achieving approximately 70% protection at a dose of 200 mg/kg BW. It must be pointed out that despite administering this dose for 28 consecutive days to the animals, there was no mortality or irreversible organ damage, as confirmed by anatomical-histological examinations and clinical biochemical analysis performed on the animals.

Consequently, DAC₂-Zn and DAC₂-Cu show promise as novel alternatives for *H. pylori* eradication therapies and as antiulcer agents. Further studies are necessary before they can be implemented for human therapeutic applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules30193849/s1>, Table S1: Effect of compounds (50 μ M) on DNA gyrase supercoil activity; Figure S1: Body weight changes; Table S2: Frequency of there being no treatment-related findings in the appearance of vital mice organs; Figure S2: No treatment-related findings; Figure S3: Representative microphotographs of cells in degeneration/necrosis in the liver of DAC₂-Cu and DAC₂-Zn treated groups.

Author Contributions: Conceptualization, A.A.-M., R.G.E., L.V., and I.R.; formal analysis A.A.-M., E.G.-C., and E.E.-M.; funding acquisition, I.R.; investigation, and methodology, A.A.-M., E.G.-C., E.H.-H. and E.E.-M.; Project administration, I.R.; resources, R.G.E., M.A.O.-M., C.E.-M., and, L.V.; supervision, I.R., R.G.E., and, L.V.; writing—original draft preparation, A.A.-M., E.G.-C., and I.R.; writing—review and editing, E.G.-C., R.G.E., L.V., and I.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by UNAM-DGAPA-PAPIIT (grant numbers IN207622, IN206625).

Institutional Review Board Statement: For the gastroprotection assays, the experimental protocols were approved by the Ethics Committee for the Care and Use of Laboratory Animals, Faculty of Medicine, UNAM, Mexico City, Mexico (CICUAL No. 006-CIC-2021). In the case of subacute toxicity procedures, the Internal Committee for the Care and Use of Laboratory Animals of Cinvestav approved the experimental protocol (CICUAL, Protocol No. 0413-25). All the experiments were conducted in accordance with the Mexican Official Norm of Animal Care and Handling and in compliance with the international guidelines on ethical standards for investigation in animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Acknowledgments: Almanelly Agabo-Martínez (A.A-M.) is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and has received CONAHCYT fellowship No. 1246957. The authors wish to thank Ximena Galicia Alba for her technical assistance in the elaboration of part of the antibacterial experiments.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DAC	Diacetylcurcumin
MIC	Minimum Inhibitory Concentration
PPI	Proton Pump Inhibitor
LD	Lethal Dose
DNA	Deoxyribonucleic acid
IC ₅₀	Half-maximal inhibitory concentration
AHA	Acetohydroxamic acid
CUR	Curcumin
SD	Standard Deviation
BW	Body weight
NSS	Isotonic sodium saline solution
VEH	Vehicle
CMC	carboxymethylcellulose
CAR	Carbenoxolone
SEM	Standard Error of the Mean
H&E	Hematoxylin & Eosin
ALT	Alanine aminotransferase
LDH	Lactate dehydrogenase
SDs	Solid dispersions
OECD	Organisation for Economic Co-operation and Development
UNAM	Universidad Nacional Autónoma de México
ATCC	American Type Culture Collection
LB	Luria–Bertani
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
CFU	Colony Forming Unit
CICUAL	Committee for the Care and Use of Laboratory Animals

References

1. Zamani, M.; Ebrahimitabar, F.; Zamani, V.; Miller, W.H.; Alizadeh-Navaei, R.; Shokri-Shirvani, J.; Derakhshan, M.H. Systematic review with meta-analysis: The worldwide prevalence of *Helicobacter pylori* infection. *Aliment. Pharmacol. Ther.* **2018**, *47*, 868–876. [CrossRef] [PubMed]

2. Kusters, J.G.; van Vliet, A.H.M.; Kuipers, E.J. Pathogenesis of *Helicobacter pylori* infection. *Clin. Microbiol. Rev.* **2006**, *19*, 449–490. [CrossRef]
3. Suzuki, S.; Kusano, C.; Horii, T.; Ichijima, R.; Ikehara, H. The Ideal *Helicobacter pylori* Treatment for the Present and the Future. *Digestion* **2022**, *103*, 62–68. [CrossRef] [PubMed]
4. Hafeez, M.; Qureshi, Z.A.; Khattak, A.L.; Saeed, F.; Asghar, A.; Azam, K.; Khan, M.A. *Helicobacter pylori* Eradication Therapy: Still a Challenge. *Cureus* **2021**, *13*, e14872. [CrossRef]
5. Kotha, R.R.; Luthria, D.L. Curcumin: Biological, Pharmaceutical, Nutraceutical, and Analytical Aspects. *Molecules* **2019**, *24*, 2930. [CrossRef] [PubMed]
6. Priyadarsini, K.I. The chemistry of curcumin: From extraction to therapeutic agent. *Molecules* **2014**, *19*, 20091–20112. [CrossRef]
7. Patel, S.S.; Acharya, A.; Ray, R.S.; Agrawal, R.; Raghuwanshi, R.; Jain, P. Cellular and molecular mechanisms of curcumin in prevention and treatment of disease. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 887–939. [CrossRef]
8. Mahady, G.B.; Pendland, S.L.; Yun, G.; Lu, Z.Z. Turmeric (*Curcuma longa*) and curcumin inhibit the growth of *Helicobacter pylori*, a group 1 carcinogen. *Anticancer Res.* **2002**, *22*, 4179–4181.
9. De, R.; Kundu, P.; Swarnakar, S.; Ramamurthy, T.; Chowdhury, A.; Nair, G.B.; Mukhopadhyay, A.K. Antimicrobial activity of curcumin against *Helicobacter pylori* isolates from India and during infections in mice. *Antimicrob. Agents Chemother.* **2009**, *53*, 1592–1597. [CrossRef]
10. Kundu, P.; De, R.; Pal, I.; Mukhopadhyay, A.K.; Saha, D.R.; Swarnakar, S. Curcumin alleviates matrix metalloproteinase-3 and -9 activities during eradication of *Helicobacter pylori* infection in cultured cells and mice. *PLoS ONE* **2011**, *6*, e16306. [CrossRef]
11. Anand, P.; Kunnumakkara, A.B.; Newman, R.A.; Aggarwal, B.B. Bioavailability of curcumin: Problems and promises. *Mol. Pharm.* **2007**, *4*, 807–818. [CrossRef]
12. Koosirirat, C.; Linpisarn, S.; Changsom, D.; Chawansuntati, K.; Wipasa, J. Investigation of the anti-inflammatory effect of *Curcuma longa* in *Helicobacter pylori*-infected patients. *Int. Immunopharmacol.* **2010**, *10*, 815–818. [CrossRef]
13. Dei Cas, M.; Ghidoni, R. Dietary Curcumin: Correlation between Bioavailability and Health Potential. *Nutrients* **2019**, *11*, 2147. [CrossRef]
14. Meza-Morales, W.; Estévez-Carmona, M.M.; Alvarez-Ricardo, Y.; Obregón-Mendoza, M.A.; Cassani, J.; Ramírez-Apan, M.T.; Escobedo-Martínez, C.; Soriano-García, M.; Reynolds, W.F.; Enríquez, R.G. Full Structural Characterization of Homoleptic Complexes of Diacetylcurcumin with Mg, Zn, Cu, and Mn: Cisplatin-level Cytotoxicity in vitro with Minimal Acute Toxicity in vivo. *Molecules* **2019**, *24*, 1598. [CrossRef]
15. Clyne, M.; Cróinín, T. Pathogenicity and virulence of *Helicobacter pylori*: A paradigm of chronic infection. *Virulence* **2025**, *16*, 2438735. [CrossRef]
16. Ruan, S.; Tu, C.H.; Bourne, C.R. Friend or Foe: Protein Inhibitors of DNA Gyrase. *Biology* **2024**, *13*, 84. [CrossRef]
17. Abd El-Hack, M.E.; El-Saadony, M.T.; Swelum, A.A.; Arif, M.; Abo Ghanima, M.M.; Shukry, M.; Noreldin, A.; Taha, A.E.; El-Tarabily, K.A. Curcumin, the active substance of turmeric: Its effects on health and ways to improve its bioavailability. *J. Sci. Food Agric.* **2021**, *101*, 5747–5762. [CrossRef] [PubMed]
18. Vetvicka, V.; Vetvickova, J.; Fernandez-Botran, R. Effects of curcumin on *Helicobacter pylori* infection. *Ann. Transl. Med.* **2016**, *4*, 479. [CrossRef] [PubMed]
19. Joshi, A.; Lehen, S.; Lasnapure, B.; Pawar, S.; Kandipati, D.; Panchal, P. Investigation of antioxidant, anti-ulcer, and analgesic potential of a metal-curcumin complex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2023**, *396*, 1043–1052. [CrossRef] [PubMed]
20. Boyanova, L.; Medeiros, J.; Yordanov, D.; Gergova, R.; Markovska, R. Turmeric and curcumin as adjuncts in controlling *Helicobacter pylori*-associated diseases: A narrative review. *Lett. Appl. Microbiol.* **2024**, *77*, ovae049. [CrossRef]
21. Wang, Y.C. Medicinal plant activity on *Helicobacter pylori* related diseases. *World J. Gastroenterol.* **2014**, *20*, 10368–10382. [CrossRef] [PubMed]
22. Moghadamtousi, S.Z.; Kadir, H.A.; Hassandarvish, P.; Tajik, H.; Abubakar, S.; Zandi, K. A review on antibacterial, antiviral, and antifungal activity of curcumin. *BioMed Res. Int.* **2014**, *2014*, 186864. [CrossRef]
23. Adamczak, A.; Ożarowski, M.; Karpiński, T.M. Curcumin, a Natural Antimicrobial Agent with Strain-Specific Activity. *Pharmaceuticals* **2020**, *13*, 153. [CrossRef]
24. Tajbakhsh, S.; Mohammadi, K.; Deilami, I.; Zandi, K.; Fouladvand, M.; Ramedani, E.; Asayesh, G. Antibacterial activity of indium curcumin and indium diacetylcurcumin. *Afr. J. Biotechnol.* **2008**, *7*, 3832–3835.
25. Changtam, C.; Hongmanee, P.; Suksamrarn, A. Isoxazole analogs of curcuminoids with highly potent multidrug-resistant antimycobacterial activity. *Eur. J. Med. Chem.* **2010**, *45*, 4446–4457. [CrossRef]
26. Souza, V.; Polaquini, C.R.; de Moraes, G.R.; Oliveira Braga, A.R.; da Silva, P.V.; da Silva, D.R.; Ribeiro Lima, F.R.; Regasini, L.O.; Cássia Orlandi Sardi, J. Diacetylcurcumin: A novel strategy against *Enterococcus faecalis* biofilm in root canal disinfection. *Future Microbiol.* **2024**, *19*, 647–654. [CrossRef]

27. Sanches, C.V.G.; Sardi, J.C.O.; Terada, R.S.S.; Lazarini, J.G.; Freires, I.A.; Polaquini, C.R.; Torrezan, G.S.; Regasini, L.O.; Fujimaki, M.; Rosalen, P.L. Diacetylcurcumin: A new photosensitizer for antimicrobial photodynamic therapy in *Streptococcus* mutants biofilms. *Biofouling* **2019**, *35*, 340–349. [CrossRef]
28. Sachs, G.; Weeks, D.L.; Wen, Y.; Marcus, E.A.; Scott, D.R.; Melchers, K. Acid acclimation by *Helicobacter pylori*. *Physiology* **2005**, *20*, 429–438. [CrossRef] [PubMed]
29. Wirth, H.P.; Beins, M.H.; Yang, M.; Tham, K.T.; Blaser, M.J. Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. *Infect. Immun.* **1998**, *66*, 4856–4866. [CrossRef]
30. Eaton, K.A.; Brooks, C.L.; Morgan, D.R.; Krakowka, S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.* **1991**, *59*, 2470–2475. [CrossRef] [PubMed]
31. Schoep, T.D.; Fulurija, A.; Good, F.; Lu, W.; Himbeck, R.P.; Schwan, C.; Choi, S.S.; Berg, D.E.; Mittl, P.R.; Benghezal, M.; et al. Surface properties of *Helicobacter pylori* urease complex are essential for persistence. *PLoS ONE* **2010**, *5*, e15042. [CrossRef] [PubMed]
32. Nugrahaeningtyas, E.; Lee, D.J.; Song, J.I.; Kim, J.K.; Park, K.H. Potential application of urease and nitrification inhibitors to mitigate emissions from the livestock sector: A review. *J. Anim. Sci. Technol.* **2022**, *64*, 603–620. [CrossRef]
33. Spencer, A.C.; Panda, S.S. DNA Gyrase as a Target for Quinolones. *Biomedicines* **2023**, *11*, 371. [CrossRef]
34. Matsuzaki, J.; Suzuki, H.; Tsugawa, H.; Nishizawa, T.; Hibi, T. Homology model of the DNA gyrase enzyme of *Helicobacter pylori*, a target of quinolone-based eradication therapy. *J. Gastroenterol. Hepatol.* **2010**, *25*, S7–S10. [CrossRef]
35. Duprey, A.; Groisman, E.A. The regulation of DNA supercoiling across evolution. *Protein Sci.* **2021**, *30*, 2042–2056. [CrossRef] [PubMed]
36. Bucciarelli, A.; Skliar, M.I. Medicinal plants from Argentina with gastroprotective activity. *Ars Pharm.* **2007**, *48*, 361–369.
37. Chattopadhyay, I.; Bandyopadhyay, U.; Biswas, K.; Maity, P.; Banerjee, R.K. Indomethacin inactivates gastric peroxidase to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen. *Free Radic. Biol. Med.* **2006**, *40*, 1397–1408. [CrossRef]
38. Morsy, M.A.; El-Moselhy, M.A. Mechanisms of the protective effects of curcumin against indomethacin-induced gastric ulcer in rats. *Pharmacology* **2013**, *91*, 267–274. [CrossRef]
39. Czekaj, R.; Majka, J.; Magierowska, K.; Sliwowski, Z.; Magierowski, M.; Pajdo, R.; Ptak-Belowska, A.; Surmiak, M.; Kwiecien, S.; Brzozowski, T. Mechanisms of curcumin-induced gastroprotection against ethanol-induced gastric mucosal lesions. *J. Gastroenterol.* **2018**, *53*, 618–630. [CrossRef]
40. Kwiecien, S.; Magierowski, M.; Majka, J.; Ptak-Belowska, A.; Wojcik, D.; Sliwowski, Z.; Magierowska, K.; Brzozowski, T. Curcumin: A Potent Protectant against Esophageal and Gastric Disorders. *Int. J. Mol. Sci.* **2019**, *20*, 1477. [CrossRef] [PubMed]
41. Mei, X.; Luo, X.; Xu, S.; Xu, D.; Zheng, Y.; Xu, S.; Lv, J. Gastroprotective effects of a new zinc (II)-curcumin complex against pylorus-ligature-induced gastric ulcer in rats. *Chem. Biol. Interact.* **2009**, *181*, 316–321. [CrossRef]
42. Mei, X.; Xu, D.; Xu, S.; Zheng, Y.; Xu, S. Gastroprotective and antidepressant effects of a new zinc (II)-curcumin complex in rodent models of gastric ulcer and depression induced by stresses. *Pharmacol. Biochem. Behav.* **2011**, *99*, 66–74. [CrossRef] [PubMed]
43. Mei, X.; Xu, D.; Xu, S.; Zheng, Y.; Xu, S. Novel role of Zn (II)-curcumin in enhancing cell proliferation and adjusting proinflammatory cytokine-mediated oxidative damage of ethanol-induced acute gastric ulcers. *Chem. Biol. Interact.* **2012**, *197*, 31–39. [CrossRef] [PubMed]
44. Organisation for Economic Co-operation and Development (OECD). Test No. 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents. In *OECD Guidelines for the Testing of Chemicals, Section 4*; OECD: Paris, France, 2025.
45. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement (Document M100-S17)*; CLSI: Wayne, PA, USA, 2007.
46. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-Dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
47. Weatherburn, M.W. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **1976**, *39*, 971–974. [CrossRef]
48. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2022.

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

Synthesis of First Copper Metal Complex of C=C Extended Curcuminoid Analogue: Structure, β -Cyclodextrin Association, and Biological Properties

Rosario Tavera-Hernández *, Rubén Sánchez-Obregón, Marco A. Obregón-Mendoza *, Antonio Nieto-Camacho, María Teresa Ramírez-Apan, Leidys L. Pérez-González and Raúl G. Enríquez *

Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, Mexico City 04510, Mexico; rubens@unam.mx (R.S.-O.); camanico2015@yahoo.com (A.N.-C.); mtrapan@yahoo.com.mx (M.T.R.-A.); leidyslaura92@gmail.com (L.L.P.-G.)

* Correspondence: rosario.tavera@gmail.com (R.T.-H.); marco.obregon@zaragoza.unam.mx (M.A.O.-M.); enriquezhabib@gmail.com or habib@unam.mx (R.G.E.); Tel.: +52-55-55562-24404 (R.T.-H. & M.A.O.-M. & R.G.E.)

Abstract: The search for bioactive compounds against chronic diseases such as cancer and diabetes includes curcuminoids as promising scaffolds. Here, we report the synthesis of a family of curcuminoid analogue compounds with an extended unsaturated central chain, as follows: difluoroboron complex **1**, the enolised curcuminoid **2**, and its homoleptic copper complex **3**, in moderate to good yields (68–90%). Additionally, their β -cyclodextrin (BCD) association complexes, **4** and **5**, were prepared through a mechanochemical method and characterised by spectroscopic techniques. Complete ^1H and ^{13}C NMR assignments and NOESY correlations revealed unique solvent effects on the conformational disposition of compound **2**, while the copper complex **3** displayed the highest extinction coefficient ($1.20 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Furthermore, the authentication of the polymorph of **1** and the new crystal structures of **2** and **3**, determined by single-crystal X-ray analysis, were highlighted. Although the copper complex **3** initially exhibited the lowest α -glucosidase inhibitory activity ($\text{IC}_{50} > 100 \mu\text{M}$), it showed a significant increase ($\text{IC}_{50} = 36.27 \mu\text{M}$) upon association with BCD, reaching values comparable to the free ligand ($\text{IC}_{50} = 45.63 \mu\text{M}$). Compounds **1**–**5** were non-toxic to healthy cells (COS-7), but compound **5** stands out as a promising candidate against this metabolic condition.

Keywords: analogue curcuminoid; copper complex; single-crystal structure; π -elongated chain; β -cyclodextrin complex; α -glucosidase

1. Introduction

The search for new compounds with biological activity points to the family of natural products, a primary source that provides unique structural scaffolds with exceptional chemical and biological properties, and curcumin stands out with particular relevance [1,2]. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are polyphenols present in the rhizome of *Curcuma longa* [3]. Currently, the extensive study of these curcuminoids is due to their various reported biological activities, including antioxidant [4], anticancer [5], anti-inflammatory [6,7], antimicrobial [8], antidiabetic [9], and neuroprotective effects against Alzheimer's disease [10,11]. Structurally, curcuminoids are composed of two aromatic rings connected by an unsaturated seven-carbon chain containing a β -diketone group, which exists in equilibrium between keto and enol tautomeric forms.

The current molecular architecture features an extended conjugated system that enables the formation of complexes with metal ions, altering its optical properties and interactions with biological targets [12]. Research has shown that modifications to the conjugation within the structure of curcuminoids, alongside their chelation with transition metals, can improve both the optical and biological properties of these compounds [13–15].

On the other hand, copper is a transition metal, and its ionic form is of physiological relevance as an essential component of many metalloenzymes involved in redox reactions [16]. Copper complexes have attracted increasing interest due to their biocompatibility and promising biological potential [17,18], since light excitation of Cu (II) complexes in the red region forms reactive species inducing cytotoxic activity, which represents an important factor in the photodynamic mechanism [19]. In addition, the importance of the π -extended ligand in the improvement of high absorption in the near-infrared region has been reported [20]. Several studies have reported the antitumor [21,22], antimicrobial [23], and antioxidant [24] properties of copper complexes, as well as their inhibitory activity toward enzymes such as α -glucosidase [25]. α -Glucosidase is an enzyme of carbohydrate degradation, and its inhibition leads to control of postprandial blood glucose levels, resulting in interest for patients undergoing type 2 diabetes treatment [26,27]. Curcumin and curcuminoids exhibit inhibitory ability against the α -glucosidase enzyme, and the development of synthetic analogues has led to compounds with an improved inhibitory potency [28–30].

Previous research has reported homoleptic copper complexes with curcumin, diacetylcurcumin, dimethoxycurcumin, diphenylcurcumin, dibenzylcurcumin, and dietoxycurcumin as ligands. These copper complexes exhibit predominantly a square-planar geometry, forming ML_2 structures through coordination with the β -diketone group of curcuminoids. Their antioxidant and antiproliferative activities and imaging properties improved in comparison with the free ligand [15,24,31].

There are limited investigations related to the synthesis, characterisation, and evaluation of the biological activity of curcuminoid analogues with extended conjugation in the unsaturated central chain. These curcuminoid analogues are particularly noteworthy for their optical properties [14,32,33] and as potential photosensitisers [34,35].

Although the biological and optical potential of curcuminoids is significant, their low aqueous solubility, susceptibility to hydrolysis, and photodegradation limit practical biological evaluation and bioavailability. Among the strategies to improve solubility in aqueous media, the formation of complexes with cyclodextrin has proven particularly effective in improving solubility and chemical stability [36,37]. β -Cyclodextrin (BCD) is efficient at forming complexes and is the most common available cyclodextrin. Structurally, BCD is composed of seven α -1,4-linked D-glucopyranose units, resulting in a toroid structure with a cavity (6–6.5 Å) of lower polarity than the surface [38,39].

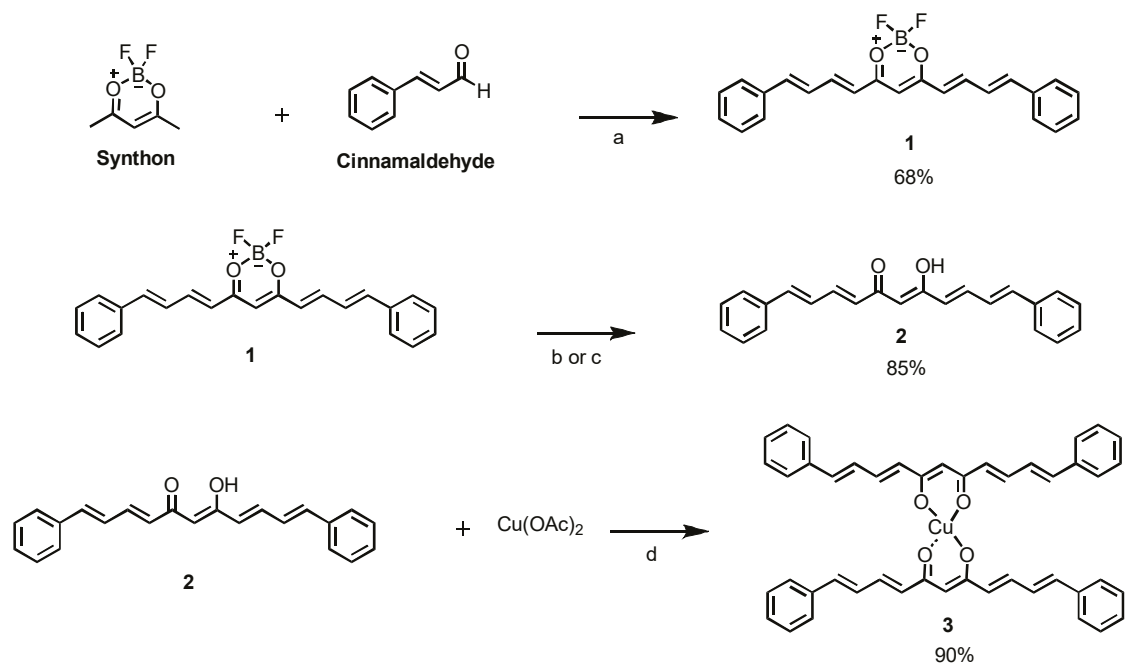
Considering the potential for exploring curcuminoids and their metal complexes, the present study aims to examine a family of analogue curcuminoids with an extended eleven-carbon unsaturated central chain, using cinnamaldehyde for synthetic convenience due to its favourable aldolic condensation reaction and the lack of functional groups in the aromatic fragment promoting efficient chelation at the β -diketone function.

The curcuminoid analogue, as well as its boron and copper complexes, were fully characterised by spectroscopic, spectrometric, and X-ray diffraction methods. Furthermore, we report the formulation of the analogue and its copper complex with BCD (compounds **4** and **5**), as well as their spectroscopic characterisation, evaluating the α -glucosidase and antiproliferative activity inhibition of these compounds.

2. Results

2.1. Synthesis

The synthon was prepared in good yield (88%) using 2,4-pentanedione and THF-boron trifluoride complex. In this compound, the $-\text{BF}_2$ group increases the acidity of methyl groups and prevents the Knoevenagel reaction in the next step. Then, the condensation reaction between the synthon and cinnamaldehyde was conducted with tributyl borate and *n*-butylamine to produce the curcuminoid- BF_2 analogue **1** as a dark purple solid in moderate yield (68%). The synthesis of ligand **2** was carried out following the reaction conditions previously reported [40], introducing minor modifications in the removal of the difluoroboron group. The cleavage of the difluoroboron group was carried out through reflux with triethylamine and methanol–DMSO–water as solvent (yield: 81%) or applying microwave irradiation using sodium oxalate in methanol–water as solvent at 140 °C for ten minutes (yield: 85%). Both methods have been reported as efficient to cleave the $-\text{BF}_2$ group in the synthesis of curcuminoids [41,42]. Finally, compound **2** with the free β -diketone group and copper (II) acetate in ethyl acetate solution produced the homoleptic copper complex (**3**) with a 90% yield. Scheme 1 shows the reactions undertaken to obtain compounds **1**–**3**.



(a) $\text{B}(\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_3$, $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$, EtOAc, t.a.; **(b)** $\text{Na}_2\text{C}_2\text{O}_4$, MeOH– H_2O , MW, 140 °C
(c) MeOH: H_2O , TEA, reflux **(d)** EtOAc, stir, t.a.

Scheme 1. Reaction conditions in the synthesis of curcuminoid analogue (**2**) and its homoleptic copper complex (**3**).

2.2. Nuclear Magnetic Resonance

Few reports document the synthesis of curcuminoid analogues (**2**). These reports provide limited information regarding their chemical shifts, with the assignments for protons and carbons largely absent. We examined the NMR spectra of **1** and **2** using 1D and 2D nuclear magnetic resonance experiments (Supplementary Material). Figure 1 presents the stacked ^1H -NMR spectra of compounds **1** and **2**, alongside the hydrogen and carbon assignments in $\text{DMSO}-d_6$ and CDCl_3 , as shown in Table 1.

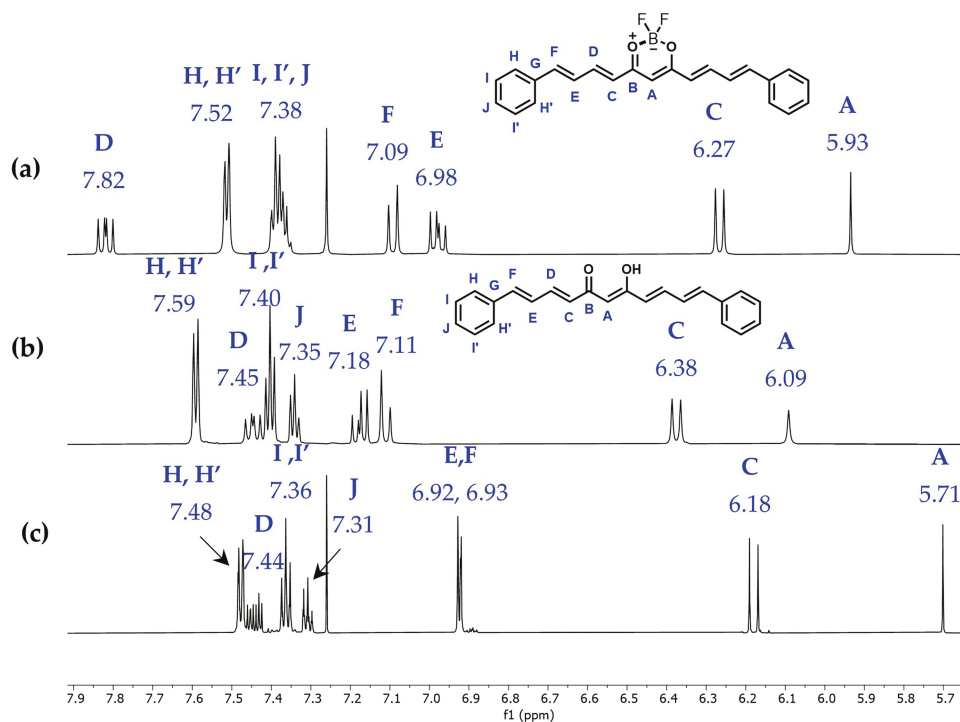


Figure 1. 700 MHz ^1H -NMR spectra of (a) curcuminoid- BF_2 **1** in CDCl_3 ; (b) curcuminoid analogue **2** in $\text{DMSO}-d_6$; and (c) compound **2** in CDCl_3 .

Table 1. ^1H and ^{13}C chemical shifts of compounds **1** and **2** (700 MHz).

	Compound 1 (CDCl_3)		Compound 2 ($\text{DMSO}-d_6$)		Compound 2 (CDCl_3)	
	^{13}C (δ , ppm)	^1H (δ , ppm)	^{13}C (δ , ppm)	^1H (δ , ppm)	^{13}C (δ , ppm)	^1H (δ , ppm)
A	102.22	5.93	101.34	6.09 (s)	101.87	5.71 (s)
B	179.59		182.88		183.24	
C	124.22	6.27	127.74	6.38 (d)	127.87	6.18 (d)
D	147.66	7.82	141.00	7.45 (dd)	140.88	7.44 (m)
E	126.64	6.98	127.36	7.18 (dd)	127.22	6.92 (d) *
F	145.13	7.09	140.34	7.11 (d)	140.38	6.93 (d) *
G	135.8		136.10		136.44	
H, H'	128.01	7.52	127.25	7.59 (m) ***	127.22	7.48 (m)
I, I'	129.14	7.38	128.87	7.40 (m) **	128.97	7.36 (m)
J	130.27	7.38	129.06	7.34 (m) **	129.15	7.31 (m)
-OH				16.06 (br, s)		15.85 (br, s)

* Superimposed signals, shifts can exchange; ** triplet-like; *** doublet-like.

The ^1H NMR spectra of **1** and **2** show their own set of signals due to magnetic equivalence. Thus, the aromatic protons in **1** (CDCl_3) appear as multiplets at $\delta 7.38$ and $\delta 7.52$ ppm, while in compound **2** (DMSO), they are multiplets at $\delta 7.35$, $\delta 7.40$, and $\delta 7.59$ ppm. Unsaturated protons H_D and H_E in **1** appear as a doublet of doublets at $\delta 7.82$ and $\delta 6.98$ ppm, while in **2**, they appear at $\delta 7.45$ and $\delta 7.18$ ppm, respectively. Coupling constants for H_D and H_E in both compounds are ca. 15 Hz due to the *trans* coupling of the unsaturated proton system. H_F and H_C hydrogens in **1** appear at $\delta 7.09$ and $\delta 6.27$ ppm with ca. $J = 15.4$ and 14.8 , respectively. The corresponding set for **2** are doublets at $\delta 7.11$ and $\delta 6.38$ ppm with $J = 15.6$ and $J = 15.1$ Hz, respectively. The methine hydrogen appears as a singlet at $\delta 5.93$ and $\delta 5.7$ ppm in **1** and **2**, respectively. In addition, the ^1H -NMR spectrum of **2** displays a broad signal at 16.06 ppm corresponding to -OH, which forms a hydrogen bond, indicating the keto-enol tautomer.

The $^1\text{H-NMR}$ of compound **2** was also acquired in CDCl_3 , and its assignments are shown in Figure 1c and Table 1. In comparison with the spectrum in DMSO-d_6 , different multiplicities for H_D , H_E , and H_F were observed as a consequence of changes in chemical shifts. In addition, the NOESY spectrum showed interactions at greater distances between $\text{H}_\text{D-H}_\text{A}$ and $\text{H}_\text{H-H}_\text{c}$ when chloroform was used as solvent. The complete set of interactions is shown in Table S1.

2.3. Electronic Paramagnetic Resonance

The electron paramagnetic resonance spectrum of compound **3** (Figure S22) exhibited two sets of quartet hyperfine lines characteristic of copper (II) coordination complexes, indicating the coexistence of two species. The experimental values obtained directly from the spectrum are $g_\parallel = 2.2819$ and 2.3040 , $g_\perp = 2.0430$ and 2.0544 , while the $A_\parallel$ values are 168.9 and $160.0 \times 10^{-4} \text{ cm}^{-1}$. In both cases, $g_\parallel > g_\perp$, suggesting an axial square-planar coordination geometry [43]. The calculated ratios $f = g_\parallel / A_\parallel$ are 135.1 and 144 cm , close to 150 cm , which is associated with a slight to moderate distortion from planar geometry [44].

2.4. Mass Spectrometry

The mass spectrum compound (**1**) showed a peak at m/z 411.6 $[\text{M} + \text{Cl}]^-$, which is in agreement with the mass expected for this compound. In the case of ligand (**2**), a peak at m/z 329.9 corresponds to a $[\text{M} + \text{H}]^+$ molecular ion, and the mass spectrum of the copper complex (**3**) showed a peak at m/z 781.538 $[\text{M} + \text{Cu}]^+$. The mass spectra are in the Supplementary Material (Figures S23–S26).

2.5. Optical Properties

The absorption and fluorescence spectra of compounds **1–3** were obtained using DMSO as solvent. The maximum absorption and emission are shown in Table 2 and the spectra are shown in Figure 2. Compound **1** showed two main maximum absorption bands in UV-Vis at 487 and 511 nm, while compounds **2** and **3** showed these at 434 and 435 nm, respectively, due to $\pi\text{-}\pi^*$ transitions.

Table 2. Maximum absorption and emission bands and extinction coefficient (ϵ) of curcuminoid analogue- BF_2 (**1**), curcuminoid analogue (**2**), and copper complex (**3**) in DMSO.

Compound	Absorption λ (nm)	ϵ ($\text{M}^{-1} \cdot \text{cm}^{-1}$) [$\log \epsilon$]	Emission λ (nm)
1	487	75,372.75 [4.9]	567.01
2	434	63,305.95 [4.8]	511.04
3	435	120,156.79 [5.1]	512.98

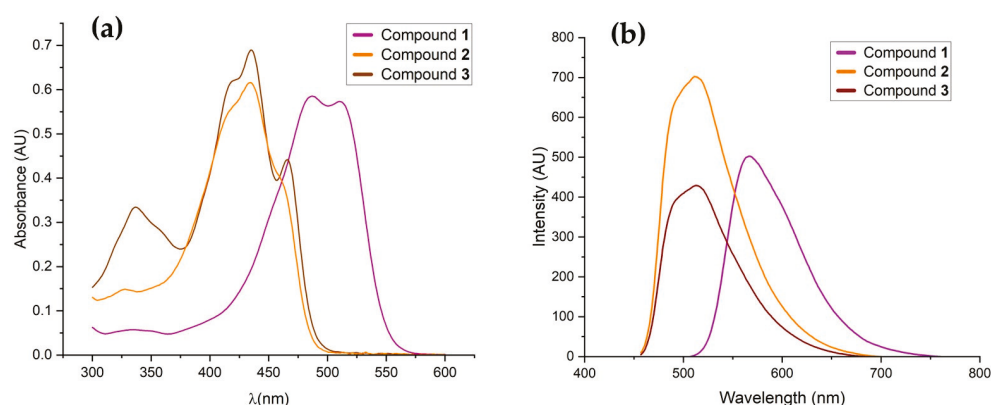


Figure 2. (a) Absorbance and (b) emission spectra of curcuminoid analogue- BF_2 (**1**) (purple), analogue curcuminoid (**2**) (orange), and copper (II) complex (**3**) (brown) in DMSO.

The absorption curve of the copper complex (**3**) shows two shoulders at 422 and 466 nm attributed to charge transfer from the ligand to Cu(II), which has been reported in curcumin copper complexes [31,45]. The UV–Vis spectra of compounds **2** and **3** are provided in detail in the Supplementary Material (Figures S33 and S34).

The fluorescence spectra of compounds **1–3** are shown in Figure 2b. The emission maxima are similar for compounds **2** and **3** (Table 2). However, **1** exhibits fluorescence at a longer wavelength than **2**. This tendency has been observed in curcuminoids-BF₂ [46].

2.6. Infrared-FT Spectroscopy

The FT-IR spectrum of compound **1** shows medium-intensity bands at 1598 and 1587 cm^{−1} corresponding to stretching vibrations of C=O and C=C; a very intense band at 1504 cm^{−1} revealing C=C bond vibrations; and medium-intensity bands at 1384 and 1050 cm^{−1} evidencing B-O vibrations. The IR spectrum of compound **2** shows intense bands at 1604 and 1488 cm^{−1} corresponding to C=O and C=C, respectively. The FT-IR spectrum of copper (II) complex (**3**) exhibits medium-intensity bands at 1612 and 1598 cm^{−1}, which correspond to C=O and C=C stretching, and at 1505 cm^{−1}, an intense band is observed due to coordination with Cu (II); furthermore, new low-intensity bands are observed at 472 and 544 cm^{−1}, indicating Cu-O bond vibrations. The FT-IR spectra of compounds **1–3** are in Supplementary Material (Figures S27–S29).

2.7. Single-Crystal X-Ray Diffraction

The crystallographic structures of compounds **1–3** were obtained, and ORTEP diagrams are shown in Figure 3; the crystallographic data are summarized in Table 3 and full details are available in the Supplementary Material (Tables S6–S21). The crystal structure of analogue-BF₂ (**1**) shows a triclinic system. Three planes are defined, with a significant deviation in planarity in the unsaturated eleven-carbon chain (3E,6Z,8E)-7-hydroxyundeca-1,3,6,8,10-pentaen-5-one (RMS = 0.135 Å), whereas the phenyl moieties remain essentially planar (RMS = 0.002 and 0.004 Å). The angles formed between the central carbon chain plane and the phenyl groups are 25.18 and 30.78°. However, the phenyl group planes have a dihedral angle of 55.90° between them. The disposition of the -BF₂ group is ca 90° with respect to the plane of the curcuminoid structure core.

Table 3. Crystal data of compounds **1**, **2**, and **3**.

Compound	1	2	3
CCDC (deposition number)	2,483,477	2,483,476	2,483,475
Empirical formula	C ₂₃ H ₁₉ BF ₂ O ₂	C ₂₇ H ₂₈ O ₃	C ₄₆ H ₃₈ CuO ₄
Formula weight	376.19	400.49	718.30
Crystal system	Triclinic	Orthorhombic	Monoclinic
Space group	P-1	Cmc2 ₁	C2/c
Unit cell dimensions			
a (Å)	a = 9.1069 (6)	a = 28.5258 (9)	a = 41.1114 (11)
b (Å)	b = 10.0955 (7)	b = 9.7389 (3)	b = 5.56190 (10)
c (Å)	c = 10.7715 (7)	c = 7.6957 (2)	c = 16.1765 (4)
α (°)	α = 74.270 (2)	α = 90	α = 90
β (°)	β = 85.586 (2)	β = 90	β = 100.2810 (10)
γ (°)	γ = 85.457 (2)	γ = 90	γ = 90
Volume (Å ³)	948.65 (11)	2137.94 (11)	3639.49 (15)
Z	2	4	4
Density (calculate) (mg/m ³)	1.317	1.244	1.311
Absorption coefficient (mm ^{−1})	0.095	0.080	0.644
F (000)	392	856	1500
Crystal size (mm ³)	0.291 × 0.239 × 0.178	0.400 × 0.280 × 0.200	0.346 × 0.106 × 0.089

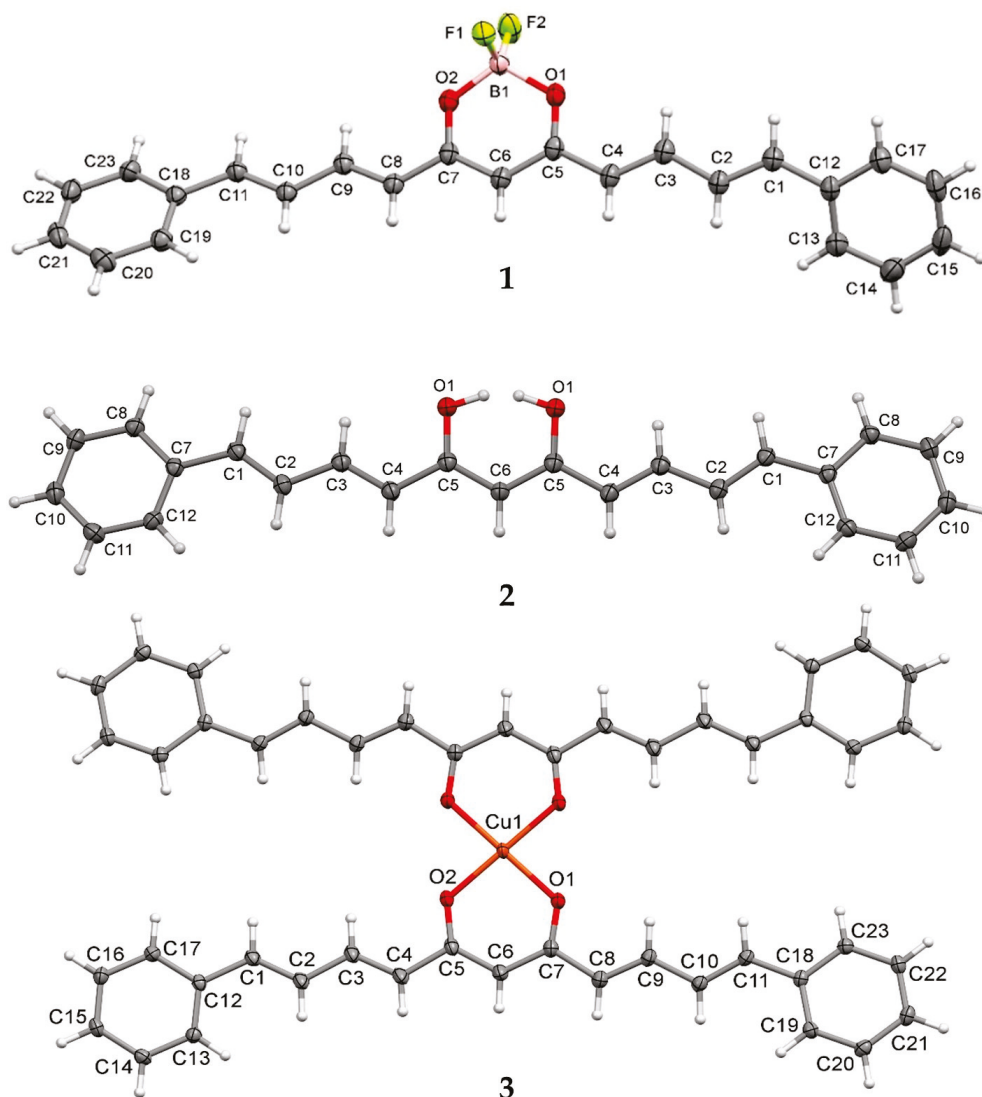


Figure 3. ORTEP representations of compounds **1**, **2**, and **3**. Displacement ellipsoids were drawn at the 50% probability level.

The analogue (**2**) crystal structure has an orthorhombic system and crystallises as a THF solvate, which interacts with three analogue molecules in the crystal packing. The asymmetric unit consists of half of a curcuminoid analogue, completed by the crystallographic mirror plane, and the linear structure exhibits a significant deviation in planarity. The following two planes are defined: a (3E,6Z,8E)-7-hydroxyundeca-1,3,6,8,10-pentaen-5-one (RMS = 0.1710 Å) and the phenyl moiety (RMS = 0.003 Å), with both at 21.45° from each other. An intramolecular hydrogen bond within the keto-enol group is observed (distance O...H 1.67 Å).

The crystal of the copper complex (**3**) shows a monoclinic system and reveals a four-coordinate number for copper, adopting a square planar geometry. The asymmetric unit includes a ligand unit binding to a copper atom, positioned on an inversion centre. The planes formed by deprotonated (3E,6Z,8E)-7-hydroxyundeca-1,3,6,8,10-pentaen-5-one (RMS = 0.082 Å) and phenyl groups (0.0020 and 0.007 Å) show a slight deviation from planarity, forming an angle of 4.33° and 5.23°. The Cu–O bond distances are approximately 1.91 Å.

Intermolecular interactions in the crystal packing of compounds **1–3** were analysed in CrystalExplorer software through the Hirshfeld surface (HS) and the related 2D fingerprint plots.

The HSs were mapped using the descriptor d_{norm} , which indicates that the intermolecular contact distances were normalised with the van der Waals radius of the atoms, involving the distance from the surface to the closest atom inside (d_i) and outside (d_e) [47,48]. In the HSs of compounds 1–3, some areas were distinguished, with red corresponding to closer contacts (strong interactions), blue to those longer than the van der Waals radius, and white to distances equal to the sum of van der Waals radii [49]. In all crystal structures, longer-distance contacts (weak interactions) were predominant. In the HS of compound 1, B-F...H-C interactions were represented by red areas (Figure 4), while in compounds 2 and 3, red areas were established by C-H... π interactions with the phenyl rings.

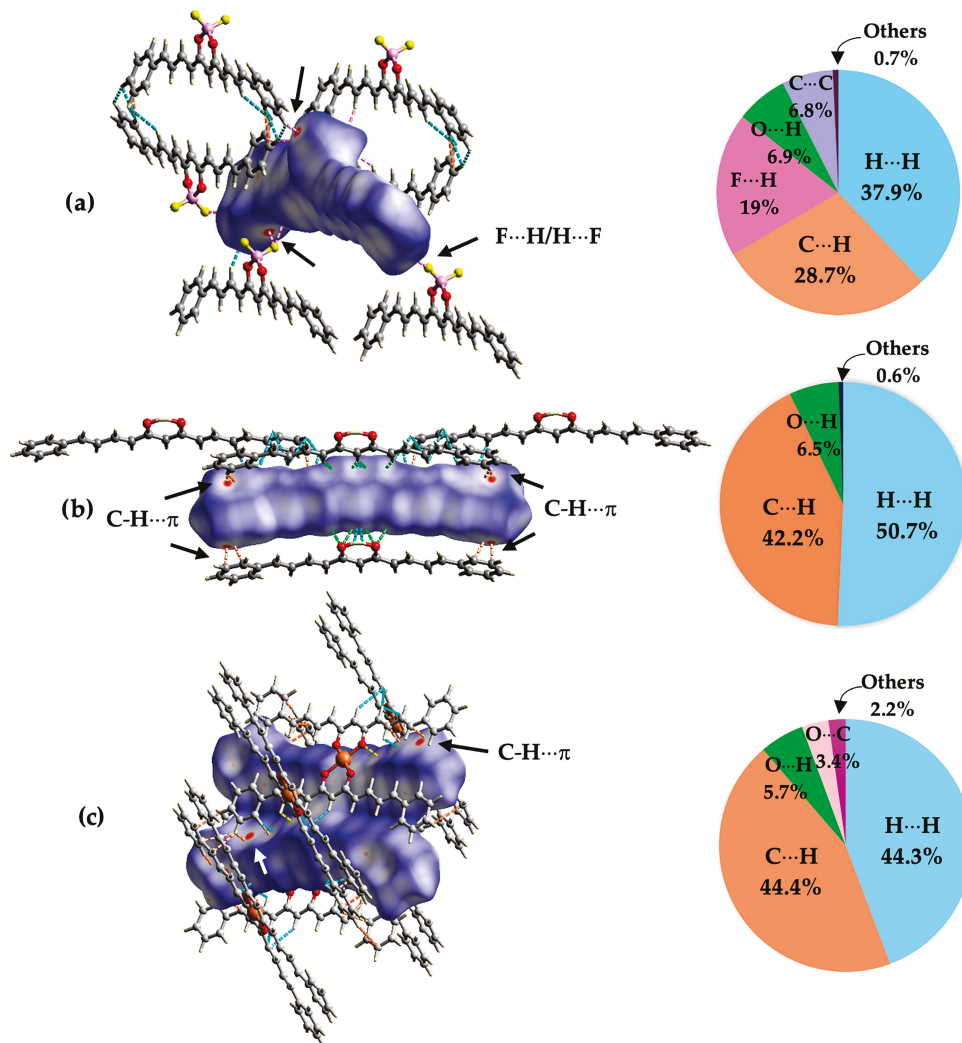


Figure 4. Hirshfeld surface and percentages of main intermolecular interaction contributions in the crystal of compounds (a) 1, (b) 2, and (c) 3. The images show interactions at 2.9 Å (carbons are in grey, hydrogens in white, oxygens in red, fluorine in yellow, copper in orange and boron in pink).

The fingerprint plots (Figure S38) reveal that the contact contributions in the crystal packing of compounds 1–3 are mainly H...H (38–51%) and C...H/H...C (29–44%) interactions, which extend along the fingerprint plots and are related to the Van der Waals forces. However, in the case of analogue-BF₂, H...F/F...H interactions are relevant (19%) and appear in the two sharp spikes extending from the bottom to the top of the 2D fingerprint plot, suggesting strong interactions when the distance is shorter than the sum of Van der Waals radii [48]. Other interactions, like C-C (π - π contacts), are scarce.

2.8. Improvement of Water Solubility Through Association with β -Cyclodextrin (BCD) Formation and Biological Activity Evaluation

The analogue curcuminoid **2** and its copper complex **3** showed poor solubility in polar solvents; therefore, formulations with dry BCD were prepared using mechanochemistry. The solid obtained directly from the mill was shaken with water and filtered to obtain the association complex as a yellow and brown solid for **4** and **5**, respectively (Figure 5), which was analysed by NMR, infrared, and UV-Vis spectroscopy.

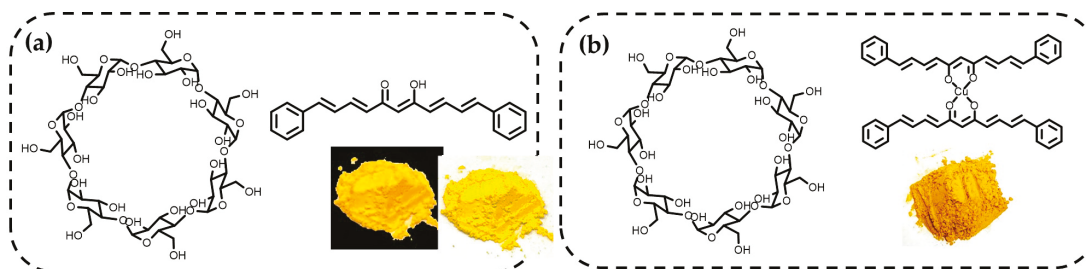


Figure 5. Formulations of (a) curcuminoid analogue with β -cyclodextrin **4** and (b) analogue copper complex with β -cyclodextrin **5**.

2.8.1. Nuclear Magnetic Resonance of the Association Complexes with BCD

The shifts in the ^1H -NMR spectrum of BCD were consistent with those reported in the literature [50]. The stacked ^1H -NMR spectra of the free BCD and compounds **2** and **4** association complex are shown in Figure 6. The signals of BCD in the ^1H -NMR spectrum of **4** are observed between 3 and 5.8 ppm. The ^1H and ^{13}C chemical shift changes defined by $\Delta\delta = \delta_{\text{complex}} - \delta_{\text{free}}$ (Tables S2 and S3) suggest interactions between BCD and **2** in the complex. The hydrogen shifts of BCD indicate a shielding effect on H3, H4, and H6 due to the anisotropy caused by π the electron of the conjugate system in **2**. A deshielding effect is observed on H1, H2, and C3-OH, with the highest $\Delta\delta$ values for H2, followed by H1, H4, and H6. Additionally, NOEs are observed between the enol-hydroxyl group of **2** and H2 and the C6-OH of BCD, indicating interactions with the exterior of BCD.

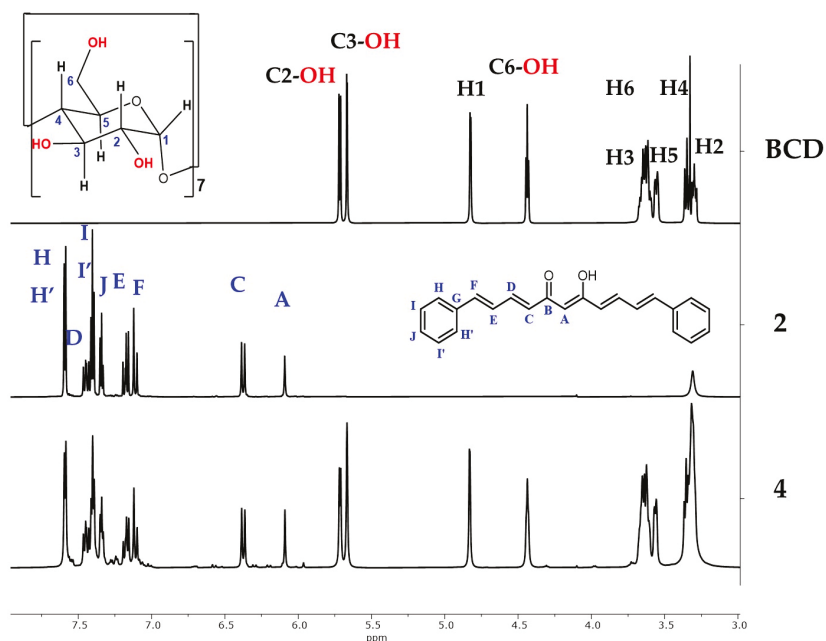


Figure 6. Stacked ^1H -NMR spectra of BCD, compound (**2**), and association complex **4** (DMSO-d_6 , 700 MHz).

2.8.2. UV-Vis and Infrared Spectroscopy of Association Complexes with BCD

The FT-IR spectrum of the **4** complex shows two intense bands at 3311 and 1026 cm^{-1} , corresponding to the -OH and C-O-C vibrations, respectively, which are related to the BCD structure (Figure 7a). Additionally, a broad and intense band absorption at 1488 cm^{-1} , attributed to the vibration of the C=C bond of compound **2** (Figure 7b), is not observed in the complex with BCD, which may be attributed to changes in band intensity and wavelength number.

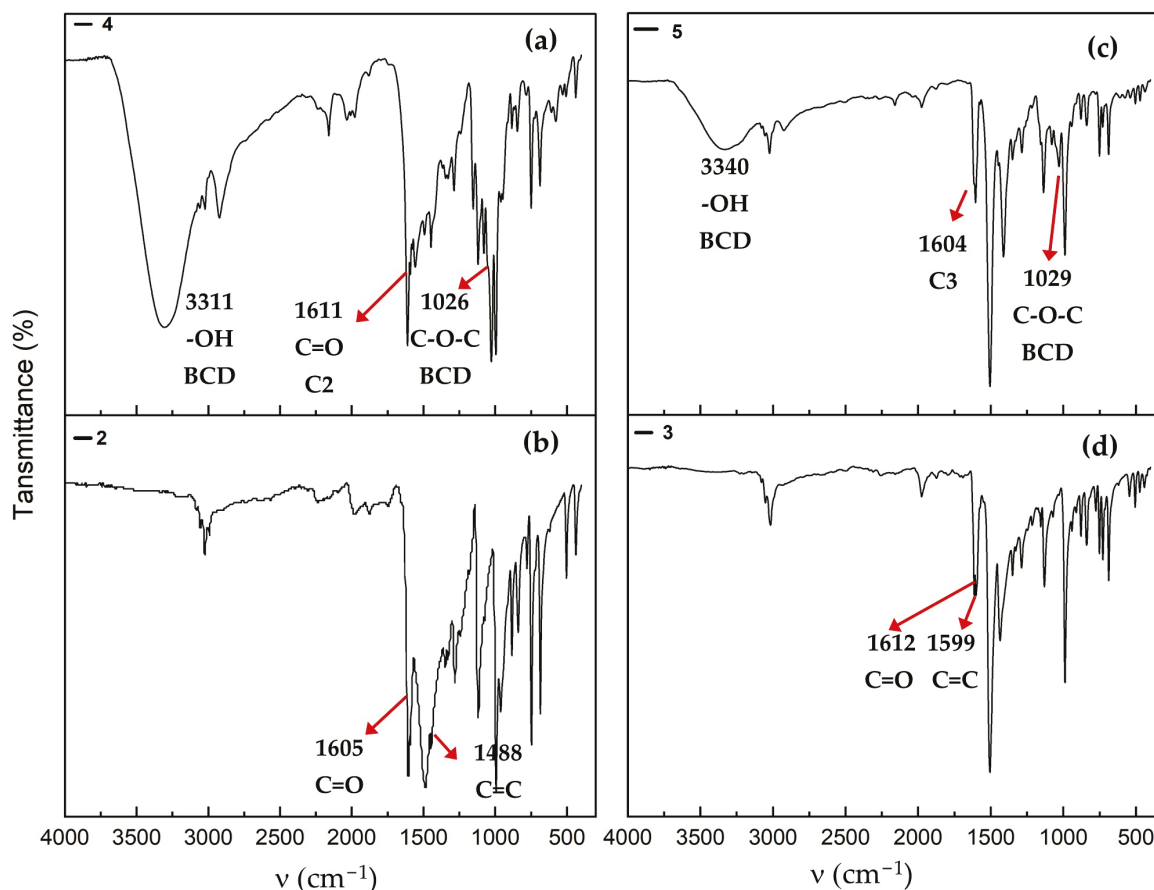


Figure 7. Prominent absorption bands in the infrared spectra of (a) association complex **4**, (b) compound **2**, (c) association complex **5**, and (d) copper complex **3**.

The FT-IR spectrum of the inclusion complex of **5** displays the characteristic BCD bands at 3340 cm^{-1} (O-H stretching) and 1029 cm^{-1} (C-O-C stretching), which shift from 3307 and 1021 cm^{-1} , respectively (see FT-IR spectrum of BCD, Figure S32). Minor shifts in wavelength number are also observed in the bands assigned to the C=O and C=C stretching vibrations, which appear at 1612 and 1599 cm^{-1} ; however, in the spectrum of the BCD complex, they collapse at 1604 cm^{-1} (Figure 7c,d). FT-IR spectra of **4** and **5** are provided in detail in the Supplementary Materials (Figures S30 and S31).

The UV-Vis absorption maxima of the association complexes **4** and **5** in DMSO were observed at 431 and 438 nm, respectively. In comparison, the free forms of compounds **2** and **3** exhibited absorption maxima at 434 and 435 nm, respectively, as shown in Table 2. These results reveal a hypsochromic shift for compound **2** upon complexation with BCD, whereas compound **3** exhibited a bathochromic shift upon BCD association.

The concentration of association complexes **4** and **5** was determined by UV-Vis spectroscopy, yielding values of 393.60 $\mu\text{g}/\text{mg}$ and 685.94 $\mu\text{g}/\text{mg}$, respectively.

Additionally, the thermal profiles obtained by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) indicated the formation of a new phase in compounds **4** and **5**, as evidenced by the disappearance of the melting points observed in the free compounds (**2** and **3**) and by temperature changes of exothermic peaks. In the Supplementary Material (Graphics S1 and S2), a detailed analysis of the thermograms is provided.

2.9. Biological Activity

The antiproliferative and α -glucosidase inhibitory activities of compounds **2** and **3**, as well as their BCD complexes **4** and **5**, were evaluated.

Antiproliferative activity was assessed in various cancer cell lines using the sulforhodamine B assay at a concentration of 10 μ M. The resulting cell growth inhibition percentages are presented in Table S4. Neither the compounds nor their BCD complexes demonstrated antiproliferative activity at this concentration.

A yeast α -glucosidase inhibition assay was performed for compounds **2**, **3**, and their BCD complexes **4** and **5** using quercetin as a positive control; complete data are shown in Table S5, and in Figure 8, the half-inhibition concentrations (IC_{50}) are shown.

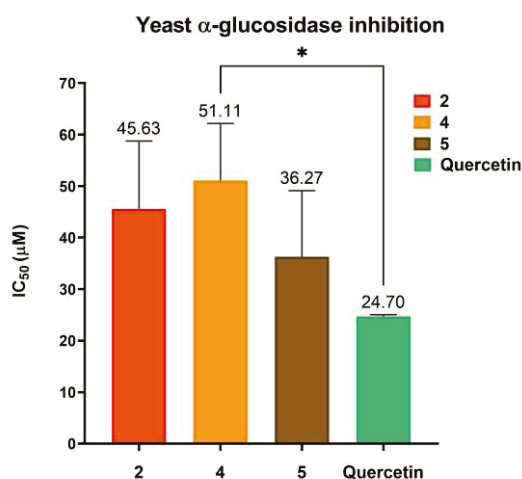


Figure 8. Yeast α -glucosidase inhibition activity of compound **2** and BCD association complexes **4** and **5**, expressed as half inhibitory concentration. $n = 3, 4, 5$; * $p < 0.05$.

The inhibitory activity of compound **2** shows no change concerning its BCD complex **4**, exhibiting IC_{50} values of 45.63 ± 13.12 and 51.11 ± 11.05 μ M, respectively. In contrast, compound **3** does not have important inhibitory activity against α -glucosidase ($16.19 \pm 3.18\%$ at 100 μ M, Table S5); however, in complex with BCD, it shows enhanced activity, exhibiting $IC_{50} = 36.27 \pm 12.85$ μ M, which is comparable with compound **2**'s inhibitory activity. The enzyme inhibitory activities of **2** and **5** do not show a significant difference from quercetin activity.

3. Discussion

Curcumin and curcuminoids are fascinating compounds due to their biological activity and applications as dyes and photosensitizers [10,35,51,52]. Research on the synthesis of analogue curcuminoids has centred on modifications around the aromatic or β -diketone group [53]; however, analogues with an extended central chain are unexplored, and a few reports describe spectroscopic and biological properties [14,32–35]. The synthesis of a curcuminoid analogue with an eleven-carbon central chain followed the previously reported methodology through a condensation reaction of cinnamaldehyde and synthon, followed by the removal of the difluoroboron group. In this final step, microwave irradiation and

reflux resulted in efficient methodologies for obtaining compound **2** in good yield (81–85%). The microwave irradiation method required less time compared to refluxing conditions. This synthetic route to synthesise compound **2** led to an improvement in time and yield compared to the reported method by Kazantzis et al., which used an acetone–boric oxide complex as the first step (63%) [35].

Overlap suppression of signals in 1D and 2D NMR experiments at 700 MHz led to a complete hydrogen and carbon assignment, observing the characteristic deshielding effect of the $-\text{BF}_2$ group over vinyl and aromatic hydrogens in compound **1**. In addition, the influence of the solvents DMSO-d_6 and CDCl_3 on the multiplicity of H_D , H_E , and H_F was evidenced in the ^1H -NMR spectrum of compound **2**. In addition, the NOESY spectrum showed distinctive hydrogen interactions between H_D – H_A and H_H – H_c when CDCl_3 was used as solvent. Such an effect can be attributed to the polarity and viscosity of the deuterated solvent, which favours different conformer populations [54]. This fact suggests that the molecular flexibility of compound **2** is due to the extended central chain, in comparison with the parent diphenylcurcuminoid, for which no changes in the multiplicity of proton signals in CDCl_3 and DMSO-d_6 have been reported in the literature [24,55].

Similarly, the EPR spectrum of compound **3** exhibits the coexistence of two species and the $g_\parallel$, $g_\perp$, and $A_\parallel$ and F values agree with the previously reported homoleptic complexes of copper with curcuminoids and curcumin, whose predominant species exhibit a square-planar geometry with distortion [24,31]. In contrast, none of the reported homoleptic copper–curcuminoid complexes have shown evidence for the coexistence of different species. Also, a change in solvent can alter the electronic distribution and geometry of the coordination sphere of copper complexes [56].

The observed optical properties show that complexation with the $-\text{BF}_2$ group in compound **1** gives rise to red-shifted absorption bands compared to the curcuminoid analogue **2**; this effect has been observed in reported curcuminoid–boron complexes [41,57,58]. Additionally, Bai and coworkers studied curcuminoids– BF_2 , finding that an extra double bond in the central chain led to a red shift compared to seven-carbon-chain curcuminoids– BF_2 [14]. On the other hand, Tahay and coworkers reported a red shift in the maximum absorption of compound **2** compared to diphenylcurcuminoid, attributed to the length of the π -conjugation [34]. The extinction coefficient of **2** exceeds that of curcumin and bisdemethoxycurcumin, agreeing with the reported value [35]. Copper complex **3** demonstrates an even higher extinction coefficient (Table 2), indicating a better interaction with light, a desirable property for applications in dyes and photosensitizers [14]. Additionally, this opens up the possibility of phototherapeutic applicability.

In the present work, the crystal structures of compounds **1–3** are reported for the first time, although Bai and coworkers previously reported a polymorphic monoclinic crystal of compound **1** [14]. Copper complex **3** showed a square planar geometry, which is predominantly observed in reported curcuminoid copper complexes [24,31]. In addition, the copper complex exhibited the best planarity, revealing efficient conjugation, as evidenced by a significant extinction coefficient, which could improve its optical properties, a phenomenon observed in copper complexes with extended conjugation [59].

Hirshfeld surfaces and the related 2D fingerprint plots of crystal structures reveal that the main interactions are governed by Van der Waals forces ($\text{H}\cdots\text{H}$ and $\text{C-H}\cdots\pi$), with similar contributions reported in the crystal packing for a copper complex of curcumin [31], an asymmetric curcuminoid [60], and zinc complexes of curcuminoids [61]. Closer contacts (strong interactions) are displayed on the extreme sides of aromatic rings through $\text{C-H}\cdots\pi$ interactions in compounds **2** and **3**. In contrast, in compound **1**, the BF_2 region, through $\text{B-F}\cdots\text{H-C}$ interactions, reveals strong interactions.

These predominant interactions described for compounds **2** and **3** are related to a reduced solubility in polar solvents, which led to the development of BCD-based formulations and further biological evaluation. The improvement in solubility enhances potential applications, particularly considering that a cytotoxic effect of compound **2** against a prostate cancer cell line has been documented, leading to increased efficacy under irradiation in photodynamic therapy experiments [28].

The spectroscopic analysis of BCD complexes of compounds **2** and **3** demonstrates guest–host interactions; ^1H -NMR shifts reveal shielding and deshielding effects attributed to guest aromatic moieties in guest molecules, an effect that has been reported in complexes with BCD [62–64]. These results suggest a partial insertion of compound **2** in the cavity of BCD through the interaction of the π -conjugated system with H3, which resides in the cavity near the wider rim of BCD [65]. In addition, NOE's correlations confirm interactions with the exterior of BCD. Infrared spectra show changes in intensity and shifts, mainly in bands related to the C=C and C=O stretching vibrations of the guest molecules and the O-H or C-O-C stretching vibrations of BCD. This effect is observed in the association of curcumin or azomethine with BCD, where the disappearance of the band corresponding to the aromatic ring is reported, suggesting the introduction of benzene rings into the BCD cavity [66]. Likewise, changes in wavenumber are a consequence of interactions between guest and host [67].

The UV-Vis spectra of **4** and **5** association complexes show both bathochromic and hypsochromic effects in the maximum absorption, respectively. The literature reports bathochromic effects for inclusion complexes of ursolic acid, oleanolic acid, and sulfabenzamide with BCD, attributed to changes in the medium's polarity and to interactions within the BCD cavity or at its outer surface [68,69]. The results provide evidence for the effective formation of complexes with BCD.

We evaluated compounds **2**, **3**, and their BCD complexes for their antiproliferative and α -glucosidase inhibition effects. All compounds showed no antiproliferative activity against different cancer cell lines; however, compound two and its BCD complex inhibited α -glucosidase without a significant difference, with comparable curcumin inhibitory activity ($\text{IC}_{50} = 37.2/78.2 \mu\text{M}$) and activities better than demetoxicurcumin ($\text{IC}_{50} = 82.4 \mu\text{M}$) and bisdemetoxicurcumin ($\text{IC}_{50} = 90.6 \mu\text{M}$) [28,70]. In the case of **3**, there was no inhibition activity at high concentrations; however, its BCD complex **5** exhibited a notable improvement in inhibition activity ($\text{IC}_{50} = 36.27 \mu\text{M}$), highlighting the importance of copper (II) and the formulation with BCD. Reports indicate that the copper ion exhibits better α -glucosidase inhibition than divalent metal ions like Zn^{2+} , Ca^{2+} , Mg^{2+} , and Mn^{2+} [71]. In the same manner, metallorganic copper complexes have also shown better enzyme inhibitory activity than free ligands [72,73]. Even the complex of hesperetin with copper exerts a synergistic effect with acarbose, and kinetic studies suggest that its copper complex could interact with the active site pocket, inhibiting carbohydrate hydrolysis [73]. The latter fact reinforces the importance of the formulation of **3** with BCD, aiming to improve its potency as inhibitor of α -glucosidase.

4. Materials and Methods

2,4-Pentanedione, boron trifluoride THF complex (CAS 462-34-0), cinnamaldehyde (CAS 080687), copper acetate (CAS 142-71-2), tributyl borate (CAS 688-74-4), *N*-butylamine (CAS 109-73-9), β -cyclodextrin (CAS 7585-39-9), triethylamine (CAS 142-71-2), and solvents of HPLC grade were commercially acquired from Sigma Aldrich® (St. Louis, MO, USA). Cinnamaldehyde (40 mL) was previously distilled under reduced pressure (10 mmHg), collecting the cinnamaldehyde fraction at 80 °C.

Melting points were measured using an Electrothermal Engineering IA9100 digital apparatus (Electrothermal Engineering Ltd, Essex, UK), and values are uncorrected.

4.1. Analytical Determinations

Infrared spectra were recorded on an FT-IR NICOLET IS-50 (Thermo Fisher Scientific, Waltham, MA, USA) using the attenuated total reflectance (ATR) technique ($4000\text{--}400\text{ cm}^{-1}$).

^1H , ^{13}C NMR, and two-dimensional spectra (COSY, HSQC, HMBC, NOESY, and ROESY) were recorded on a Bruker Avance III HD 700 MHz spectrometer (Bruker, Rheinstetten, Germany) using DMSO- d_6 as a solvent and reference. Spectra were processed using MestReNova software 14.2.0 (Mestrelab Research S.L., Santiago de Compostela, Spain) [74].

The mass spectrum of compound **3** was acquired on a Bruker Microflex MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337\text{ nm}$). Analyses were performed in positive ion mode with an acceleration voltage of 20 kV, using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

The electron paramagnetic resonance (EPR) spectrum was recorded on a JEOL JES-TE300 (JEOL Ltd., Akishima, Tokyo, Japan) spectrometer operating in the X-band at 9.5 GHz. Magnetic field calibration was performed using an ES-FC5 NMR field meter (JEOL Ltd., Tokyo, Japan). Spectral acquisition and data processing were carried out with the ES-IPRITS data system (v.3, Jeol Ltd., Tokyo, Japan, 1997). The sample was prepared as a 3 mM solution in THF and placed in a 5 mm quartz tube. Measurements were conducted at 77 K, with the magnetic field centred at 300.0 mT.

The mass spectra of compounds **1** and **2** were recorded on a Bruker Esquire 6000 spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation (ESI) source, operated in both positive and negative ion modes. The drying temperature was set at $300\text{ }^\circ\text{C}$, and the nebuliser pressure was maintained at 10 psi.

HPLC-UV was performed on an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) with a UV-Vis diode array detector Waters 2996. Separation was performed on Eclipse Plus C18 ($2.1 \times 100\text{ mm} \times 3.5\text{ }\mu\text{m}$) column using a gradient of methanol and water starting at 60:40 and ending with 100% of methanol at a flow rate of 0.2 mL/min. Samples were dissolved in DMSO.

Single-crystal X-ray diffraction was performed on a Bruker D8 Venture diffractometer (Bruker, Karlsruhe, Germany), CCD equipped with a graphite monochromator, and MoK α as the source of radiation ($\lambda = 0.71073$) at 150 K for compounds **1** and **2** and 100 K for compound **3**. Direct methods by SHELXS (v2019/3) solved the structures, [75], which were refined by full-matrix least-squares on F^2 using SHELXL (v2014/2) [76]. All non-hydrogen atoms were refined anisotropically. For compound **2**, the symmetry transformation $\#1-x+1,y,z$ was used to generate equivalent atoms. The structure was visualised using the MERCURY software (v.2025.1.1, Cambridge Crystallographic Data Centre, Cambridge, UK) [77] and RMS values were calculated in Olex2 (v.1.5, OlexSys Ltd., Durham, UK) [78].

Intermolecular interactions present in the crystal packing were analysed using Crystal-Explorer (v.21.5, University of Western Australia, Perth, Australia) [79]. Hirshfeld surfaces and finger 2D plots were generated from the crystallographic information files (CIFs) obtained by single-crystal X-ray diffraction.

UV-Vis spectra were determined on Shimadzu UV-160 and Shimadzu UV-1800 spectrophotometers (Shimadzu, Kyoto, Japan) in the range of 200–800 nm. Measurements were performed using quartz cuvettes of 1.0 cm, and DMSO was used as a blank.

Fluorescence spectra were recorded on an Agilent Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA, USA) using quartz cuvettes with a length of 1.0 cm. Samples were prepared at a concentration of 3 μM using DMSO as the solvent and

measured at 25 °C. Each compound was excited at its maximum absorption wavelength (compound 1: 487 nm; compound 2: 434 nm; and compound 3: 435 nm). Excitation and emission slit widths were set to 5 nm, and the scan rate was 600 nm/min.

Complexes with β -cyclodextrin were prepared in a planetary ball mill PQ-N04 (Across International, Livingston, NJ, USA) using zirconia jars of 100 mL and balls of 0.5 cm in diameter.

4.2. Synthetic Procedures

Synthon was prepared using 2,4-pentanedione and boron trifluoride–THF complex, as previously reported [40].

Compound 1: Distilled cinnamaldehyde (7.000 g, 53 mmol) was placed in a 125 mL Erlenmeyer flask and dissolved in ethyl acetate (20 mL). Tributyl borate (6.70 mL, 28 mmol) was added, and the mixture was stirred to ensure complete homogenization. The resulting solution was added to a 250 mL round-bottom flask containing the synthon (4.70 g, 53 mmol) previously dissolved in ethyl acetate (47 mL). Separately, *n*-butylamine (2.80 mL, 28 mmol) was dissolved in ethyl acetate (6 mL) and placed in an addition funnel. This amine solution was added dropwise to the reaction mixture under continuous stirring. The reaction was maintained at room temperature with stirring for 4 h to afford a purple precipitate, which was filtered and washed with a 90:10 water/acetone mixture. Crystallisation was performed in an acetone–tetrahydrofuran mixture. Yield: 68%, m.p. = 220 °C, ^1H NMR (700 MHz, Chloroform-*d*) δ 7.82 (t, J = 14.8 Hz, 2H), 7.53–7.49 (m, 4H), 7.41–7.31 (m, 6H), 7.09 (d, J = 15.4 Hz, 2H), 6.98 (t, J = 15.3 Hz, 2H), 6.27 (d, J = 14.8 Hz, 2H), 5.93 (s, 1H). ^{13}C NMR (176 MHz, CDCl_3) δ 179.59, 147.66, 145.13, 135.80, 130.27, 129.14, 128.01, 126.64, 124.22, 102.22. FT-IR (ATR) 3024, 2158, 11972, 1172, 1667, 1599 cm^{-1} , 1586.55 cm^{-1} , 1503, 1383, 1130, 1050, 984 885, 849, 752, 689. MS (ESI-) m/z 411.6 $[\text{M} + \text{Cl}]^-$.

Compound 2: Reflux: In a 1 L round-bottom flask, compound 1 (3.000 g, 8 mmol) was dissolved in DMSO (7.5 mL), then methanol (400 mL) and water (2.5 mL) were added. Triethylamine (2.20 mL, 16 mmol) was added to the solution, and the reaction mixture was stirred under reflux for 10 h. The orange precipitate was filtered and washed with a solution of acetone–water (90:10, *v/v*). Yield: 81%, orange powder, mp: 189–191 °C. Crystallisation was performed in tetrahydrofuran.

Microwave irradiation: The experimental procedure was carried out according to the methodological conditions described in the literature [80,81], and some variations were made. Compound 1 (0.400 g, 1 mol) and sodium oxalate (0.250 g, 1.8 mol) were placed in a sealed microwave reaction vessel, and 20 mL of methanol–water (75:25, *v/v*) solution was added. The reaction mixture was subjected to microwave irradiation at 140 °C for 10 min. The precipitate was collected by vacuum filtration and washed with water. Recrystallisation from ethyl acetate–hexane afforded the orange solid product. Yield: 85%, orange powder, mp: 189–191 °C.

^1H NMR (700 MHz, DMSO- d_6) δ 16.06 (br s, 1H), 7.59 (d, J = 7.5 Hz, 4H), 7.45 (t, J = 14.9 Hz, 2H), 7.40 (t, J = 7.6 Hz, 4H), 7.34 (t, J = 7.3 Hz, 2H), 7.18 (t, J = 15.4 Hz, 2H), 7.11 (d, J = 15.6 Hz, 2H), 6.38 (d, J = 15.1 Hz, 2H), 6.09 (s, 1H). ^{13}C NMR (176 MHz, DMSO- d_6) δ 182.88, 141.00, 140.34, 136.10, 129.06, 128.87, 127.74, 127.36, 127.25, 101.3. FT-IR (ATR) 3023 cm^{-1} , 2992 cm^{-1} , 2239 cm^{-1} , 1985 cm^{-1} , 1605 cm^{-1} , 1488 cm^{-1} , 1281 cm^{-1} , 1119 cm^{-1} , 993 cm^{-1} , 747 cm^{-1} , 684 cm^{-1} , 502 cm^{-1} , 436 cm^{-1} . MS (ESI+) m/z = 329.9 $[\text{M} + \text{H}]^+$. Purity 99.85% (HPLC).

Compound 3: Compound 2 (0.400 g, 1.20 mmol) was dissolved in 70 mL of ethyl acetate in a round-bottom flask. Separately, copper (II) acetate (0.1320 g, 0.7 mmol) was dissolved in a methanol–water solution. The resulting solution was added dropwise to the reaction mixture, which was stirred at room temperature for 36 h. The precipitate was

filtered and washed with a water–acetone solution (90:10, *v/v*). Crystallisation was carried out in acetonitrile and acetonitrile–tetrahydrofuran mixture. Brown powder, yield: 90%, decomposition temperature: 269–270 °C. FT-IR (ATR) 3052 cm^{−1}, 3017 cm^{−1}, 1975 cm^{−1}, 1873 cm^{−1}, 1794 cm^{−1}, 1691.05 cm^{−1}, 1612 cm^{−1}, 1599 cm^{−1}, 1505 cm^{−1}, 1435 cm^{−1}, 1130 cm^{−1}, 988 cm^{−1}, 686 cm^{−1}, 544 cm^{−1}, 504 cm^{−1}, 472 cm^{−1} and 441 cm^{−1}. MS (MALDI-TOF) *m/z* = 781.538 [M + Cu]⁺. Purity 100% (HPLC).

4.3. Preparation of Association Complexes with β -Cyclodextrin

The formulations with β -cyclodextrin were prepared using a planetary mill ball.

Compounds **2** (0.120 g, 0.365 mmol) or **3** (0.120 g, 0.160 mmol) and β -cyclodextrin (2 equivalents), previously dried in a conventional microwave, were placed in separate zirconia jars with 15 balls (0.5 cm in diameter). The mixtures were subjected to four 30 min mill cycles at 500 rpm, alternating between clockwise and counterclockwise rotation. The unreacted β -cyclodextrin was removed by extraction with water (10 mL) under stirring for 10 min, and the solids were collected by vacuum filtration.

Compound 4: yellow powder, yield 45%, m.p. 281–285 °C. ¹H NMR (700 MHz, DMSO-*d*₆) δ 16.06 (br s, 1H), 7.59 (d, *J* = 7.6 Hz, 4H), 7.45 (t, *J* = 14.9 Hz, 2H), 7.40 (t, *J* = 7.6 Hz, 4H), 7.34 (t, *J* = 7.4 Hz, 2H), 7.17 (t, *J* = 15.6 Hz, 2H), 7.11 (d, *J* = 15.5 Hz, 2H), 6.37 (d, *J* = 15.1 Hz, 2H), 6.09 (s, 1H), 5.71 (d, *J* = 6.9 Hz, 4H), 5.67 (d, *J* = 2.55 Hz, 3H), 4.83 (d, *J* = 3.64 Hz, 3H), 4.44 (t, *J* = 5.60, 3H), 3.63 (m, 10H), 3.56 (m, 3H), 3.35 (m, 4H), 3.30 (m, 4H). ¹³C NMR (176 MHz, DMSO-*d*₆) δ 182.89, 141.02, 140.35, 136.10, 129.07, 128.88, 127.74, 127.36, 127.26, 101.93, 101.36, 81.53, 73.03, 72.41, 72.03, 59.90. FT-IR (ATR) 3312 cm^{−1}, 2922 cm^{−1}, 2160 cm^{−1}, 2033 cm^{−1}, 1979 cm^{−1}, 1611 cm^{−1}, 1557 cm^{−1}, 1447 cm^{−1}, 1026 cm^{−1}, 996 cm^{−1}, 749 cm^{−1}, 688 cm^{−1}, 577 cm^{−1}, 505 cm^{−1}, 438 cm^{−1}. Concentration by UV/VIS = 393.60 μ g/mg.

Compound 5: brown powder, yield 35%, decomposition temperature = 300 °C, FT-IR (ATR) 3340 cm^{−1}, 3022 cm^{−1}, 2159 cm^{−1}, 1976 cm^{−1}, 1604 cm^{−1}, 1504 cm^{−1}, 1411 cm^{−1}, 1135 cm^{−1}, 1029 cm^{−1}, 988 cm^{−1}, 750 cm^{−1}, 687 cm^{−1}, 504 cm^{−1}, 471 cm^{−1}, 436 cm^{−1}. Concentration by UV/VIS = 685.94 μ g/mg.

4.4. Antiproliferative Activity

Antiproliferative activity was evaluated by Sulforhodamine B assay against colon cancer (HCT-15), breast cancer (MCF-7), leukaemia (K-262 CLM), central nervous system glial (U-251), and prostate cancer (PC-3) cell lines, which the National Cancer Institute supplied. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10,000 units per mL penicillin G, 100 μ g/mL streptomycin sulfate, and 0.25 μ g/mL amphotericin B Gibco (Thermo Fisher Scientific, Grand Island, NY, USA). They were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. For the assay, cells were suspended in a 0.1% trypsin-EDTA solution (Gibco). Cell viability was counted using a hemacytometer and diluted with medium to achieve the following densities: 5 $\times$ 10⁴ cells/mL (SKLU-1, K562) and 7.5 $\times$ 10⁴ cells/mL (U251, PC-3). Then, 100 μ L/well of the aforementioned cell suspensions were seeded in 96-well plates and incubated at 37 °C to allow for cell adhesion. A control plate (time zero evaluation) was used, containing the same volumes of each cell line and the target (RPMI-1640 growth medium).

After 24 h, the cells were treated with 100 μ L of the compounds to be evaluated, previously dissolved in RPMI-1640 medium and 1% DMSO, and then incubated for 48 h at 37 °C in a 5% CO₂ atmosphere. Only 100 μ L of RPMI-1640 culture medium was added to the control plate.

After 48 h, the adherent cells were fixed by adding 50 μ L of a cold 50% (m/vol) trifluoroacetic acid solution and incubated at 4 °C for 60 min. The supernatant was then removed, washed three times with water, and allowed to dry at room temperature. The trifluoroacetic acid-fixed cells were then treated with 100 μ L of an SRB solution (0.4% m/vol in 1% acetic acid) for 30 min at room temperature. Unbound SRB was removed with four washes with 1% acetic acid, and bound SRB was extracted with 100 μ L of unbuffered 10 mM Tris base. The optical density of the treated samples was determined in a Bio Kinetics microplate reader at a wavelength of 515 nm (Synergy HT, BioTek, Winooski, VT, USA). The colour intensity was directly proportional to the number of live cells.

4.5. Inhibition of Yeast α -Glucosidase

The inhibition of α -glucosidase was evaluated using a previously adapted method [82,83]. Briefly, a solution (25 μ L) of tested samples in DMSO-H₂O 1:1 was added to 150 μ L of phosphate buffer (PBS, 67 mM, pH 6.8) and incubated at 37 °C for 10 min with 25 μ L of reduced glutathione (3 mM in PBS) and 25 μ L of α -glucosidase type I solution (0.2 U/mL in PBS, CAS G5003-100UN) (Sigma Aldrich, Toluca, Estado de México, Mexico). Subsequently, 25 μ L of the substrate solution (23.2 mM *p*-nitrophenyl- α -D-glucopyranoside, CAS N1377-1G, in PBS) (Sigma Aldrich, Toluca, Estado de México, Mexico) was added, and the mixture was incubated at 37 °C for 15 min under shaking. The reaction mixture was stopped by the addition of a CaCO₃ 1M solution (50 μ L), followed by agitation for 5 min. The optical density was measured at 405 nm, and quercetin was used as a positive standard. The percentage of inhibition was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A is the absorbance at 405 nm of the sample and the control.

5. Conclusions

The successful synthesis of a group of extended-chain curcuminoid analogues containing eleven carbon atoms as the primary component is achieved with commendable yields. The alternative use of microwave radiation or reflux demonstrates the effectiveness of these methods in the final stages of the synthesis process. Moreover, we obtain the crystal structures of ligand **2** and its BF₂ complex, as well as those of the first copper complexes, **1** and **3**. We also provide the ¹H and ¹³C NMR assignments of compounds **1** and **2** in detail. Notably, the conformational changes of compound **2** are dependent on the solvent, highlighting a degree of flexibility within the unsaturated central chain. All compounds (**1–3**) exhibit promising optical properties, showing a red shift in maximum absorption and a high extinction coefficient compared to their seven-carbon curcuminoid counterparts.

Additionally, we report a formulation of compounds **2** and **3** with BCD, underscoring the effectiveness of the mechanochemical method. Compounds **2** and **3** exhibit moderate/good inhibitory activity against α -glucosidase and are comparable to the positive control. Notably, the formulation of a copper complex with BCD **5** is crucial in enhancing enzyme inhibition activity, highlighting the significance of solubility improvement in these compounds for biological activity evaluation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules30193943/s1>, Figure S1: ¹H-NMR spectrum of compound **1** (DMSO-d₆, 700 MHz); Figure S2: ¹³C-NMR spectrum of compound **1** (DMSO-d₆, 175 MHz); Figure S3: COSY spectrum of compound **1** (DMSO-d₆); Figure S4: HSQC spectrum of compound **1** (DMSO-d₆); Figure S5: HMBC spectrum of compound **1** (DMSO-d₆); Figure S6: ¹H-NMR spectrum of compound **2** (DMSO-d₆, 700 MHz); Figure S7: ¹³C-NMR spectrum of compound **2** (DMSO-d₆, 175 MHz); Figure S8: COSY spectrum of compound **2** (DMSO-d₆); Figure S9: HSQC spectrum of

compound **2** (DMSO- d_6); Figure S10: HMBC spectrum of compound **2** (DMSO- d_6); Figure S11: NOESY spectrum of compound **2** (DMSO- d_6 , 700 MHz); Figure S12: ^1H -NMR spectrum of compound **2** (CDCl_3 , 700 MHz); Figure S13: ^{13}C -NMR spectrum of compound **2** (CDCl_3 , 175 MHz); Figure S14: COSY spectrum of compound **2** (CDCl_3); Figure S15: HSQC spectrum of compound **2** (CDCl_3); Figure S16: HMBC spectrum of compound **2** (CDCl_3); Figure S17: NOESY spectrum of compound **2** (CDCl_3 , 700 MHz); Figure S18: ^1H -NMR spectrum of association complex **4** (DMSO- d_6 , 700 MHz); Figure S19: ^{13}C -NMR spectrum of spectrum of association complex **4** (DMSO- d_6 , 175 MHz); Figure S20: NOESY spectrum of spectrum of association complex **4** (DMSO- d_6 , 700 MHz); Figure S21: NOESY spectrum expansion of spectrum of association complex **4** (DMSO- d_6 , 700 MHz); Figure S22: Electron paramagnetic resonance spectrum of compound **3** (THF); Figure S23: Mass spectrometry spectrum of compound **1** (ESI-); Figure S24: Mass spectrometry spectrum of compound **2** (ESI+); Figure S25: Mass spectrometry spectrum of compound **3** (MALDI-TOF); Figure S26: Mass spectrometry spectrum of compound **3**, expansion (MALDI-TOF); Figure S27: Infrared spectrum of compound **1** (ATR); Figure S28: Infrared spectrum of compound **2** (ATR); Figure S29: Infrared spectrum of compound **3** (ATR); Figure S30: Infrared spectrum of spectrum of association complex **4** (ATR); Figure S31: Infrared spectrum of spectrum of association complex **5** (ATR); Figure S32: Infrared spectrum of β -cyclodextrin (ATR); Figure S33: UV-Vis spectrum of compound **2** (DMSO); Figure S34: UV-Vis spectrum of compound **3** (DMSO); Figure S35: UV-Vis spectra of association complexes **4** and **5** in DMSO; Figure S36: HPLC-UV chromatogram of compound **2**; Figure S37: HPLC-UV chromatogram of compound **2**; Figure S38: 2D-Fingerprint of Hirshfeld surface of compounds **1–3** and percentage of intermolecular interaction contributions in the crystal; Table S1: NOESY correlations of compound **2** in DMSO- d_6 and CDCl_3 (700 MHz); Table S2: Differences in ^1H and ^{13}C chemical shifts of free compound **2** and in complex; Table S3: Differences in ^1H and ^{13}C chemical shifts of free BCD and in complex; Table S4: Cellular growth inhibition (%); Table S5: Inhibition of yeast α -glucosidase. Determination of half-maximal inhibitory concentration (IC_{50}); Table S6: Crystal data and structure refinement for **1**; Table S7: Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **1**: $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U^{ij} tensor; Table S8: Bond lengths [$\text{\AA}$] and angles [$^\circ$] for **1**; Table S9: Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **1**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2}U^{11} + \dots + 2hka^*b^*U^{12}]$; Table S10: Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **1**; Table S11: Crystal data and structure refinement for **2**; Table S12: Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **2**. $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U^{ij} tensor; Table S13: Bond lengths [$\text{\AA}$] and angles [$^\circ$] for **2**; Table S14: Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **2**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2}U^{11} + \dots + 2hka^*b^*U^{12}]$; Table S15: Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **2**; Table S16: Hydrogen bonds for **1** [$\text{\AA}$ and $^\circ$]; Table S17: Crystal data and structure refinement for **3**; Table S18: Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **3**. $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U^{ij} tensor; Table S19: Bond lengths [$\text{\AA}$] and angles [$^\circ$] for **3**; Table S20: Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **3**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2}U^{11} + \dots + 2hka^*b^*U^{12}]$; Table S21: Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for **3**; Graphic S1. TGA and DSC thermograms of compounds **2**, **4** and dry BCD [84–86]; Graphic S2. TGA and DSC thermograms of compounds **3**, **5** and dry BCD. The crystallographic data of compounds **1–3** were deposited in the CCDC with numbers 2483475–2483477, <https://www.ccdc.cam.ac.uk/> (accessed on 28 August 2025).

Author Contributions: Conceptualisation, R.G.E., R.T.-H. and M.A.O.-M.; methodology, R.T.-H. and R.S.-O.; formal analysis, R.T.-H. and M.A.O.-M.; investigation R.T.-H., R.S.-O., M.T.R.-A., A.N.-C. and R.G.E.; resources, R.G.E.; writing—original draft preparation, R.T.-H.; writing—review and editing, R.T.-H., M.A.O.-M., L.L.P.-G. and R.G.E.; supervision, R.G.E.; project administration, R.G.E.; funding acquisition, R.G.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCyT, Mexico), now Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) through project CONAHCyT-FOINS-PRONACES, Grant number 307152 and PAPIIT DGAPA, Universidad Nacional Autónoma de México, Grant number IT202125.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Acknowledgments: R.T.-H. is thankful for a post-doctoral fellowship (CVU 662794) from Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) and Universidad Nacional Autónoma de México, Instituto de Química, UNAM. M.A.O.-M. CVU 599367 is thankful to SECIHTI. L.L.P.-G is thankful for a doctoral scholarship to SECIHTI CVU 1234478 and Programa de Maestría y Doctorado en Ciencias Químicas, UNAM. The authors are grateful to: Simón Hernández-Ortega, Virginia Gómez Vidales, Martha Elena García Aguilera, María de los Ángeles Peña González, Isabel Chávez, Rubén Gaviño Ramírez, Adriana Romo Pérez, Eréndira García, Lucero Ríos, Jorge Jesús Villa Rivera and Alejandra Núñez Pineda for technical assistance. The authors acknowledge IA (Grammarly IA) for improving the clarity and grammar of this manuscript. The authors present the results, analysis, and conclusions.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NOESY	Nuclear Overhauser Effect Spectroscopy
NMR	Nuclear magnetic resonance
FT-IR	Fourier Transform Infrared spectroscopy
UV-Vis	Ultraviolet–Visible spectroscopy
BDC	β -Cyclodextrin
ORTEP	Oak Ridge Thermal Ellipsoid Plot
RMS	Root Mean Square
CCDC	Cambridge Crystallographic Data Centre
MS	Mass Spectrometry

References

1. Mir, R.H.; Mohi-ud-din, R.; Mir, P.A.; Shah, A.J.; Banday, N.; Sabreen, S.; Maqbool, M.; Jan, R.; Shafi, N.; Masoodi, M.H. Chapter 9—Curcumin as a Privileged Scaffold Molecule for Various Biological Targets in Drug Development. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, The Netherlands, 2022; Volume 73, pp. 405–434, ISBN 1572-5995.
2. Yang, H.; Zeng, F.; Luo, Y.; Zheng, C.; Ran, C.; Yang, J. Curcumin Scaffold as a Multifunctional Tool for Alzheimer’s Disease Research. *Molecules* **2022**, *27*, 3879. [CrossRef]
3. Li, S.; Yuan, W.; Deng, G.; Wang, P.; Yang, P.; Aggarwal, B.B. Chemical Composition and Product Quality Control of Turmeric (*Curcuma longa* L.). *Pharm. Crops* **2011**, *5*, 28–54. [CrossRef]
4. Inoue, K.; Nomura, C.; Ito, S.; Nagatsu, A.; Hino, T.; Oka, H. Purification of Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin by High-Speed Countercurrent Chromatography. *J. Agric. Food Chem.* **2008**, *56*, 9328–9336. [CrossRef]
5. Huang, C.; Lu, H.-F.; Chen, Y.-H.; Chen, J.-C.; Chou, W.-H.; Huang, H.-C. Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin Induced Caspase-Dependent and -Independent Apoptosis via Smad or Akt Signaling Pathways in HOS Cells. *BMC Complement. Med. Ther.* **2020**, *20*, 68. [CrossRef]
6. Ramsewak, R.S.; DeWitt, D.L.; Nair, M.G. Cytotoxicity, Antioxidant and Anti-Inflammatory Activities of Curcumins I–III from *Curcuma Longa*. *Phytomedicine* **2000**, *7*, 303–308. [CrossRef]
7. Guo, L.Y.; Cai, X.F.; Lee, J.J.; Kang, S.S.; Shin, E.M.; Zhou, H.Y.; Jung, J.W.; Kim, Y.S. Comparison of Suppressive Effects of Demethoxycurcumin and Bisdemethoxycurcumin on Expressions of Inflammatory Mediators In Vitro and In Vivo. *Arch. Pharm. Res.* **2008**, *31*, 490–496. [CrossRef]

8. Hung, S.-J.; Hong, Y.-A.; Lin, K.-Y.; Hua, Y.-W.; Kuo, C.-J.; Hu, A.; Shih, T.-L.; Chen, H.-P. Efficient Photodynamic Killing of Gram-Positive Bacteria by Synthetic Curcuminoids. *Int. J. Mol. Sci.* **2020**, *21*, 9024. [CrossRef] [PubMed]
9. Den Hartogh, D.J.; Gabriel, A.; Tsiani, E. Antidiabetic Properties of Curcumin II: Evidence from In Vivo Studies. *Nutrients* **2019**, *12*, 58. [CrossRef] [PubMed]
10. Amalraj, A.; Pius, A.; Gopi, S.; Gopi, S. Biological Activities of Curcuminoids, Other Biomolecules from Turmeric and Their Derivatives—A Review. *J. Tradit. Complement. Med.* **2017**, *7*, 205–233. [CrossRef]
11. Zhang, H.A.; Kitts, D.D. Turmeric and Its Bioactive Constituents Trigger Cell Signaling Mechanisms That Protect Against Diabetes and Cardiovascular Diseases. *Mol. Cell. Biochem.* **2021**, *476*, 3785–3814. [CrossRef]
12. Bhagat, K.K.; Cheke, R.S.; Gavali, V.D.; Kharkar, P.S.; Arote, N.D. A Brief Review on Metal-Curcumin Complexes: Synthesis Approaches and Their Pharmaceutical Applications. *Discov. Chem.* **2025**, *2*, 119. [CrossRef]
13. Liu, Y.; Zhang, C.; Pan, H.; Li, L.; Yu, Y.; Liu, B. An Insight into the In Vivo Imaging Potential of Curcumin Analogues as Fluorescence Probes. *Asian J. Pharm. Sci.* **2021**, *16*, 419–431. [CrossRef]
14. Bai, G.; Yu, C.; Cheng, C.; Hao, E.; Wei, Y.; Mu, X.; Jiao, L. Syntheses and Photophysical Properties of BF₂ Complexes of Curcumin Analogues. *Org. Biomol. Chem.* **2014**, *12*, 1618–1626. [CrossRef]
15. Xu, G.; Wang, J.; Liu, T.; Wang, M.; Zhou, S.; Wu, B.; Jiang, M. Synthesis and Crystal Structure of a Novel Copper (II) Complex of Curcumin-Type and Its Application in In Vitro and In Vivo Imaging. *J. Mater. Chem. B* **2014**, *2*, 3659–3666. [CrossRef] [PubMed]
16. Festa, R.A.; Thiele, D.J. Copper: An Essential Metal in Biology. *Curr. Biol.* **2011**, *21*, R877–R883. [CrossRef]
17. Esmaeili, L.; Perez, M.G.; Jafari, M.; Paquin, J.; Ispas-Szabo, P.; Pop, V.; Andruh, M.; Byers, J.; Mateescu, M.A. Copper Complexes for Biomedical Applications: Structural Insights, Antioxidant Activity and Neuron Compatibility. *J. Inorg. Biochem.* **2019**, *192*, 87–97. [CrossRef] [PubMed]
18. Ashraf, J.; Riaz, M.A. Biological Potential of Copper Complexes: A Review. *Turk. J. Chem.* **2022**, *46*, 595–623. [CrossRef]
19. Rani, J.J.; Roy, S. Recent Development of Copper (II) Complexes of Polypyridyl Ligands in Chemotherapy and Photodynamic Therapy. *ChemMedChem* **2023**, *18*, e202200652. [CrossRef] [PubMed]
20. Mani, A.; Feng, T.; Gandioso, A.; Vinck, R.; Notaro, A.; Gourdon, L.; Burckel, P.; Saubaméa, B.; Blaque, O.; Cariou, K.; et al. Structurally Simple Osmium(II) Polypyridyl Complexes as Photosensitizers for Photodynamic Therapy in the Near Infrared. *Angew. Chem.* **2023**, *135*, e202218347. [CrossRef]
21. Shoaib, A.F.; El-Bindary, A.A.; El-Ghamaz, N.A.; Rezk, G.N. Synthesis, Characterization, DNA Binding and Antitumor Activities of Cu(II) Complexes. *J. Mol. Liq.* **2018**, *269*, 619–638. [CrossRef]
22. Choroba, K.; Machura, B.; Erfurt, K.; Casimiro, A.R.; Cordeiro, S.; Baptista, P.V.; Fernandes, A.R. Copper(II) Complexes with 2,2':6',2''-Terpyridine Derivatives Displaying Dimeric Dichloro- μ -Bridged Crystal Structure: Biological Activities from 2D and 3D Tumor Spheroids to In Vivo Models. *J. Med. Chem.* **2024**, *67*, 5813–5836. [CrossRef]
23. Ngece, K.; Khwaza, V.; Paca, A.M.; Aderibigbe, B.A. The Antimicrobial Efficacy of Copper Complexes: A Review. *Antibiotics* **2025**, *14*, 516. [CrossRef] [PubMed]
24. Meza-Morales, W.; Machado-Rodriguez, J.C.; Alvarez-Ricardo, Y.; Obregón-Mendoza, M.A.; Nieto-Camacho, A.; Toscano, R.A.; Soriano-García, M.; Cassani, J.; Enríquez, R.G. A New Family of Homoleptic Copper Complexes of Curcuminoids: Synthesis, Characterization and Biological Properties. *Molecules* **2019**, *24*, 910. [CrossRef] [PubMed]
25. Sohrabi, M.; Binaeizadeh, M.R.; Iraj, A.; Larijani, B.; Saeedi, M.; Mahdavi, M. A Review on α -Glucosidase Inhibitory Activity of First Row Transition Metal Complexes: A Futuristic Strategy for Treatment of Type 2 Diabetes. *RSC Adv.* **2022**, *12*, 12011–12052. [CrossRef] [PubMed]
26. Dirir, A.M.; Daou, M.; Yousef, A.F.; Yousef, L.F. A Review of Alpha-Glucosidase Inhibitors from Plants as Potential Candidates for the Treatment of Type-2 Diabetes. *Phytochem. Rev.* **2022**, *21*, 1049–1079. [CrossRef]
27. Gong, L.; Feng, D.; Wang, T.; Ren, Y.; Liu, Y.; Wang, J. Inhibitors of α -amylase and α -glucosidase: Potential Linkage for Whole Cereal Foods on Prevention of Hyperglycemia. *Food Sci. Nutr.* **2020**, *8*, 6320–6337. [CrossRef]
28. Du, Z.; Liu, R.; Shao, W.; Mao, X.; Ma, L.; Gu, L.; Huang, Z.; Chan, A.S.C. α -Glucosidase Inhibition of Natural Curcuminoids and Curcumin Analogues. *Eur. J. Med. Chem.* **2006**, *41*, 213–218. [CrossRef]
29. Mehrabi, M.; Esmaeili, S.; Ezati, M.; Abassi, M.; Rasouli, H.; Nazari, D.; Adibi, H.; Khodarahmi, R. Antioxidant and Glycohydrolase Inhibitory Behavior of Curcumin-Based Compounds: Synthesis and Evaluation of Anti-Diabetic Properties In Vitro. *Bioorg. Chem.* **2021**, *110*, 104720. [CrossRef]
30. Liu, Y.; Zhu, J.; Yu, J.; Chen, X.; Zhang, S.; Cai, Y.; Li, L. Curcumin as a Mild Natural α -glucosidase Inhibitor: A Study on Its Mechanism In Vitro. *Int. J. Food Sci. Technol.* **2022**, *57*, 2689–2700. [CrossRef]
31. Arenaza-Corona, A.; Obregón-Mendoza, M.A.; Meza-Morales, W.; Ramírez-Apan, M.T.; Nieto-Camacho, A.; Toscano, R.A.; Pérez-González, L.L.; Sánchez-Obregón, R.; Enríquez, R.G. The Homoleptic Curcumin–Copper Single Crystal (ML2): A Long Awaited Breakthrough in the Field of Curcumin Metal Complexes. *Molecules* **2023**, *28*, 6033. [CrossRef]
32. Cai, L.; Du, H.; Wang, D.; Lyu, H.; Wang, D. Synthesis and Photophysical Properties of Ditrifluoroacetoxyboron Complexes with Curcumin Analogues. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2021**, *249*, 119297. [CrossRef]

33. Lyu, H.; Wang, D.; Cai, L.; Wang, D.-J.; Li, X.-M. Synthesis, Photophysical and Solvatochromic Properties of Diacetoxyboron Complexes with Curcumin Derivatives. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2019**, *220*, 117126. [CrossRef]
34. Tahay, P.; Parsa, Z.; Zamani, P.; Safari, N. A Structural and Optical Study of Curcumin and Curcumin Analogs. *J. Iran. Chem. Soc.* **2022**, *19*, 3177–3188. [CrossRef]
35. Kazantzis, K.T.; Koutsounikoli, K.; Mavroidi, B.; Zachariadis, M.; Alexiou, P.; Pelecanou, M.; Politopoulos, K.; Alexandratou, E.; Sagnou, M. Curcumin Derivatives as Photosensitizers in Photodynamic Therapy: Photophysical Properties and In Vitro Studies with Prostate Cancer Cells. *Photochem. Photobiol. Sci.* **2020**, *19*, 193–206. [CrossRef] [PubMed]
36. Urošević, M.; Nikolić, L.; Gajić, I.; Nikolić, V.; Dinić, A.; Miljković, V. Curcumin: Biological Activities and Modern Pharmaceutical Forms. *Antibiotics* **2022**, *11*, 135. [CrossRef]
37. Tønnesen, H.H.; Másson, M.; Loftsson, T. Studies of Curcumin and Curcuminoids. XXVII. Cyclodextrin Complexation: Solubility, Chemical and Photochemical Stability. *Int. J. Pharm.* **2002**, *244*, 127–135. [CrossRef]
38. Arruda, T.R.; Marques, C.S.; Soares, N.F.F. Native Cyclodextrins and Their Derivatives as Potential Additives for Food Packaging: A Review. *Polysaccharides* **2021**, *2*, 825–842. [CrossRef]
39. Del Valle, E.M.M. Cyclodextrins and Their Uses: A Review. *Process Biochem.* **2004**, *39*, 1033–1046. [CrossRef]
40. Obregón-Mendoza, M.A.; Meza-Morales, W.; Alvarez-Ricardo, Y.; Estévez-Carmona, M.M.; Enríquez, R.G. High Yield Synthesis of Curcumin and Symmetric Curcuminoids: A “Click” and “Unclick” Chemistry Approach. *Molecules* **2022**, *28*, 289. [CrossRef]
41. Weiss, H.; Reichel, J.; Görls, H.; Schneider, K.R.A.; Micheel, M.; Pröhl, M.; Gottschaldt, M.; Dietzek, B.; Weigand, W. Curcuminoid–BF₂ Complexes: Synthesis, Fluorescence and Optimization of BF₂ Group Cleavage. *Beilstein J. Org. Chem.* **2017**, *13*, 2264–2272. [CrossRef]
42. Bakun, P.; Kucinska, M.; Kobyłka, P.; Kuźmińska, J.; Koczorowski, T.; Mlynarczyk, D.T.; Popena, L.; Górka, K.; Kasperkowiak, M.; Murias, M.; et al. Morpholinated Curcuminoids against Urinary Bladder Cancer Cells: Synthesis and Anticancer Evaluation. *Med. Chem. Res.* **2024**, *33*, 944–963. [CrossRef]
43. Karunakaran, C.; Balamurugan, M.; Karthikeyan, M. Applications of Electron Paramagnetic Resonance. In *Spin Resonance Spectroscopy*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 281–347, ISBN 978-0-12-813608-9.
44. Łabanowska, M.; Bidzińska, E.; Para, A.; Kurdziel, M. EPR Investigation of Cu(II)-Complexes with Nitrogen Derivatives of Dialdehyde Starch. *Carbohydr. Polym.* **2012**, *87*, 2605–2613. [CrossRef]
45. Barik, A.; Mishra, B.; Shen, L.; Mohan, H.; Kadam, R.M.; Dutta, S.; Zhang, H.-Y.; Priyadarsini, K.I. Evaluation of a New Copper(II)–Curcumin Complex as Superoxide Dismutase Mimic and Its Free Radical Reactions. *Free Radic. Biol. Med.* **2005**, *39*, 811–822. [CrossRef] [PubMed]
46. Gál, E.; Nagy, L.C. Photophysical Properties and Electronic Structure of Symmetrical Curcumin Analogues and Their BF₂ Complexes, Including a Phenothiazine Substituted Derivative. *Symmetry* **2021**, *13*, 2299. [CrossRef]
47. Feddaoui, I.; Abdelbaky, M.S.M.; García-Granda, S.; Essalah, K.; Ben Nasr, C.; Mrad, M.L. Synthesis, Crystal Structure, Vibrational Spectroscopy, DFT, Optical Study and Thermal Analysis of a New Stannate(IV) Complex Based on 2-Ethyl-6-Methylanilinium (C₉H₁₄N)₂[SnCl₆]. *J. Mol. Struct.* **2019**, *1186*, 31–38. [CrossRef]
48. Li, S.; Bu, R.; Gou, R.; Zhang, C. Hirshfeld Surface Method and Its Application in Energetic Crystals. *Cryst. Growth Des.* **2021**, *21*, 6619–6634. [CrossRef]
49. Surface Properties | CrystalExplorer. Available online: <https://crystalexplorer.net/docs/manual/isosurfaces/properties/> (accessed on 17 July 2025).
50. Schneider, H.-J.; Hacket, F.; Rüdiger, V.; Ikeda, H. NMR Studies of Cyclodextrins and Cyclodextrin Complexes. *Chem. Rev.* **1998**, *98*, 1755–1786. [CrossRef]
51. Han, W.; You, J.; Li, H.; Zhao, D.; Nie, J.; Wang, T. Curcuminoid-Based Difluoroboron Dyes as High-Performance Photosensitizers in Long-Wavelength (Yellow and Red) Cationic Photopolymerization. *Macromol. Rapid Commun.* **2019**, *40*, 1900291. [CrossRef]
52. Kaur, K.; Al-Khazaleh, A.K.; Bhuyan, D.J.; Li, F.; Li, C.G. A Review of Recent Curcumin Analogues and Their Antioxidant, Anti-Inflammatory, and Anticancer Activities. *Antioxidants* **2024**, *13*, 1092. [CrossRef]
53. Kuzminska, J.; Szyk, P.; Mlynarczyk, D.T.; Bakun, P.; Muszalska-Kolos, I.; Dettlaff, K.; Sobczak, A.; Goslinski, T.; Jelinska, A. Curcumin Derivatives in Medicinal Chemistry: Potential Applications in Cancer Treatment. *Molecules* **2024**, *29*, 5321. [CrossRef] [PubMed]
54. Sobornova, V.V.; Belov, K.V.; Krestyaninov, M.A.; Khodov, I.A. Influence of Solvent Polarity on the Conformer Ratio of Bicalutamide in Saturated Solutions: Insights from NOESY NMR Analysis and Quantum-Chemical Calculations. *Int. J. Mol. Sci.* **2024**, *25*, 8254. [CrossRef] [PubMed]
55. Leow, P.-C.; Bahety, P.; Boon, C.P.; Lee, C.Y.; Tan, K.L.; Yang, T.; Ee, P.-L.R. Functionalized Curcumin Analogs as Potent Modulators of the Wnt/β-Catenin Signaling Pathway. *Eur. J. Med. Chem.* **2014**, *71*, 67–80. [CrossRef]
56. Lutskii, A.E.; Kotelevskii, N.M.; Osipov, O.A.; Zamaraev, K.I. Effect of Solvent on EPR Spectra of Cu(II) Complexes. *Theor. Exp. Chem.* **1971**, *4*, 296–300. [CrossRef]

57. Kamada, K.; Namikawa, T.; Senatore, S.; Matthews, C.; Lenne, P.; Maury, O.; Andraud, C.; Ponce-Vargas, M.; Le Guennic, B.; Jacquemin, D.; et al. Boron Difluoride Curcuminoid Fluorophores with Enhanced Two-Photon Excited Fluorescence Emission and Versatile Living-Cell Imaging Properties. *Chem.-Eur. J.* **2016**, *22*, 5219–5232. [CrossRef]
58. Ferreira, J.R.M.; Alves, M.; Sousa, B.; Vieira, S.I.; Silva, A.M.S.; Guieu, S.; Cunha, Â.; Nunes da Silva, R. Curcumin-Based Molecular Probes for Fluorescence Imaging of Fungi. *Org. Biomol. Chem.* **2023**, *21*, 1531–1536. [CrossRef]
59. Bessho, T.; Constable, E.C.; Graetzel, M.; Hernandez Redondo, A.; Housecroft, C.E.; Kylberg, W.; Nazeeruddin, M.K.; Neuburger, M.; Schaffner, S. An Element of Surprise—Efficient Copper-Functionalized Dye-Sensitized Solar Cells. *Chem. Commun.* **2008**, *32*, 3717–3719. [CrossRef] [PubMed]
60. Abbo, H.; Ashfaq, M.; Feizi-Dehnyebi, M.; Titinchi, S. Asymmetrical Curcumin Derivative: Synthesis, Structural Exploration, Hirshfeld Surface Analysis, and Computational Study. *Struct. Chem.* **2025**, *36*, 1855–1870. [CrossRef]
61. Meza-Morales, W.; Alvarez-Ricardo, Y.; Obregón-Mendoza, M.A.; Arenaza-Corona, A.; Ramírez-Apan, M.T.; Toscano, R.A.; Poveda-Jaramillo, J.C.; Enríquez, R.G. Three New Coordination Geometries of Homoleptic Zn Complexes of Curcuminoids and Their High Antiproliferative Potential. *RSC Adv.* **2023**, *13*, 8577–8585. [CrossRef]
62. Zhao, R.; Tan, T.; Sandström, C. NMR Studies on Puerarin and Its Interaction with Beta-Cyclodextrin. *J. Biol. Phys.* **2011**, *37*, 387–400. [CrossRef]
63. Maheshwari, A.; Sharma, M.; Sharma, D. Complexation of Sodium Picosulphate with Beta Cyclodextrin: NMR Spectroscopic Study in Solution. *J. Incl. Phenom. Macrocycl. Chem.* **2013**, *77*, 337–342. [CrossRef]
64. Jiang, H.; Sun, H.; Zhang, S.; Hua, R.; Xu, Y.; Jin, S.; Gong, H.; Li, L. NMR Investigations of Inclusion Complexes between β -Cyclodextrin and Naphthalene/Anthraquinone Derivatives. *J. Incl. Phenom. Macrocycl. Chem.* **2007**, *58*, 133–138. [CrossRef]
65. Saha, S.; Roy, A.; Roy, K.; Roy, M.N. Study to Explore the Mechanism to Form Inclusion Complexes of β -Cyclodextrin with Vitamin Molecules. *Sci. Rep.* **2016**, *6*, 35764. [CrossRef] [PubMed]
66. Rachmawati, H.; Edityaningrum, C.A.; Mauludin, R. Molecular Inclusion Complex of Curcumin- β -Cyclodextrin Nanoparticle to Enhance Curcumin Skin Permeability from Hydrophilic Matrix Gel. *AAPS PharmSciTech* **2013**, *14*, 1303–1312. [CrossRef] [PubMed]
67. Sambasevam, K.; Mohamad, S.; Sarih, N.; Ismail, N. Synthesis and Characterization of the Inclusion Complex of β -Cyclodextrin and Azomethine. *Int. J. Mol. Sci.* **2013**, *14*, 3671–3682. [CrossRef]
68. Yuan, H.; Peng, Q.; Yongwei, W.; Dongsheng, Z.; Mingwan, Z.; Rui, L.; Nan, J. Host-Guest Interaction of β -Cyclodextrin with Isomeric Ursolic acid and Oleanolic Acid: Physicochemical Characterization and Molecular Modeling Study. *J. Biomed. Res.* **2017**, *31*, 395. [CrossRef]
69. Alizadeh, N.; Poorbagher, N. Host-Guest Inclusion Complexes of Sulfabenzamide with β - and Methyl- β -Cyclodextrins: Characterization, Antioxidant Activity and DFT Calculation. *J. Mol. Struct.* **2022**, *1260*, 132809. [CrossRef]
70. Nguyen, A.-T.; Pham, M.Q.; Nguyen, P.-H.; To, D.C.; Dang, N.Q.; Nguyen, T.-H.; Nguyen, H.-T.; Nguyen, T.-D.; Thi Pham, K.-H.; Tran, M.-H. Identification of Natural Curcumins as Potential Dual Inhibitors of PTP1B and α -Glucosidase through Experimental and Computational Study. *Kuwait J. Sci.* **2025**, *52*, 100312. [CrossRef]
71. Yoshikawa, Y.; Hirata, R.; Yasui, H.; Hattori, M.; Sakurai, H. Inhibitory Effect of CuSO₄ on α -Glucosidase Activity in ddY Mice. *Metallomics* **2010**, *2*, 67–73. [CrossRef]
72. Malik, N.P.; Ashiq, U.; Jamal, R.A.; Gul, S.; Lateef, M. Design, Synthesis, In Vitro and In Silico Alpha Glucosidase and Lipoxxygenase Inhibition Studies of Copper(II) Oxamide Complexes. *ChemistrySelect* **2024**, *9*, e202400398. [CrossRef]
73. Peng, X.; Liu, K.; Hu, X.; Gong, D.; Zhang, G. Hesperetin-Cu(II) Complex as Potential α -Amylase and α -Glucosidase Inhibitor: Inhibition Mechanism and Molecular Docking. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2023**, *290*, 122301. [CrossRef]
74. Mestrelab Research S.L. *MestReNova*®, Version 2020 v14.2.0; Mestrelab Research S.L.: Santiago de Compostela, Spain, 2020.
75. Sheldrick, G.M. A Short History of SHELX. *Acta Crystallogr. A* **2008**, *64*, 112–122. [CrossRef]
76. Sheldrick, G.M. Crystal Structure Refinement with SHELXL. *Acta Crystallogr. C Struct. Chem.* **2015**, *71*, 3–8. [CrossRef]
77. Macrae, C.F.; Sovago, I.; Cottrell, S.J.; Galek, P.T.A.; McCabe, P.; Pidcock, E.; Platings, M.; Shields, G.P.; Stevens, J.S.; Towler, M.; et al. Mercury 4.0: From Visualization to Analysis, Design and Prediction. *J. Appl. Crystallogr.* **2020**, *53*, 226–235. [CrossRef] [PubMed]
78. Dolomanov, O.V.; Bourhis, L.J.; Gildea, R.J.; Howard, J.A.K.; Puschmann, H. OLEX2: A Complete Structure Solution, Refinement and Analysis Program. *J. Appl. Crystallogr.* **2009**, *42*, 339–341. [CrossRef]
79. Spackman, P.R.; Turner, M.J.; McKinnon, J.J.; Wolff, S.K.; Grimwood, D.J.; Jayatilaka, D.; Spackman, M.A. CrystalExplorer: A Program for Hirshfeld Surface Analysis, Visualization and Quantitative Analysis of Molecular Crystals. *J. Appl. Crystallogr.* **2021**, *54*, 1006–1011. [CrossRef]
80. Laali, K.K.; Rathman, B.M.; Bunge, S.D.; Qi, X.; Borosky, G.L. Fluoro-Curcuminoids and Curcuminoid-BF₂ Adducts: Synthesis, X-Ray Structures, Bioassay, and Computational/Docking Study. *J. Fluor. Chem.* **2016**, *191*, 29–41. [CrossRef]

81. Abonia, R.; Laali, K.K.; Raja Somu, D.; Bunge, S.D.; Wang, E.C. A Flexible Strategy for Modular Synthesis of Curcuminoid-BF₂/Curcuminoid Pairs and Their Comparative Antiproliferative Activity in Human Cancer Cell Lines. *ChemMedChem* **2020**, *15*, 354–362. [CrossRef]
82. Zhou, T.; Zhang, S.; Liu, S.; Cong, H.; Xuan, L. Daphnodorin Dimers from Edgeworthia Chrysantha with α -Glucosidase Inhibitory Activity. *Phytochem. Lett.* **2010**, *3*, 242–247. [CrossRef]
83. Ye, X.-P.; Song, C.-Q.; Yuan, P.; Mao, R.-G. α -Glucosidase and α -Amylase Inhibitory Activity of Common Constituents from Traditional Chinese Medicine Used for Diabetes Mellitus. *Chin. J. Nat. Med.* **2010**, *8*, 349–352. [CrossRef]
84. Abarca, R.L.; Rodríguez, F.J.; Guarda, A.; Galotto, M.J.; Bruna, J.E. Characterization of Beta-Cyclodextrin Inclusion Complexes Containing an Essential Oil Component. *Food Chem.* **2016**, *196*, 968–975. [CrossRef]
85. Kim, J.-S. Study of Flavonoid/Hydroxypropyl- β -Cyclodextrin Inclusion Complexes by UV-Vis, FT-IR, DSC, and X-Ray Diffraction Analysis. *Prev. Nutr. Food Sci.* **2020**, *25*, 449–456. [CrossRef] [PubMed]
86. Zhang, W.; Zhou, T.-P.; Zou, W.; Wang, Y.; Wang, K.; Yang, Y.; Liu, C.; Tu, Z.; Liu, Q.; Yuan, Y. Formation of β -Cyclodextrin Inclusion Complexes with a Series of Structurally Related Parabens: Preparation, Physicochemical Characterization and Antifungal Properties. *Carbohydr. Polym. Technol. Appl.* **2025**, *11*, 100924. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

Phenolic-Rich Wild Edible Macrofungi: Antimicrobial Activity and Antioxidant Potential

Elif Ildız and Elif Yürümez Canpolat *

Department of Biotechnology, Faculty of Science, Niğde Ömer Halisdemir University, Niğde 51240, Türkiye

* Correspondence: elif.yurumez@ohu.edu.tr

Abstract

This study evaluated the antimicrobial and antioxidant activities and chemical properties of four wild edible macrofungi—*Tuber aestivum* (Wulfen) Spreng., *Terfezia clavaryi* Chatin, *Agaricus arvensis* Schaeff. and *Bovistella utriformis* (Bull.) Demoulin & Rebriev—collected from different regions of Türkiye, with particular emphasis on the role of phenolic compounds. Methanol and hexane extracts were assessed for antimicrobial activity against Gram-positive, Gram-negative, multidrug-resistant (MDR) bacterial strains, and *Candida albicans* using minimum inhibitory concentration (MIC) assays. Total phenolic content (TPC) was determined, and antioxidant capacities were evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power), and CUPRAC (cupric ion reducing antioxidant capacity) assays. The chemical profiles of hexane extracts were characterized by GC–MS analysis, whereas methanol extracts were analyzed by LC–MS/MS. Methanol extracts with high content of phenolic compounds exhibited markedly higher antimicrobial activity than hexane extracts, especially against Gram-positive bacteria. *B. utriformis* and *A. arvensis* displayed the highest phenolic contents (29.61 ± 0.6 and 27.14 ± 0.59 mg GAE/g DW, respectively) and antioxidant activities, revealing a strong positive correlation between TPC and antioxidant capacity. LC–MS/MS analysis revealed catechin, cinnamic acid, and caffeic acid as prominent phenolic constituents, highlighting the role of polyphenols in the observed bioactivity. GC–MS profiling predominantly identified fatty acid methyl esters, particularly linoleic and oleic acids, together with minor phenolic derivatives, suggesting a possible synergistic interaction contributing to the overall biological potential. The results highlight phenolic-rich macrofungi as valuable natural sources of antioxidant and antimicrobial agents with potential applications.

Keywords: edible macrofungi; phenolic compounds; antioxidant activity; antimicrobial activity; multidrug-resistant bacteria; GC–MS; LC–MS/MS

1. Introduction

The emergence of antibiotic-resistant bacterial strains has become one of the most severe public health threats of the 21st century, as many infectious diseases that were once easily treated are becoming increasingly unmanageable [1,2]. This crisis is driven by the genetic ability of pathogens to acquire or mutate resistance genes through horizontal gene transfer, including processes such as conjugation, transformation, and transduction. The World Health Organization (WHO) identifies antimicrobial resistance as a worldwide challenge directly associated with high morbidity and mortality [3,4]. It is conservatively estimated that antibiotic resistance results in at least 23,000 deaths annually in the United

States alone, with global predictions suggesting this figure could reach 10 million deaths per year by 2050. Furthermore, the pharmaceutical pipeline for new antibiotics has largely run dry, leaving a critical shortage of effective therapies and necessitating the development of novel antimicrobial alternatives [5].

Natural products have gained increasing attention as potential sources of novel bioactive compounds. Mushrooms are valuable organisms that have been used in both cuisine and traditional medicine since ancient times, due to their unique aroma and high nutritional value. Despite their low calorie and fat content, they are a rich source of proteins, vitamins, minerals, and dietary fiber [6–8]. Beyond these basic nutritional properties, mushrooms offer a wide range of health benefits through secondary metabolites such as phenolic compounds. The growing interest in mushroom polyphenols in the field of functional foods and nutraceuticals today stems from the potential of these bioactive compounds to prevent chronic diseases and improve human health [9–11]. The phenolic compounds found in mushrooms combat oxidative stress in a multifaceted manner by acting as both primary and secondary antioxidants [12]. These compounds break the chain reactions of auto-oxidation by neutralizing free radicals through hydrogen atom donation or electron transfer [13]. They protect cells from tissue damage or death by preventing DNA, RNA, protein, and lipid damage caused by reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals [14,15]. The antioxidant capacity of phenolics protects cells beyond their radical scavenging effect; it also defends cells by chelating ROS-producing metals (such as Fe and Cu) and inhibiting oxidase enzymes like NADH oxidase or lipoxygenase that lead to radical formation. Research confirms a direct and strong correlation between total phenolic content (TPC) in mushrooms and antioxidant capacity, with phenolic acids (gallic, caffeic, p-coumaric acid, etc.) being the main components responsible for this activity [12,16,17].

In addition to their antioxidant effects, mushroom polyphenols exhibit a broad spectrum of bioactivity. They have anti-inflammatory effects by suppressing the production of inflammatory mediators such as nitric oxide (NO) and reactive oxygen species [18–20]. Furthermore, they exhibit antitumor activity by inhibiting the proliferation of cancer cells and triggering apoptosis, while displaying antihyperglycemic properties by inhibiting α -glucosidase and α -amylase enzymes, thereby balancing blood sugar levels [21–25]. These compounds, which provide antimicrobial protection against microorganisms by inhibiting cell wall, protein, and nucleic acid synthesis, have also been scientifically documented to inhibit the tyrosinase enzyme responsible for melanin synthesis and to offer anti-osteoporotic effects that prevent bone loss [26,27]. Due to these multifaceted biological activities, fungal phenolics are recognized as valuable active compounds with applications in a wide range of fields.

Several genera included in the present study, including *Terfezia*, *Tuber*, *Agaricus*, and *Bovistella*, are known to contain phenolic compounds contributing to antioxidant and antimicrobial activities. While previous studies have reported bioactive properties of these genera, comparative evaluations integrating total phenolic content, antioxidant capacity, and antimicrobial activity of wild edible representatives collected from Türkiye remain scarce. The chemical diversity of wild edible fungi, particularly those collected from different ecological regions, represents an underexplored reservoir of bioactive molecules [28,29].

The application of fungus extracts against multidrug-resistant (MDR) bacterial strains has yielded promising results in recent clinical studies. Extracts from species such as *Pleurotus ostreatus*, *Fistulina hepatica*, and *Russula delica* have demonstrated potent inhibitory effects against high-priority pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Pseudomonas aeruginosa*. Specifically, methanolic extracts of the fungi, edible macrofungi in particular, were found to be more effective than conven-

tional antibiotics against clinical isolates of vancomycin-resistant *S. aureus* and *Salmonella typhi*. These extracts contain a complex cocktail of molecules that can directly inhibit bacterial growth by targeting cell walls, mRNA, and protein synthesis [5,30,31]. Despite these promising findings, most previous studies have focused on single species or single extraction solvents, and comprehensive evaluations combining chemical profiling and activity assessment remain scarce.

In this context, the present study aimed to comparatively evaluate the chemical composition and biological activities of four species of wild edible fungi belonging to different families of Ascomycota and Basidiomycota collected from Türkiye between 2022 and 2023. Unlike previous reports focusing on single species or limited bioactivity screening, this study integrates (i) solvent-based extraction (methanol and hexane), (ii) determination of total phenolic content, (iii) assessment of antioxidant capacity, and (iv) determination of minimum inhibitory concentration (MIC) values against Gram-positive, Gram-negative, and multidrug-resistant bacterial strains.

2. Results and Discussion

2.1. Identification of the Macrofungi Samples

The four wild edible macrofungi collected during field studies were identified based on morphological characteristics according to the current literature [32–34]. Figure 1 shows the ascocarps and basidiocarps of the samples collected from their natural environment.



Figure 1. Macrofungi samples collected from field studies: (A) *Tuber aestivum*, (B) *Terfezia clavaryi*, (C) *Agaricus arvensis*, and (D) *Bovistella utriformis*.

A further identification based on extraction, amplification and sequencing of the internal transcribed spacer (ITS) region of the rDNA, which is a highly conserved and widely used fragment for identification of the fungal samples due to its interspecific variations, was also performed to avoid possible confusion at the species level [35,36]. The sequence data was compared with the GenBank database via Basic Local Alignment Search

Tool (BLAST) (nucleotide BLAST, accessed on https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), and the results revealed that the fungal samples were *Tuber aestivum* (Wulfen) Spreng. (1827), *Terfezia clavaryi* Chatin (1892), *Agaricus arvensis* Schaeff. (1774) and *Bovistella utriformis* (Bull.) Demoulin & Rebriv (2017), which are in accordance with the morphological identification. The sequence data of the identified samples were also deposited in GenBank, and the accession numbers are listed in Table 1, which also indicate the GPS coordinates, locations, average altitudes and the collection dates of the samples.

Table 1. The collection dates, geographical positions, and GenBank accession numbers of macrofungi samples collected during the field studies.

Sample Name	Locations	Coordinates	Average Altitude	Collection Date	Accession No.
<i>Tuber aestivum</i>	Vize/Kırklareli	41°33'54" N 27°43'09" E	170 m	June, 2022	PX912447
<i>Terfezia clavaryi</i>	Mazıdağı/Mardin	37°31'05" N 40°26'32" E	950 m	June, 2023	PX925061
<i>Agaricus arvensis</i>	Özyurt/Niğde	37°57'41" N 34°51'24" E	1870 m	May, 2022	PX907866
<i>Bovistella utriformis</i>	Çamardı/Niğde	37°54'55" N 34°53'32" E	1750 m	May, 2022	PX907867

2.2. Antioxidant Capability

One of the most important parameters in determining the bioactive potential of mushroom extracts is the total phenolic content (TPC) and the associated antioxidant activities. When these were examined, statistically significant differences were observed between species ($p < 0.05$). Based on the data presented in Table 2, the highest total phenolic content was detected in the *B. utriformis* sample with a value of 29.61 ± 0.6 mg GAE/g dry weight. This was followed by *A. arvensis* with 27.14 ± 0.59 mg GAE/g DW. In contrast, the hypogeous fungi (truffle group) *T. aestivum* (18.52 ± 1.06 mg GAE/g DW) and *T. clavaryi* (16.66 ± 0.59 mg GAE/g DW) exhibited lower phenolic content.

Table 2. The content of total phenolic compounds and antioxidant activities of fungal extracts with 100 mg/mL concentration.

Sample	TPC	DPPH	ABTS	FRAP	CUPRAC
<i>T. aestivum</i>	18.52 ± 1.06 c	2.38 ± 0.15 b	1.46 ± 0.06 c	5.34 ± 0.08 c	3.91 ± 0.01 c
<i>T. clavaryi</i>	16.66 ± 0.59 d	2.01 ± 0.23 c	1.30 ± 0.02 d	3.41 ± 0.05 d	2.57 ± 0.04 d
<i>A. arvensis</i>	27.14 ± 0.59 b	4.92 ± 0.01 a	1.90 ± 0.05 a	11.16 ± 0.27 a	8.04 ± 0.18 b
<i>B. utriformis</i>	29.61 ± 0.6 a	4.93 ± 0.04 a	1.66 ± 0.02 b	7.30 ± 0.14 b	10.45 ± 0.39 a

TPC: total phenolic compounds (mg GAE/g DW); DPPH: DPPH scavenging activity (mmol TE/g DW); ABTS: ABTS⁺ cation removal activity (mmol TE/g DW); FRAP: ferric reducing antioxidant power (mmol AAE/g DW); CUPRAC: cupric reducing antioxidant capacity (mmol TE/g DW). All data shown in the table is presented as the mean $\pm$ SE of three independent measurements. Different lowercase letters in each column indicate a significant difference at $p < 0.05$.

These findings are consistent with recent studies in the literature. Several studies have reported that members of Basidiomycota (especially members of the Agaricaceae and Lycoperdaceae families) generally have a higher secondary metabolite synthesis capacity compared to hypogeous fungi [37,38]. The high phenolic content of *B. utriformis* supports the scientific basis for the use of this species and its close relatives (*Calvatia* sp.) as wound healers in traditional medicine. The results of antioxidant activity tests (DPPH, ABTS,

FRAP, and CUPRAC) showed a strong positive correlation with total phenolic content. *B. utrififormis* and *A. arvensis* exhibited the highest activity in all tested methods.

In particular, *B. utrififormis* and *A. arvensis* performed higher free radical scavenging activity for the DPPH assay with 4.93 ± 0.04 and 4.92 ± 0.01 mmol TE/g DW, respectively. On the other hand, their ABTS⁺ cation-degrading values were 1.66 ± 0.02 and 1.90 ± 0.05 mmol TE/g DW, respectively. For both antioxidant assays investigated with free radicals, *T. aestivum* and *T. claveryi* exhibited lower scavenging activities. The FRAP test, which measures metal chelation and reduction capacity, yielded notable values for *A. arvensis* with 11.16 ± 0.27 mmol AAE/g DW, and the CUPRAC test yielded notable values for *B. utrififormis* with 10.45 ± 0.39 mmol TE/g DW, while *T. aestivum* and *T. claveryi* exhibited lower values again for both assays.

In Figure 2, the DPPH and ABTS free radical scavenging activities of the fungal samples were demonstrated according to the extract concentrations. All samples exhibited an increasing antioxidant activity with regard to the increasing extract concentration. Interestingly, for DPPH scavenging activity, it was observed that the extract of *A. arvensis* reached a plateau at the lowest dose of 20 mg/mL, exhibiting values close to the antioxidant activity values displayed at the highest concentration. Another noteworthy point is that no significant difference was observed between the DPPH scavenging effects of *A. arvensis* and *B. utrififormis* extracts at concentrations of 40 mg/mL and above. On the other hand, a significant difference was clearly observed at all concentrations between *T. claveryi* and *T. aestivum* and these two fungi. In terms of ABTS cation-degrading activity, a statistically significant difference ($p < 0.05$) was found between *A. arvensis* and all other fungi at all concentrations tested. In the study conducted with six wild edible fungi, a 10.92 ± 0.60 mg TE/g extract DPPH scavenging and 51.91 ± 2.72 mg TE/g extract ABTS degrading value were reported for *Lycoperdon utrifforme* (syn. *B. utrififormis*) methanol extract [39], which supports the findings of this study. Similarly, the DPPH radical scavenging value of *A. arvensis* collected from nature was reported as 65.73% for 20 mg/mL extract [40], while the EC₅₀ ABTS⁺ degrading activity of this fungus was reported as 3.19 ± 0.3 mg/mL [41].

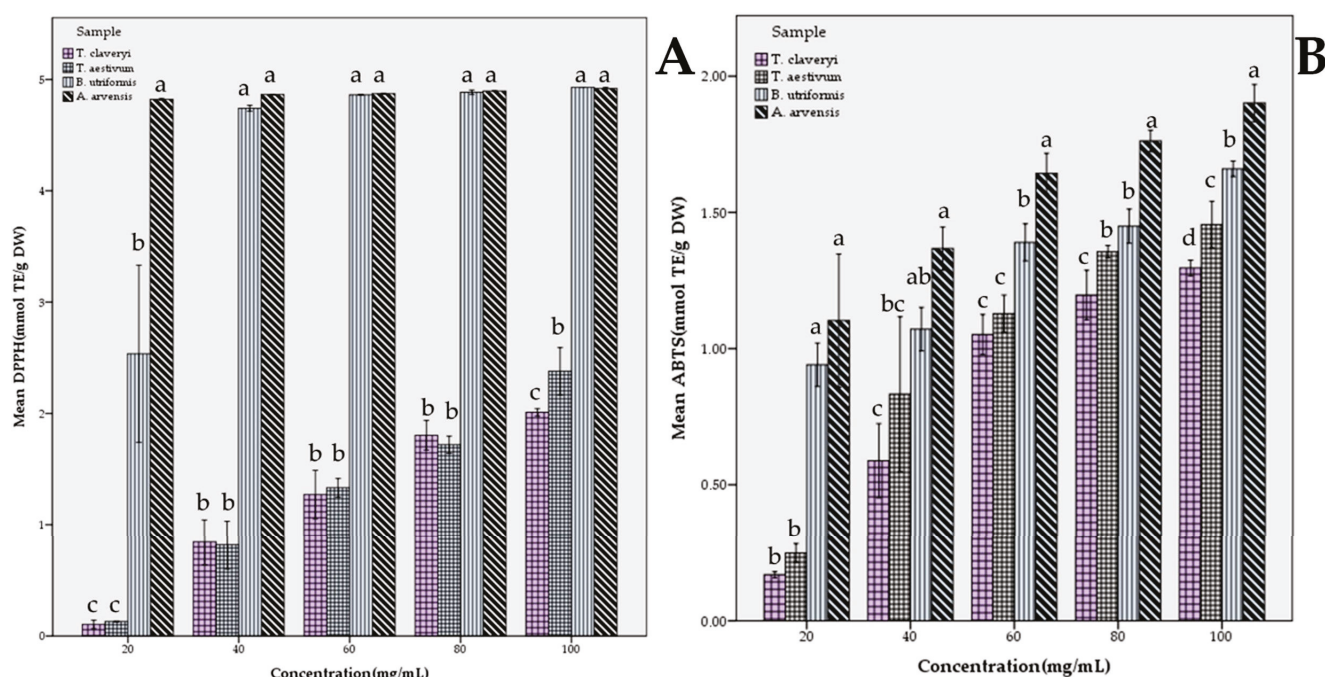


Figure 2. Dose-dependent free radical scavenging activity of fungal extracts: (A) DPPH radical scavenging activity and (B) ABTS⁺ cation-degrading activity. Different lowercase letters at each concentration indicate a significant difference at $p < 0.05$.

Similar to the free radical scavenging activity assays, metal ion reducing capacity assays (FRAP and CUPRAC) revealed that all samples demonstrated an increasing pattern of antioxidant capacity with the increase in extract concentration (Figure 3). In the FRAP test, *A. arvensis* showed a significant difference at the $p < 0.05$ level at all tested concentrations compared to the other fungal samples investigated. This is also the case for *B. utrifomis* in the CUPRAC test. For all concentrations tested, a significant difference was found compared to the other samples.

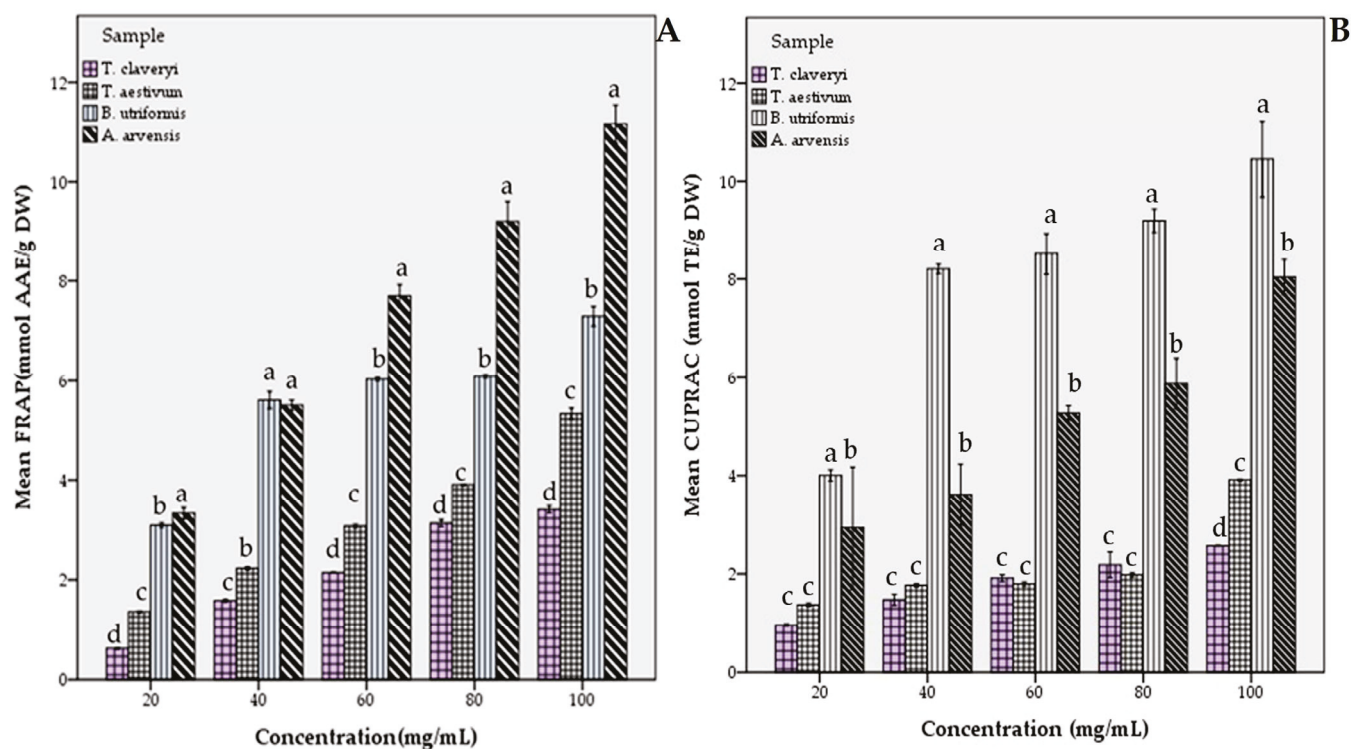


Figure 3. Dose-dependent metal ion reducing capability of fungal extracts: (A) ferric reducing antioxidant power (FRAP) and (B) cupric reducing antioxidant capacity (CUPRAC). Different lowercase letters at each concentration indicate a significant difference at $p < 0.05$.

In a study on *T. claveryi*, the aqueous extract exhibited markedly higher total phenolic (176.67 mg GAE/g DW) content compared to other solvent extracts, and this value was positively correlated with antioxidant activity [42]. The authors further emphasized that antioxidant and antibacterial activities were strongly solvent-dependent, with aqueous and methanolic extracts showing different bioactivity profiles. In contrast, the present study employed methanolic extracts and revealed substantially lower TPC and antioxidant activities for *T. claveryi*, which is consistent with the solvent effect reported in the literature. The lower FRAP and CUPRAC values observed in methanolic *T. claveryi* extract likely reflect a reduced extraction efficiency of hydrophilic phenolic compounds, which dominate the antioxidant profile of this species. Therefore, the discrepancy between the high bioactivity reported for aqueous extracts of *T. claveryi* and the comparatively lower antioxidant performance observed in the present study can be directly attributed to differences in the extraction solvent rather than species-specific limitations.

In the present study, the methanolic extract of *Tuber aestivum* exhibited lower total phenolic content and antioxidant activity compared to epigeous Basidiomycota species (*B. utrifomis* and *A. arvensis*). This finding is consistent with the literature reporting the relatively limited antioxidant potential of *T. aestivum*. In a comprehensive study conducted by Beara et al. [43], the total phenolic content of methanolic and aqueous extracts of *T. aestivum* was reported to range between 11.72 and 18.68 mg GAE/g dry weight, while

the antioxidant activities assessed by DPPH, FRAP, and lipid peroxidation inhibition assays were found to be at moderate levels. Although the total phenolic content values obtained for *T. aestivum* in the present study are consistent with this range, the FRAP capacity reported in the same study (maximum 13.4 mg AAE/g dry weight) is noticeably lower than the FRAP and CUPRAC values determined for *A. arvensis* and *B. utrifomis*. Beara et al. [43] emphasized that the limited antioxidant activity of *T. aestivum* may be associated with its hypogeous growth habit, lower phenolic density, and the restricted structural diversity of phenolic compounds.

In a previous study evaluating the antioxidant properties of *Lycoperdon utriforme*, high total antioxidant capacity (203.61 mg TE/g extract) and notable FRAP (10.85 mg TE/g extract) and CUPRAC (39.66 mg TE/g extract) values were reported. When these findings are compared with the present study, *B. utrifomis* (*syn. Lycoperdon utriforme*) exhibited comparable reducing power in the FRAP assay, with a value of 11.159 ± 0.27 mmol AAE/g DW, indicating a similar metal-reducing capacity despite differences in extraction protocol and expression units. However, CUPRAC activity in the mentioned study was considerably higher, which may be attributed to the differences in solvent polarity and extraction efficiency [39]. In the present study, methanolic extracts were used, whereas the solvent type was not standardized across studies, which is known to strongly influence the recovery of redox-active compounds. Nevertheless, the consistent observation of strong FRAP responses in both studies supports the conclusion that *B. utrifomis* possesses an inherently high electron-donating and metal-reducing antioxidant potential.

In the study evaluating phenolic profiles and antioxidant properties of several *Agaricus* species, *Agaricus arvensis* was reported to exhibit one of the highest DPPH radical scavenging activities within the genus, ranking second after *A. bitorquis* and exceeding commonly consumed species such as *A. bisporus* and *A. campestris*. This finding indicates a relatively strong antiradical capacity for *A. arvensis* based on DPPH assay results [10]. In agreement with these findings, the present study demonstrated that *A. arvensis* showed high antioxidant activity not only in DPPH but also consistently across ABTS, FRAP, and CUPRAC assays. Notably, the FRAP value obtained for *A. arvensis* methanol extract with 100 mg/mL concentration in the present study (11.16 ± 0.27 mmol AAE/g DW) confirms its strong reducing capacity, extending the single-assay DPPH results reported previously to multiple antioxidant mechanisms. Therefore, while the mentioned study characterized *A. arvensis* primarily by its DPPH scavenging ability, the current results demonstrate that this species exhibits a broad and robust antioxidant profile across different assay systems.

Phenolic compounds are known to form a protective shield against oxidative stress due to their ability to stabilize free radicals (DPPH and ABTS cations) and reduce metals (FRAP/CUPRAC). The data obtained in our study reveal that Basidiomycota samples have stronger free radical scavenging potential compared to the investigated Ascomycota members. In agreement with the present findings, several studies have shown that phenolic compounds in mushrooms are closely linked to antioxidant activities across multiple assay systems. LC-MS/MS profiling of edible and wild mushrooms has identified various phenolic acids and flavonoids, such as catechin, caffeic acid, and ferulic acid, which correlate with strong antioxidant potential [29,44]. Our LC-MS results confirmed the presence of these compounds in *Tuber aestivum*, *Terfezia clavaryi*, *Agaricus arvensis*, and *Bovistella utrifomis*, showing that phenolic content underpins the DPPH, ABTS, FRAP, and CUPRAC antioxidant activities observed. Furthermore, GC-MS analysis of the same mushroom samples revealed a diverse profile of fatty acids, including both saturated and unsaturated species, which are also known to contribute to antioxidant and health-promoting properties. The integration of LC-MS and GC-MS data indicates that the

antioxidant capacity of these mushrooms arises not only from phenolic compounds but also from fatty acids, highlighting the multi-component nature of their bioactive potential.

2.3. Chemical Compositions of the Fungal Samples

LC–MS analysis was conducted to determine the concentrations of selected phenolic compounds in *T. aestivum*, *T. claveryi*, *A. arvensis*, and *B. utrififormis*. Phenolic compounds were detected using LC–MS/MS in Multiple Reaction Monitoring (MRM) mode. Precursor and product ions, ionization modes, and retention times are shown in Table 3. Catechin hydrate and cinnamic acid were detected in positive ion mode, while other compounds were analyzed in negative ion mode. Catechin was detected in all species, reaching the highest concentrations in *B. utrififormis* (18.657 µg/g DW) and *A. arvensis* (16.359 µg/g DW). Cinnamic acid was only detected in epigeous species, with concentrations of 65.178 µg/g DW in *A. arvensis* and 69.981 µg/g DW in *B. utrififormis*; it was not detected in hypogeous species (*T. aestivum* and *T. claveryi*). Caffeic acid showed the highest levels in *T. aestivum* (0.952 µg/g DW) and *T. claveryi* (0.804 µg/g DW), while it was present at very low levels in *A. arvensis* and *B. utrififormis*. 2,5-Dihydroxybenzoic acid was only detected in *T. aestivum* at 0.041 µg/g DW. Trans-ferulic acid was detected at low concentrations in all four species, with the highest level observed in *T. aestivum* (0.098 µg/g DW). Among flavonoids, quercetin was detected at low levels only in hypogeous species, while luteolin was found exclusively in *T. aestivum* at a very low concentration (0.002 µg/g dw). Myricetin, naringenin, chrysin, tannic acid, and ellagic acid were not detected in any of the species. Overall, hypogeous truffle species (*T. aestivum* and *T. claveryi*) exhibited low to moderate phenolic profiles in terms of catechin and caffeic acid, whereas epigeous species (*A. arvensis* and *B. utrififormis*) showed higher concentrations of catechin and cinnamic acid. These results indicate species-specific metabolic differences and habitat-related phenolic diversity. These findings are in agreement with previous reports indicating the presence of various phenolic acids and flavonoids, such as gallic acid, protocatechuic acid, hydroxycinnamic acid derivatives, catechin, and myricetin [45,46].

Table 3. Content of phenolic compounds in four macrofungi by LC–MS/MS (µg/g DW).

Compound	RT (min)	Precursor (m/z)	Product (m/z)	Ionization Mode	Content of Selected Phenolics (µg/g DW)			
					<i>T. aestivum</i>	<i>T. claveryi</i>	<i>A. arvensis</i>	<i>B. utrififormis</i>
Catechin	2.279	291.1	138.9; 122.9	[M + H] ⁺	14.724	3.268	16.359	18.657
Cinnamic acid	3.78	149.1	130.9; 103.2	[M + H] ⁺	-	-	65.178	69.981
Caffeic acid	2.649	178.9	135; 134; 89	[M – H] [–]	0.952	0.804	0.033	0.027
2-5 dihydroxybenzoic acid	2.399	153.1	107.9; 109	[M – H] [–]	0.041	-	-	-
Trans-ferulic acid	2.946	192.8	132.9; 178	[M – H] [–]	0.098	0.061	0.047	0.040
Myricetin	3.356	316.8	178.9; 151; 137	[M – H] [–]	-	-	-	-
Naringenin	3.608	270.8	150.9; 118.9; 92.9	[M – H] [–]	-	-	-	-
Quercetin	3.628	300.8	150.8; 121.4; 106.9	[M – H] [–]	0.013	0.011	-	-
Luteolin	3.737	284.8	217; 198.8; 174.9	[M – H] [–]	0.002	-	-	-
Chrysin	4.346	252.8	142.9; 119; 209.1; 106.9	[M – H] [–]	-	-	-	-
Tannic acid	2.18	182.9	123.6; 78.2	[M – H] [–]	-	-	-	-
Ellagic acid	3.624	300.8	229.1; 257.1	[M – H] [–]	-	-	-	-

RT: retention time; “-”: not detected. [M + H]⁺: The protonated molecular ion formed by the addition of a proton to the molecule in positive electrospray ionization (ESI) mode.

In the study by [10], *A. arvensis* was reported to contain phenolic acids including gallic, caffeic, and ferulic acids, while flavonoids were generally not detected; total phenolic content was correlated with high antioxidant activity. The results of the present study are

consistent with these findings regarding the predominance of phenolic acids and the contribution of catechin and cinnamic acid to antioxidant potential. The detection of catechin in both hypogeous and epigeous species in our study further supports the role of flavan-3-ols in contributing to antioxidant potential. Dundar et al. [40] investigated methanol extracts of seven mushroom species from Türkiye and found significant antioxidant activity, along with the presence of phenolic acids including protocatechuic, caffeic, syringic, and p-coumaric acids. Çayan et al. [47] analyzed 26 mushroom species for their phenolic acid content and reported a total of 16 phenolic and organic acid compounds. The majority of the samples contained gallic acid, fumaric acid, barrosprotocatechuic acid, catechin hydrate, and trans-cinnamic acid. Barros et al. [48] examined sixteen wild Portuguese mushroom species and identified phenolic acids as the predominant compounds. They also reported that *Lycoperdon* species (*L. molle* and *L. perlatum*, currently classified as *Bovistella*) exhibited the highest total phenolic contents, which is consistent with the results obtained in our study.

The GC-MS analysis of hexane extracts from *Tuber aestivum*, *Terfezia claveryi*, *Agaricus arvensis*, and *Bovistella utriformis* revealed a complex chemical profile composed primarily of fatty acid esters, along with a diverse array of alkanes, alcohols, ketones, and phenols. Fatty acid esters constituted the most abundant class of compounds across all four species, though their specific distributions varied significantly. *T. claveryi* was characterized by a high concentration of hexadecanoic acid methyl ester (26.58%), whereas *T. aestivum* was dominated by 9,12-octadecadienoic acid methyl ester (19.9%). In contrast, the profiles of *A. arvensis* and *B. utriformis* were distinctively rich in octadecanoic acid methyl ester, which accounted for 22.78% and 26.18% of their relative concentrations, respectively. Notably, the unsaturated fatty acid ester 9-octadecenoic acid methyl ester acted as a distinguishing marker between the groups; it was prominent in the truffle species (*T. aestivum* and *T. claveryi*, >11%) but present only in trace amounts in *A. arvensis* and *B. utriformis* (~1%).

Beyond the dominant fatty acid esters, the extracts contained various hydrocarbons and volatile organic compounds. Alkanes represented the most chemically diverse group, with compounds such as tetradecane, hexadecane, and octadecane detected across the species, generally ranging from trace amounts to approximately 5%. *B. utriformis*, in particular, showed a slightly higher accumulation of specific long-chain alkanes like octadecane (4.99%) and hexadecane (4.16%). Other bioactive constituents included ketones, specifically 2,6-di-*t*-butyl-4-methylene-2,5-cyclohexadiene-1-one, found in all samples (1.75–4.02%), and minor concentrations of phenolic compounds and alcohols (Table 4). Overall, while the compositions were generally similar, the variations in relative concentrations, especially within the fatty acid ester profiles, highlighted distinct chemical differences between the investigated Ascomycota and Basidiomycota species (Figure 4).

Several studies have reported that the lipid fraction of mushrooms and truffles is dominated by long-chain fatty acids, particularly oleic and linoleic acids, usually detected as methyl esters in GC-MS analyses. Kalač [49] comprehensively reviewed mushroom lipid composition and demonstrated that linoleic acid is the predominant unsaturated fatty acid in most edible mushrooms and truffles, followed by oleic acid. Similarly, Reis et al. [50] reported that methanolic extracts of wild mushrooms were characterized by high proportions of unsaturated fatty acids, with linoleic acid frequently accounting for more than 50% of total fatty acids.

Table 4. Chemical composition of fungal extracts determined by GC-MS.

Compound	CF	MW	RT	RI	CT	Relative Concentration (%)			
						<i>T. aestivum</i>	<i>T. claveryi</i>	<i>A. arvensis</i>	<i>B. utrififormis</i>
3-Hexanol	C ₆ H ₁₄ O	102	5.123	780	Alcohol	0.76	0.31	0.71	-
3,7-Dimethyldecane	C ₁₂ H ₂₆	170	12.814	1086	Alkane	0.59	0.32	0.75	-
1-Iodononane	C ₉ H ₁₉ I	254	12.826	1330	Alkane	-	-	-	0.75
2,3,6,7-Tetramethyloctane	C ₁₂ H ₂₆	170	14.249	958	Alkane	-	-	0.43	-
Dodecane	C ₁₂ H ₂₆	170	17.26	1214	Alkane	0.74	0.43	0.55	0.54
5-Methyltetradecane	C ₁₅ H ₃₂	212	19.177	1448	Alkane	0.68	-	-	0.74
4,6-Dimethyldodecane	C ₁₄ H ₃₀	198	19.182	1285	Alkane	-	1.3	2.84	-
5-Butylnonane	C ₁₃ H ₂₈	184	19.662	1249	Alkane	1.53	-	0.92	1.01
Hexadecane	C ₁₆ H ₃₄	226	19.665	1612	Alkane	-	-	-	2.05
1-Ethyl-2-propylcyclohexane	C ₁₁ H ₂₂	154	20.431	1140	Cycloalkane	0.5	-	-	-
1-Tridecanol	C ₁₃ H ₂₈ O	200	20.661	1556	Alcohol	0.5	0.29	-	1.41
7-Methyl-1-undecene	C ₁₂ H ₂₄	168	20.668	1140	Alkene	-	-	0.52	-
Tetradecane	C ₁₄ H ₃₀	198	23.018	1413	Alkane	3.96	2.45	3.41	3.08
2,6,10,14-Tetramethylpentadecane	C ₁₉ H ₄₀	268	25.021	1707	Alkane	1.2	-	-	-
2,6-di- <i>t</i> -butyl-4-methylene-2,5-cyclohexadiene-1-one	C ₁₅ H ₂₂ O	218	25.285	1582	Ketone	3.35	1.75	4.02	3.3
Heptadecane	C ₁₇ H ₃₆	240	25.617	1711	Alkane	3.84	0.58	3.93	1.51
7-Propyltridecane	C ₁₆ H ₃₄	226	25.697	1548	Alkane	-	-	-	1.34
Octadecane	C ₁₈ H ₃₈	254	25.788	1800	Alkane	-	-	1.36	2.99
3,5-bis(1,1-Dimethylethyl) phenol	C ₁₄ H ₂₂ O	206	26.113	1555	Phenol	0.35	0.37	-	1.51
2-Hexyl-1-octanol	C ₁₄ H ₃₀ O	214	26.745	1591	Alcohol	-	-	1.81	-
2-Hexyl-1-decanol	C ₁₆ H ₃₄ O	242	27.645	1790	Alcohol	0.75	-	0.49	1.01
Hexadecane	C ₁₆ H ₃₄	226	28.902	1612	Alkane	2.83	3.14	3.05	4.16
2,6-Bis(1,1-dimethylethyl)-4-(methoxymethyl)phenol	C ₁₆ H ₂₆ O ₂	250	31.963	1803	Phenol	-	0.81	1.09	2.08
Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	C ₁₇ H ₃₄ O ₂	270	32.749	1686	Fatty acid ester	-	-	0.77	-
Tridecanoic acid, 12-methyl-, methyl ester	C ₁₅ H ₃₀ O ₂	242	32.76	1615	Fatty acid ester	-	0.47	-	-
Octadecane	C ₁₈ H ₃₈	254	34.802	1810	Alkane	5.26	3.01	3.16	4.99
9-Hexadecenoic acid, methyl ester	C ₁₇ H ₃₂ O ₂	268	37.429	1886	Fatty acid ester	-	0.57	-	-
Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O	270	37.916	1878	Fatty acid ester	11.55	26.58	14.98	12.72
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	C ₁₈ H ₂₈ O ₃	292	38.403	2134	Ester	0.78	0.6	1.39	1.45
Nonadecane	C ₁₉ H ₄₀	268	39.574	1900	Alkane	1.49	0.92	1.81	1.44
1-Octadecanol	C ₁₈ H ₃₈ O	270	41.36	2053	Alcohol	-	-	0.51	0.88
9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	294	41.622	2093	Fatty acid ester	19.9	17.07	18.08	12.1
9-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296	41.737	2085	Fatty acid ester	12.69	11.54	1.34	1.11
Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	298	42.246	2077	Fatty acid ester	17.48	17.52	22.78	26.18
Pentadecanal	C ₁₅ H ₃₀ O	226	42.407	1701	Aldehyde	0.64	-	-	-
Docosane	C ₂₂ H ₄₆	310	43.299	2200	Alkane	2.36	1.23	1.7	2.69
2-Propenoic acid, pentadecyl ester	C ₁₈ H ₃₄ O ₂	282	45.468	1968	Ester	1.62	1.3	1.44	1.22
Cyclopentanetridecanoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296	46.034	2120	Fatty acid ester	0.33	-	-	-
Eicosanoic acid, methyl ester	C ₂₁ H ₄₂ O ₂	326	46.045	2276	Fatty acid ester	-	0.94	1.01	-
Hexadeca-2,4-dienoic acid, methyl ester	C ₁₇ H ₃₀ O ₂	266	46.322	1875	Fatty acid ester	-	0.47	-	-
6,9,12,15-Docosatetraenoic acid, methyl ester	C ₂₃ H ₃₈ O ₂	346	47.088	2507	Fatty acid ester	-	0.63	-	-
2,6,11,15-Tetramethylhexadecane	C ₂₀ H ₄₂	282	47.357	1753	Alkane	-	-	0.69	-
Tetracosane	C ₂₄ H ₅₀	338	47.363	2400	Alkane	1.27	0.77	-	2.52
9,12-Octadecadienoic acid, ethyl ester	C ₂₀ H ₃₆ O ₂	308	48.998	2193	Fatty acid ester	-	0.47	-	-
Docosanoic acid, methyl ester	C ₂₃ H ₄₆ O ₂	354	50.305	2475	Fatty acid ester	-	0.88	-	-

RT: retention time (min); RI: Kovats' retention index; CF: chemical formula; MW: molecular weight; CT: compound type.

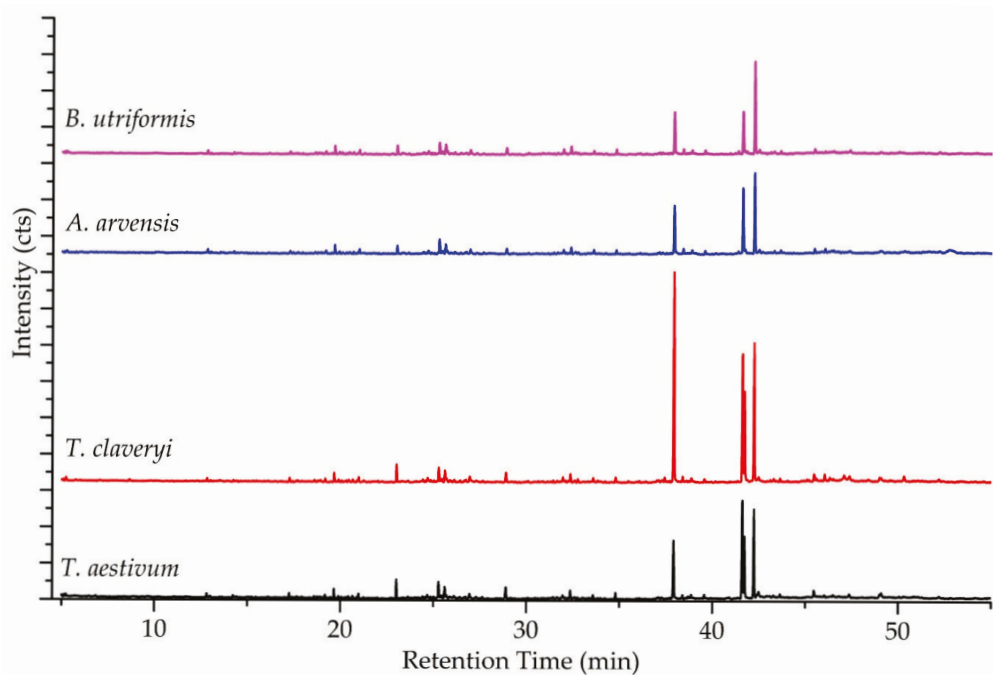


Figure 4. The GC-MS chromatograms of hexane extracts obtained from the studied mushroom species.

In agreement with these reports, the present study revealed that all four investigated mushroom species (*T. aestivum*, *T. claveryi*, *A. arvensis*, and *B. utriformis*) contained substantial amounts of linoleic and oleic acid methyl esters, confirming the predominance of unsaturated fatty acids. Comparable fatty acid patterns have also been described for truffle species by Tejedor-Calvo et al. [51], who reported that *Tuber* spp. exhibit lipid profiles rich in unsaturated fatty acids, contributing to both their nutritional quality and aroma characteristics. The fatty acid profiles reported in this study are in agreement with previously published data for truffle species. In a chemical characterization of multiple *Tuber* and *Terfezia* truffles, oleic and linoleic acids were identified as the predominant fatty acids, with unsaturated fatty acids constituting a major fraction of the total lipid content (38.2–79.3%) in most species studied.

The fatty acid profile obtained for *B. utriformis* in the present study shows a strong similarity with previously reported data for *Lycoperdon utriformis* (syn. *Bovistella utriformis*). In both species, long-chain C16–C18 fatty acids constitute the dominant lipid fraction, with palmitic, stearic, oleic and linoleic acids being the major components. The predominance of unsaturated fatty acids over saturated ones reported for *L. utriformis* is consistent with the high relative abundance of oleic and linoleic acid methyl esters detected in *B. utriformis* by GC–MS analysis. Although differences in relative percentages were observed, these variations can be attributed to differences in geographical origin, extraction solvent and analytical methodology. Overall, the comparable lipid patterns support the notion that members of the Lycoperdaceae family share conserved fatty acid biosynthetic characteristics. Similarly, in the present GC–MS analysis, linoleic acid, oleic acid, stearic acid and palmitic acid methyl esters were among the most abundant lipid-derived constituents across the four mushroom species. This concordance with the literature not only reinforces the reproducibility of fatty acid distribution patterns in truffles and related fungi but also provides a solid chemical basis for interpreting biological activities linked to lipid composition in these taxa.

2.4. Antimicrobial Activity

The antimicrobial activities of methanol and hexane extracts obtained from *T. aestivum*, *T. claveryi*, *A. arvensis*, and *B. utrifomis* were evaluated against a panel of Gram-positive (+), Gram-negative (−), and multidrug-resistant (MDR) bacterial strains and *Candida albicans*. The minimum inhibitory concentration (MIC) values are presented in Tables 5 and 6. Overall, methanol extracts exhibited substantially stronger antimicrobial activity compared to hexane extracts. Among the tested fungi, *T. claveryi* and *B. utrifomis* methanol extracts demonstrated the broadest and most potent inhibitory effects. The lowest MIC values were observed against *E. faecalis* ATCC 29212, with MICs of 0.8 mg/mL for *T. aestivum*, *T. claveryi*, and *B. utrifomis*, and 1.6 mg/mL for *A. arvensis*. Similarly, *B. cereus* RSKK 863 and *B. subtilis* DSMZ 1971 showed high susceptibility to methanol extracts, particularly those of *T. aestivum* and *T. claveryi* (MICs ranging from 1.6 to 3.125 mg/mL). Notably, the methanol extracts exhibited variable inhibitory activity against multidrug-resistant (MDR) strains. The methanol extract of *A. arvensis* showed the lowest MIC value against *S. pneumoniae* MDR (12.5 mg/mL) and moderate activity against *E. coli* MDR and *K. pneumoniae* MDR (25 mg/mL). In contrast, the methanol extract of *T. claveryi* exhibited inhibitory activity against *K. pneumoniae* MDR and methicillin-resistant *S. aureus* (MRSA), with MIC values of 25 mg/mL. The methanol extract of *B. utrifomis* showed activity against *S. pneumoniae* MDR and MRSA with MIC values of 25 and 50 mg/mL, respectively, whereas the methanol extract of *T. aestivum* did not exhibit inhibitory activity against MRSA and showed higher MIC values against other MDR strains.

Table 5. Minimum inhibitory concentrations (MICs) of methanol extracts against bacterial and yeast strains (mg/mL).

Strains	MIC (mg/mL)			
	<i>T. aestivum</i>	<i>T. claveryi</i>	<i>A. arvensis</i>	<i>B. utrifomis</i>
<i>Enterococcus faecalis</i> ATCC 29212	0.8	0.8	1.6	0.8
<i>Bacillus subtilis</i> DSMZ 1971	1.6	3.125	25	50
<i>Staphylococcus aureus</i> ATCC 25923	200	100	100	50
<i>Pseudomonas aeruginosa</i> DSMZ 50071	200	100	100	100
<i>Escherichia coli</i> ATCC 25922	200	100	50	100
<i>Staphylococcus epidermidis</i> DSMZ 20044	200	200	50	25
<i>Enterobacter aerogenes</i> ATCC 13048	100	50	100	50
<i>Micrococcus luteus</i> M41	25	25	100	12.5
<i>Escherichia coli</i> MDR	200	50	25	100
<i>Klebsiella pneumoniae</i> MDR	200	25	25	100
<i>Salmonella typhimurium</i> SL1344	200	50	100	100
<i>Streptococcus pneumoniae</i> MDR	25	50	12.5	25
<i>Staphylococcus aureus</i> MRSA	-	25	-	50
<i>Candida albicans</i> DSMZ 1386	6.25	3.125	200	100
<i>Bacillus cereus</i> RSKK 863	3.125	1.6	25	50

All MIC values are expressed in mg/mL. “-” indicates no inhibition was observed at the tested concentration range.

Table 6. Minimum inhibitory concentrations (MICs) of hexane extracts against bacterial and yeast strains (mg/mL).

Strains	MIC (mg/mL)			
	<i>T. aestivum</i>	<i>T. claveryi</i>	<i>A. arvensis</i>	<i>B. utrifomis</i>
<i>Enterococcus faecalis</i> ATCC 29212	50	25	50	50
<i>Bacillus subtilis</i> DSMZ 1971	100	50	25	50
<i>Staphylococcus aureus</i> ATCC 25923	200	100	-	100
<i>Pseudomonas aeruginosa</i> DSMZ 50071	-	-	-	-
<i>Escherichia coli</i> ATCC 25922	-	-	-	-
<i>Staphylococcus epidermidis</i> DSMZ 20044	-	-	100	200
<i>Enterobacter aerogenes</i> ATCC 13048	-	-	-	-
<i>Micrococcus luteus</i> M41	50	50	50	100
<i>Escherichia coli</i> MDR	-	-	-	-
<i>Klebsiella pneumoniae</i> MDR	-	-	100	200
<i>Salmonella typhimurium</i> SL1344	-	-	-	-
<i>Streptococcus pneumoniae</i> MDR	-	-	-	-
<i>Staphylococcus aureus</i> MRSA	-	-	-	-
<i>Candida albicans</i> DSMZ 1386	-	-	-	-
<i>Bacillus cereus</i> RSKK 863	50	25	25	50

All MIC values are expressed in mg/mL. “-” indicates no inhibition was observed at the tested concentration range.

Hexane extracts exhibited limited antimicrobial activity overall. Inhibitory effects were mainly restricted to certain Gram-positive bacteria, particularly *Bacillus* species and *M. luteus* M41, with MIC values generally ranging from 25 to 100 mg/mL. Most Gram-negative bacteria, MDR strains, and *C. albicans* showed no detectable inhibition in response to hexane extracts. In the hexane extracts, inhibitory activity was detected only against a limited number of bacterial strains, including *E. faecalis*, *B. subtilis*, *M. luteus*, *S. epidermidis*, *K. pneumoniae* MDR, and *B. cereus*, with MIC values ranging from 25 to 200 mg/mL, whereas no inhibition was observed for several other tested strains, including *E. coli* ATCC 25922 and *E. aerogenes* ATCC 13048.

In the present study, the antimicrobial potential of hexane and methanol extracts obtained from four wild edible fungal species was systematically evaluated against a broad spectrum of microorganisms, including Gram-positive, Gram-negative, multidrug-resistant (MDR) bacteria, and *C. albicans*. The results clearly demonstrated that methanol extracts exhibited significantly higher antimicrobial activity than hexane extracts, suggesting that bioactive compounds extracted by polar solvents play a major role in the observed inhibitory effects. The superior activity of methanol extracts is consistent with previous studies reporting that phenolic compounds, flavonoids, organic acids, and other secondary metabolites extracted using polar solvents are primarily responsible for antimicrobial effects in fungi [52,53]. In particular, truffles and other macrofungi are known to be rich in phenolic acids and bioactive peptides, which can disrupt microbial cell membranes and interfere with metabolic pathways [54]. These results are supported by recent studies showing that mushroom extracts often display higher antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria, likely due to structural differences in the bacterial cell envelope that limit antimicrobial penetration in Gram-negative organisms.

Previous studies have demonstrated that *Terfezia* species possess notable antimicrobial potential, although the magnitude of activity varies considerably depending on the extraction solvent and tested microorganisms [42,55–57]. For instance, Doğan and Aydın reported strong antimicrobial activity of *Terfezia boudieri*, particularly with acetone and chloroform extracts, showing low MIC values ($<100\text{ }\mu\text{g/mL}$) against both Gram-positive and Gram-negative bacteria as well as *Candida albicans*. In contrast, methanol extracts in that study generally exhibited moderate activity, with MIC values ranging between 156 and 625 $\mu\text{g/mL}$. In the present study, methanol extracts of *T. claveryi* and *T. aestivum* exhibited measurable antimicrobial activity against a broad spectrum of bacteria, including MDR strains, although at higher MIC values (mg/mL range) compared to those reported by Doğan and Aydın [58]. This difference is likely attributable to variations in extraction protocols, solvent polarity, fungal species, and test strains. Notably, the antifungal activity observed against *Candida albicans* in the present study, particularly for *T. claveryi* (MIC: 3.125 mg/mL), aligns with earlier findings demonstrating the susceptibility of *Candida* species to *Terfezia* extracts.

In line with the findings of the present study, previous research has also demonstrated the antimicrobial potential of *Handkea utrififormis* (*syn. Bovistella utrififormis*). Petrović et al. [59] reported that the methanol extract of *B. utrififormis* exhibited inhibitory activity at varying concentrations against a range of clinically relevant microorganisms, including *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*, and *C. albicans*. The consistency between these findings and our results further supports the notion that methanol is an effective solvent for extracting bioactive compounds from *B. utrififormis* and highlights this species as a promising source of antimicrobial agents with broad-spectrum activity. Sevindik et al. [60] reported that the methanol extract of *B. utrififormis* exhibited notable antimicrobial activity against *E. coli* at a concentration of 200 $\mu\text{g/mL}$. These findings are in agreement with the results of the present study, in which the methanol extract of *B. utrififormis* demonstrated inhibitory activity against *E. coli*, although higher MIC values were observed. The differences in effective concentrations may be attributed to variations in experimental design, microbial strains, and antimicrobial testing conditions. Nevertheless, both studies consistently support the antimicrobial potential of *B. utrififormis*, highlighting this species as a promising source of bioactive compounds with antibacterial activity.

In the study conducted by Asgharpour et al. [61], the hexane extract of *L. pyriforme* exhibited a highly selective antibacterial profile, showing inhibitory activity only against *S. aureus* ATCC 25923 with a MIC value of 125 $\mu\text{g/mL}$, while no detectable activity was observed against *E. coli*, *P. aeruginosa*, or *B. subtilis*. This narrow spectrum of activity was attributed to the apolar nature of the hexane solvent, which predominantly extracts lipophilic compounds such as sterols and fatty acid derivatives. In comparison, the hexane extracts evaluated in the present study also demonstrated limited and strain-dependent antimicrobial activity, supporting the notion that hexane is generally less effective than polar solvents for extracting broad-spectrum antimicrobial compounds from mushrooms. While selective inhibition was observed against certain bacterial strains in our study, the overall antimicrobial potency of hexane extracts was lower than that of methanol extracts and comparable to the restricted activity reported for *L. pyriforme*.

The antimicrobial activity observed in the present study can be partially attributed to the presence of phenolic compounds identified by GC–MS analysis, including substituted phenols such as 3,5-bis(1,1-dimethylethyl)phenol, 2,6-Bis(1,1-dimethylethyl)-4-(methoxymethyl)phenol and related derivatives. Phenolic compounds are well known for their ability to disrupt microbial cell membranes and inhibit essential enzymatic processes. In addition, the detection of fatty acid methyl esters, such as hexadecanoic, octadecanoic, and octadecadienoic acid methyl esters, may further contribute to the antimicrobial effect

through membrane-targeting mechanisms, suggesting a synergistic role of phenolic and lipophilic constituents.

3. Materials and Methods

3.1. Chemicals and Reagents

Methanol, potassium persulfate, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Cu(II) chloride, DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium ferri-cyanide, trichloroacetic acid, ABTS (2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), disodium hydrogen phosphate, and sodium sulfate anhydrous were purchased from Sigma Aldrich (St. Louis, MO, USA); Folin–Ciocalteu’s phenol reagent, sodium dihydrogen phosphate, ammonium acetate, ascorbic acid, ferric chloride, neocuproine and HCl were purchased from Merck (Damstadt, Germany); sodium carbonate was purchased from Tekkim Lab (Bursa, Türkiye); and hexane was purchased from Supelco (Sigma-Aldrich, St. Louis, MO, USA). All the chemicals and reagents used in the present study were of analytical grade.

3.2. Fungal Material

The macrofungi samples comprising the material for this study were collected at appropriate intervals from various locations where ecological conditions were suitable for fungal growth. Information about the locations (region, coordinates, and altitude) where the samples were collected and the photographs of the samples are provided in Table 1 and Figure 1.

The samples were identified based on the morphological characteristics such as color, size, number and shape of the cap, stipe, gill, basidiocarp, gleba, ascocarp and spores according to the current literature [32–34]. The samples were then air dried below 40 °C using a commercial vegetable drier, until the samples reached a constant weight, and kept in zipper bags away from direct sunlight in the laboratory. A further identification based on the isolation, amplification and sequencing of the internal transcribed spacer (ITS) region of the rDNA was also performed.

In this context, the DNA extraction of the samples was performed using a Macharey Nagel Plant II DNA extraction kit (Düren, Germany) according to the manufacturer’s instructions. The ITS region of the DNA was amplified by PCR using universal ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [62]. The obtained PCR products were sent to BM Labosis (Ankara, Türkiye) for sequence analysis. The data was compared with the NCBI GenBank database via BLAST for identification of the samples.

3.3. Extract Preparation

For preparing the extracts, 2 g of dried mushroom material, previously ground into a fine powder using a porcelain mortar and pestle, was mixed with 30 mL of methanol and homogenized at 9000 rpm for 5 min using a homogenizer (HG-15D, Daihan Scientific, Gangwon-do, Korea). The homogenate was incubated overnight at room temperature in the dark, followed by sonication for 40 min at 35 °C in an ultrasonic bath (Sonorex, Bandelin, Berlin, Germany). The resulting extract was filtered through Whatman No. 1 filter paper and subsequently concentrated to dryness using a rotary evaporator (HeiVap Value, Heidolph, Berlin, Germany). The obtained residue was re-dissolved in methanol to prepare a stock extract at a concentration of 200 mg/mL. These extracts were used to determine the total phenolic content and antioxidant properties of the fungal samples. The same extraction procedure was also applied using hexane as the solvent. Both methanol

and hexane extracts were reconstituted in 5% dimethyl sulfoxide (DMSO) and used for antimicrobial activity assays.

3.4. Total Phenolic Compounds

The total phenolic compound contents of the methanol extracts of the fungal samples were determined using the Folin–Ciocalteu reagent method [63]. A total of 100 µL was taken from the extracts with a concentration of 100 mg/mL, and 1 mL of Folin–Ciocalteu's phenol reagent (10-fold diluted) was added to it. After incubation at room temperature for 5 min, 1 mL of 7.5% Na₂CO₃ solution was added to the mixture and mixed with a vortex. The mixture was incubated again at room temperature in the dark for 90 min, and the absorbance of the resulting blue color was measured at 760 nm using a spectrophotometer (MultiSkan GO, Thermo Fisher Scientific, Waltham, MA, USA). The results obtained were calculated according to the gallic acid standard and presented as mg GAE/g dry weight.

3.5. Antioxidant Assays

3.5.1. DPPH Radical Scavenging Activity

One of the commonly used methods for determining the free radical scavenging activity of mushroom extracts is the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity method [64]. To generate a DPPH scavenging pattern, a five-step dilution series was prepared from 100 mg/mL methanol extracts of fungal samples. Briefly, 100 µL of each dilution was added to 2.9 mL of a 0.1 mM DPPH solution prepared with methanol. The mixture was vortexed and incubated at room temperature for 30 min. The absorbance of the resulting color was measured at 517 nm using a spectrophotometer. The DPPH scavenging activities of the fungal extracts were calculated according to the following formula:

$$\%DPPH = [(A_0 - A_{\text{sample}})/A_0] \times 100 \quad (1)$$

where %DPPH is DPPH scavenging activity, A₀ is the absorbance value of the blank tube at 517 nm wavelength, and A_{sample} is the absorbance value of the tube containing the fungal extract at 517 nm wavelength. The DPPH radical scavenging activities of the mushroom extracts were calculated using the Trolox standard, and the results are presented as mmol TE/g dry weight.

3.5.2. ABTS⁺ Cation Removal Activity

2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) is another stable radical commonly used to measure the radical inhibition capacity of samples with antioxidant properties. For the ABTS assay, a mixture of 7.5 mM ABTS and 2.45 mM potassium persulfate (K₂S₂O₈) solutions was prepared and incubated in the dark for 12–16 h to form the ABTS⁺ reaction solution. This solution was diluted to reach an absorbance of 0.700 ± 0.02 at 734 nm to obtain the ABTS⁺ working solution. In a 96-well plate, 10 µL of extracts or Trolox solutions at various concentrations was added to 200 µL of ABTS⁺ working solution, thoroughly mixed, and incubated in the dark for 6 min. The absorbance of the plate was measured at 734 nm, and the ABTS inhibition percentages of the samples were calculated using the following formula:

$$\%ABTS = [(A_0 - A_{\text{sample}})/A_0] \times 100 \quad (2)$$

where %ABTS is ABTS scavenging activity, A₀ is the absorbance of the ABTS⁺ working solution at 734 nm, and A_{sample} is the absorbance of the sample at 734 nm [65,66]. A standard curve was established using the inhibition values of the Trolox solutions, and the results were expressed as mmol TE/g dry weight.

3.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The increase in absorbance of ferric ferrocyanide, a blue-colored complex exhibiting maximum absorbance at 700 nm, can be associated with the antioxidant activity of the sample, which reacts with potassium ferricyanide ($K_3[Fe(CN)_6]$) to form potassium ferrocyanide ($K_4[Fe(CN)_6]$). For the ferric reducing antioxidant power (FRAP) test, 30 μ L of 0.2 M PBS at pH 6.6, 10 μ L of extract or standard ascorbic acid solution at various concentrations, and 30 μ L of 1% potassium ferricyanide ($K_3[Fe(CN)_6]$) solution were mixed and incubated at 50 °C for 20 min. After incubation, 30 μ L of 10% trichloroacetic acid solution was added to the wells and mixed. The absorbance measurement of the plate was performed at 700 nm after adding 100 μ L of distilled water and 20 μ L of iron (III) chloride ($FeCl_3$) [67]. A standard curve was created using ascorbic acid solution values, and the results were expressed as mmol AAE/g dry weight.

3.5.4. Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

The color change from bright yellow to orange in the presence of compounds acting as electron donors in the neocuproine and $Cu(II)Cl_2$ mixture indicates the antioxidant capacity of a sample by reducing cupric (Cu^{2+}) ions to cuprous (Cu^+) ions. The CUPRAC assay was performed according to the method originally reported by Apak et al. [68]. A total of 50 μ L of 0.1 M ammonium acetate buffer (pH 7.0), 50 μ L of 10 mM $Cu(II)Cl_2$, 50 μ L of 7.5 mM neocuproine solution, 50 μ L of distilled water, and 5 μ L of extract or Trolox solution at different concentrations were added to a 96-well plate and incubated in the dark for 30 min. The absorbance of the samples was then measured at 450 nm using a spectrophotometer. A standard curve was created using the absorbance values of the Trolox solutions, and the results were expressed as mmol TE/g dry weight.

3.6. Chromatographic Analyses

3.6.1. GC-MS

The chemical composition of the fungal samples was determined using Gas Chromatography–Mass Spectrometry (GC-MS). Forty milliliters of hexane was added to 1 g of dried and powdered fungal sample, homogenized at 8000 rpm for 5 min, and then tightly sealed and left to incubate for 24 h. The homogenate was then incubated in an ultrasonic bath at 40 °C for 30 min and centrifuged at 4000 rpm for 5 min. The extract was filtered through Whatman No. 1 filter paper, and the solvent was removed using a rotary evaporator. The resulting residue was resuspended in 5 mL of hexane. Then, 2 M KOH in methanol and 1 N HCl solutions were used for saponification of the extracts (IUPAC Method 2.301). After phase separation, the upper layer was dried with anhydrous Na_2SO_4 and passed through a 0.45 μ m nylon filter before injection into the GC-MS.

The composition of the hexane extracts was determined using a Shimadzu QP2010 Ultra GC-MS system equipped with a Restek Rxi 5MS column (30 m $\times$ 0.25 mm ID $\times$ 0.25 μ m df). The injection block temperature was set to 240 °C. A 1 μ L sample was injected into the system in split mode at a 1:40 ratio using an AOC2.0i autosampler. Helium was used as the carrier gas at a constant flow rate of 1.1 mL/min. The oven temperature program was set as follows: initial temperature 40 °C for 1 min, heated to 160 °C at a rate of 5 °C/min and held for 3 min, and finally heated to 250 °C at a rate of 5 °C/min and held for 11 min. The total analysis time was 57 min. The interface and ion source temperatures were 270 and 200 °C, respectively. All spectra were obtained in electron impact (EI) mode at 70 eV, and the mass range was set to 40–650 amu in full scan mode. The Wiley mass spectrum library (W9N11) and the Flavour and Fragrance Natural and Synthetic Compounds library (FFNSC 1.2) were used to identify the components detected by MS [69].

3.6.2. LC–MS/MS

The phenolic profiles of the methanolic extracts obtained from the fungal samples were analyzed using a LC–MS/MS (Shimadzu 8040 system, Kyoto, Japan) equipped with an Inertsil ODS4 column (2.1 mm × 50 mm, 3 µm particle size; GL Science, Tokyo, Japan), maintained at 40 °C. Chromatographic separation of phenolic constituents was performed by injecting 10 µL of the sample and applying a gradient elution program with solvent A (1% formic acid in distilled water) and solvent B (1% formic acid in methanol) at a constant flow rate of 0.4 mL/min. The gradient conditions were set as follows: 95% A and 5% B at 0 min, gradually reaching 5% A and 95% B at 7 min. The total run time for each analysis was 12 min. Identification of individual phenolic compounds was achieved by comparing their retention times with those of authentic standards and by monitoring precursor and product ions in both positive and negative electrospray ionization modes. The operating parameters of the mass spectrometer were set as follows: an interface temperature of 350 °C, a desolvation line (DL) temperature of 250 °C, and a heat block temperature of 400 °C. Nitrogen was used as both the nebulizing and drying gas, with flow rates of 3 L/min and 15 L/min, respectively [70].

3.7. Microbiological Analyses

3.7.1. Microorganisms

In this study, a total of 14 bacterial strains were utilized, including 4 multidrug-resistant strains. Additionally, the antifungal activity of the plant sample was tested against the standard yeast *Candida albicans* DSMZ 1386. The bacteria and yeast strains were obtained from the Department of Biotechnology at Niğde Ömer Halisdemir University's Faculty of Science (Niğde, Türkiye).

3.7.2. Microdilution Method (MIC)

Microdilution is a widely used and reliable method for evaluating antimicrobial susceptibility. This technique is based on the preparation of serial two-fold dilutions of the tested antimicrobial agent, typically ranging from 1 to 200 mg/mL, in an appropriate liquid growth medium. The assay can be performed either in 96-well microtiter plates (microdilution), which allows the use of smaller volumes while enabling high-throughput analysis. The antimicrobial activities of methanol and hexane extracts obtained from *B. utrifomis*, *A. arvensis*, *T. claveryi*, and *T. aestivum*—which were recovered with 5% dimethylsulfoxide—with a final concentration of 200 mg/mL were evaluated by determining the minimum inhibitory concentration (MIC) using the microdilution method, in accordance with minor modifications. Pathogen microorganisms were adjusted to a turbidity equivalent to the 0.5 McFarland standard (approximately 1×10^8 CFU/mL) and subsequently diluted to obtain the working inoculum. Serial two-fold dilutions of the mushroom extracts were prepared in LB broth, and 100 µL of each extract dilution was dispensed into sterile 96-well microtiter plates. Subsequently, 50 µL of fresh LB broth and 50 µL of the standardized microbial inoculum were added to each well, resulting in a final volume of 200 µL per well. Wells containing LB broth supplemented with microbial inoculum but without mushroom extract served as the positive growth control, whereas wells containing LB broth alone were used as the negative control. The MIC was defined as the minimum concentration of extracts (methanol and hexane) obtained from the mushrooms required to inhibit visible bacterial growth after 24 h of incubation. The results were expressed as mg/mL based on the mean values of three independent experiments [71,72].

3.8. Statistical Analysis

Group mean comparisons were performed using one-way ANOVA followed by Duncan's multiple range test, considering $p < 0.05$ as statistically significant. Statistical analyses were carried out using IBM SPSS Statistics version 24.0 (SPSS Inc., Chicago, IL, USA).

4. Conclusions

The present study demonstrates that wild edible macrofungi represent promising natural sources of bioactive compounds with significant antimicrobial and antioxidant potential. Among the investigated species, methanol extracts consistently showed superior antimicrobial activity compared to hexane extracts, emphasizing the key role of polar bioactive constituents, particularly phenolic compounds. *B. utriformis* and *A. arvensis* exhibited the highest total phenolic contents and antioxidant capacities, while *T. claveryi* showed notable antimicrobial efficacy, including activity against selected multidrug-resistant bacterial strains. Chemical profiling of the investigated fungal samples using LC–MS/MS and GC–MS revealed that all four species were characterized by phenolic compounds including catechin, cinnamic acid and caffeic acid, and lipid profiles rich in unsaturated fatty acid methyl esters, especially linoleic and oleic acids, which may contribute synergistically to their biological activities. The strong correlation observed between phenolic content and antioxidant assays further supports the contribution of phenolic compounds to the overall bioactive potential of these fungi. Collectively, these findings underline the nutritional and pharmacological relevance of wild edible macrofungi and suggest their potential use as natural antioxidant and antimicrobial agents. Future studies focusing on compound isolation, mechanism of action, and in vivo evaluations are warranted to further explore their applicability in the food and pharmaceutical industries.

Author Contributions: Conceptualization, E.Y.C. and E.I.; methodology, E.Y.C. and E.I.; software, E.Y.C.; validation, E.Y.C. and E.I.; formal analysis, E.Y.C. and E.I.; investigation, E.Y.C. and E.I.; resources, E.Y.C. and E.I.; data curation, E.Y.C. and E.I.; writing—original draft preparation, E.Y.C. and E.I.; writing—review and editing, E.Y.C.; visualization, E.Y.C.; supervision, E.Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article material; further inquiries can be directed to the corresponding author.

Acknowledgments: The authors would like to thank Cemil İşlek for scientific support and criticism during the antioxidant assays. This study is partially based on a Master's thesis. The authors have reviewed and edited the final manuscript and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

The following abbreviations are used in this manuscript:

ITS	Internal Transcribed Spacer
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
FRAP	Ferric Reducing Antioxidant Power
CUPRAC	Cupric Reducing Antioxidant Capacity

TPCs	Total Phenolic Compounds
LC-MS	Liquid Chromatography–Mass spectrometry
GC-MS	Gas Chromatography–Mass Spectrometry
MIC	Minimum Inhibitory Concentration

References

- Ren, L.; Hemar, Y.; Perera, C.O.; Lewis, G.; Krissansen, G.W.; Buchanan, P.K. Antibacterial and antioxidant activities of aqueous extracts of eight edible mushrooms. *Bioact. Carbohydr. Diet. Fibre* **2014**, *3*, 41–51. [CrossRef]
- Munita, J.M.; Arias, C.A. Mechanisms of Antibiotic Resistance. In *Virulence Mechanisms of Bacterial Pathogens*, 5th ed.; Kudva, I.T., Cornick, N.A., Plummer, P.J., Zhang, Q., Nicholson, T.L., Bannantine, J.P., Bellaire, B.H., Eds.; ASM Press: Washington, DC, USA, 2016; pp. 481–511. [CrossRef]
- Frieri, M.; Kumar, K.; Boutin, A. Antibiotic resistance. *J. Infect. Public Health* **2017**, *10*, 369–378. [CrossRef]
- MacGowan, A.; Macnaughton, E. Antibiotic resistance. *Medicine* **2017**, *45*, 622–628. [CrossRef]
- Pandey, A.T.; Pandey, I.; Hachenberger, Y.; Krause, B.-C.; Haidar, R.; Laux, P.; Luch, A.; Singh, M.P.; Singh, A.V. Emerging paradigm against global antimicrobial resistance via bioprospecting of mushroom into novel nanotherapeutics development. *Trends Food Sci. Technol.* **2020**, *106*, 333–344. [CrossRef]
- Aprotosoiaie, A.C.; Zavastin, D.E.; Mihai, C.T.; Voichita, G.; Gherghel, D.; Sillion, M.; Trifan, A.; Miron, A. Antioxidant and antigenotoxic potential of *Ramaria larentii* Marr & DE Stuntz, a wild edible mushroom collected from Northeast Romania. *Food Chem. Toxicol.* **2017**, *108*, 429–437. [CrossRef]
- Rathore, H.; Prasad, S.; Sharma, S. Mushroom nutraceuticals for improved nutrition and better human health: A review. *PharmaNutrition* **2017**, *5*, 35–46. [CrossRef]
- Altaf, U.; Lalotra, P.; Sharma, Y.P. Nutritional and mineral composition of four wild edible mushrooms from Jammu and Kashmir, India. *Indian Phytopathol.* **2020**, *73*, 313–320. [CrossRef]
- Kalač, P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. *J. Sci. Food Agric.* **2013**, *93*, 209–218. [CrossRef] [PubMed]
- Gąsecka, M.; Magdziak, Z.; Siwulski, M.; Mleczek, M. Profile of phenolic and organic acids, antioxidant properties and ergosterol content in cultivated and wild growing species of *Agaricus*. *Eur. Food Res. Technol.* **2018**, *244*, 259–268. [CrossRef]
- Abdelshafy, A.M.; Belwal, T.; Liang, Z.; Wang, L.; Li, D.; Luo, Z.; Li, L. A comprehensive review on phenolic compounds from edible mushrooms: Occurrence, biological activity, application and future prospective. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 6204–6224. [CrossRef]
- Tellez-Tellez, M. Wild edible mushrooms as an alternative for the consumption of antioxidants and phenolic compounds: An overview. *BioResources* **2024**, *19*, 3945–3978. [CrossRef]
- Ahmad, N.; Mahmood, F.; Khalil, S.A.; Zamir, R.; Fazal, H.; Abbasi, B.H. Antioxidant activity via DPPH, gram-positive and gram-negative antimicrobial potential in edible mushrooms. *Toxicol. Ind. Health* **2014**, *30*, 826–834. [CrossRef] [PubMed]
- Ferreira, I.C.; Barros, L.; Abreu, R. Antioxidants in wild mushrooms. *Curr. Med. Chem.* **2009**, *16*, 1543–1560. [CrossRef]
- Jeong, E.M.; Liu, M.; Sturdy, M.; Gao, G.; Varghese, S.T.; Sovari, A.A.; Dudley, S.C., Jr. Metabolic stress, reactive oxygen species, and arrhythmia. *J. Mol. Cell. Cardiol.* **2012**, *52*, 454–463. [CrossRef] [PubMed]
- Bach, F.; Zielinski, A.A.F.; Helm, C.V.; Maciel, G.M.; Pedro, A.C.; Stafussa, A.P.; Avila, S.; Haminiuk, C.W.I. Bio compounds of edible mushrooms: In vitro antioxidant and antimicrobial activities. *LWT* **2019**, *107*, 214–220. [CrossRef]
- Contato, A.G.; Inácio, F.D.; de Araújo, C.A.V.; Brugnari, T.; Maciel, G.M.; Haminiuk, C.W.I.; Bracht, A.; Peralta, R.M.; de Souza, C.G.M. Comparison between the aqueous extracts of mycelium and basidioma of the edible mushroom *Pleurotus pulmonarius*: Chemical composition and antioxidant analysis. *J. Food Meas. Charact.* **2020**, *14*, 830–837. [CrossRef]
- Palacios, I.; Lozano, M.; Moro, C.; D'Arrigo, M.; Rostagno, M.A.; Martínez, J.A.; Garcia-Lafuente, A.; Guillamón, E.; Villares, A. Antioxidant properties of phenolic compounds occurring in edible mushrooms. *Food Chem.* **2011**, *128*, 674–678. [CrossRef]
- Sadi, G.; Kaya, A.; Yalcin, H.A.; Emsen, B.; Kocabas, A.; Kartal, D.I.; Altay, A. Wild edible mushrooms from Turkey as possible anticancer agents on HepG2 cells together with their antioxidant and antimicrobial properties. *Int. J. Med. Mushrooms* **2016**, *18*, 83–95. [CrossRef] [PubMed]
- Lee, S.; Park, J.Y.; Lee, D.; Seok, S.; Kwon, Y.J.; Jang, T.S.; Kang, K.S.; Kim, K.H. Chemical constituents from the rare mushroom *Calvatia nipponica* inhibit the promotion of angiogenesis in HUVECs. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4122–4127. [CrossRef]
- Orhan, I.; Üstün, O. Determination of total phenol content, antioxidant activity and acetylcholinesterase inhibition in selected mushrooms from Turkey. *J. Food Compos. Anal.* **2011**, *24*, 386–390. [CrossRef]
- Liu, K.; Xiao, X.; Wang, J.; Chen, C.Y.O.; Hu, H. Polyphenolic composition and antioxidant, antiproliferative, and antimicrobial activities of mushroom *Inonotus sanghuang*. *LWT-Food Sci. Technol.* **2017**, *82*, 154–161. [CrossRef]

23. Yan, H.; Che, X.; Lv, Q.; Zhang, L.; Dongol, S.; Wang, Y.; Sun, H.; Jiang, J. Grifolin induces apoptosis and promotes cell cycle arrest in the A2780 human ovarian cancer cell line via inactivation of the ERK1/2 and Akt pathways. *Oncol. Lett.* **2017**, *13*, 4806–4812. [CrossRef] [PubMed]
24. Saltarelli, R.; Palma, F.; Gioacchini, A.M.; Calcabrini, C.; Mancini, U.; De Bellis, R.; Stocchi, V.; Potenza, L. Phytochemical composition, antioxidant and antiproliferative activities and effects on nuclear DNA of ethanolic extract from an Italian mycelial isolate of *Ganoderma lucidum*. *J. Ethnopharmacol.* **2019**, *231*, 464–473. [CrossRef]
25. Stojkovic, D.; Smiljkovic, M.; Ciric, A.; Glamoclija, J.; Van Griensven, L.; Ferreira, I.C.; Sokovic, M. An insight into antidiabetic properties of six medicinal and edible mushrooms: Inhibition of α -amylase and α -glucosidase linked to type-2 diabetes. *S. Afr. J. Bot.* **2019**, *120*, 100–103. [CrossRef]
26. Smolskaitė, L.; Venskutonis, P.R.; Talou, T. Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species. *LWT-Food Sci. Technol.* **2015**, *60*, 462–471. [CrossRef]
27. Tanaka, T.; Onuma, H.; Shigihara, T.; Kimura, E.; Fukuta, Y.; Shirasaka, N.; Moriyama, T.; Homma, Y. Anti-osteoporotic effects of syringic acid and vanilic acid in the extracts of waste beds after mushroom cultivation. *J. Biosci. Bioeng.* **2019**, *128*, 622–629. [CrossRef]
28. Moniruzzaman, M.; Tuong, D.T.C.; Chin, S.; Anjana, S.; Karthikeyan, A.; Min, T.; Kim, I.J. A review on pharmacological insights of edible and medicinal mushroom based β -glucans. *Appl. Biol. Chem.* **2025**, *68*, 41. [CrossRef]
29. Nowacka, N.; Nowak, R.; Drozd, M.; Olech, M.; Los, R.; Malm, A. Analysis of phenolic constituents, antiradical and antimicrobial activity of edible mushrooms growing wild in Poland. *LWT-Food Sci. Technol.* **2014**, *59*, 689–694. [CrossRef]
30. Alves, M.J.; Ferreira, I.C.; Martins, A.; Pintado, M. Antimicrobial activity of wild mushroom extracts against clinical isolates resistant to different antibiotics. *J. Appl. Microbiol.* **2012**, *113*, 466–475. [CrossRef]
31. Skariyachan, S.; Prasanna, A.; Manjunath, S.P.; Karanth, S.S.; Nazre, A. Exploring the medicinal potential of the fruit bodies of oyster mushroom, *Pleurotus ostreatus* (Agaricomycetes), against multidrug-resistant bacterial isolates. *Int. J. Med. Mushrooms* **2016**, *18*, 245–252. [CrossRef]
32. Breitenbach, J.; Kränzlin, F. *Fungi of Switzerland 3: Boletales and Agaricales (Bolets and Agarics Pt. 1)*; Verlag Mykologia: Luzern, Switzerland, 1991; 361p.
33. Cannon, P.F.; Kirk, P.M. *Fungal Families of the World*; CAB International: Wallingford, UK, 2007.
34. Hansen, L.; Knudsen, H. *Nordic Macromycetes, Volume 2, Polyporales, Boletales, Agaricales, Russulales*; Nordsvamp: Copenhagen, Denmark, 1992; 474p.
35. Lücking, R.; Aime, M.C.; Robbertse, B.; Miller, A.N.; Ariyawansa, H.A.; Aoki, T.; Cardinali, G.; Crous, P.W.; Druzhinina, I.S.; Geiser, D.M.; et al. Unambiguous identification of fungi: Where do we stand and how accurate and precise is fungal DNA barcoding? *IMA Fungus* **2020**, *11*, 14. [CrossRef] [PubMed]
36. Giusti, A.; Ricci, E.; Gasperetti, L.; Galgani, M.; Polidori, L.; Verdigi, F.; Narducci, R.; Armani, A. Molecular identification of mushroom species in Italy: An ongoing project aimed at reinforcing the control measures of an increasingly appreciated sustainable food. *Sustainability* **2020**, *13*, 238. [CrossRef]
37. Kozarski, M.; Klaus, A.; Jakovljevic, D.; Todorovic, N.; Vunduk, J.; Petrović, P.; Niksic, M.; Vrvic, M.M.; Van Griensven, L. Antioxidants of edible mushrooms. *Molecules* **2015**, *20*, 19489–19525. [CrossRef]
38. Reis, F.S.; Martins, A.; Vasconcelos, M.H.; Morales, P.; Ferreira, I.C. Functional foods based on extracts or compounds derived from mushrooms. *Trends Food Sci. Technol.* **2017**, *66*, 48–62. [CrossRef]
39. Akata, I.; Zengin, G.; Picot, C.M.N.; Mahomoodally, M.F. Enzyme inhibitory and antioxidant properties of six mushroom species from the Agaricaceae family. *S. Afr. J. Bot.* **2019**, *120*, 95–99. [CrossRef]
40. Dundar, A.; Okumus, V.; Ozdemir, S.; Celik, K.S.; Boğa, M.; Ozcagli, E. Determination of cytotoxic, anticholinesterase, antioxidant and antimicrobial activities of some wild mushroom species. *Cogent Food Agric.* **2016**, *2*, 1178060. [CrossRef]
41. Sharma, S.K.; Gautam, N. Chemical, bioactive, and antioxidant potential of twenty wild culinary mushroom species. *BioMed Res. Int.* **2015**, *2015*, 346508. [CrossRef]
42. Hadjira, G.; Bakchiche, B.; Erenler, R.; Yildiz, İ.; Mohamed, A.S.; El-Shazly, M.A. Mineral contents, antioxidant and antimicrobial activities of algerian terfezia clavaryi extracts. *Fabard J. Pharm. Sci.* **2024**, *49*, 19–36. [CrossRef]
43. Beara, I.N.; Lesjak, M.M.; Četojević-Simin, D.D.; Marjanović, Ž.S.; Ristić, J.D.; Mrkonjić, Z.O.; Mimica-Dukić, N.M. Phenolic profile, antioxidant, anti-inflammatory and cytotoxic activities of black (*Tuber aestivum* Vittad.) and white (*Tuber magnatum* Pico) truffles. *Food Chem.* **2014**, *165*, 460–466. [CrossRef]
44. Chu, M.; Khan, R.D.; Zhou, Y.; Agar, O.T.; Barrow, C.J.; Dunshea, F.R.; Suleria, H.A. LC-ESI-QTOF-MS/MS characterization of phenolic compounds in common commercial mushrooms and their potential antioxidant activities. *Processes* **2023**, *11*, 1711. [CrossRef]
45. Yahia, E.M.; Gutiérrez-Orozco, F.; Moreno-Pérez, M.A. Identification of phenolic compounds by liquid chromatography-mass spectrometry in seventeen species of wild mushrooms in Central Mexico and determination of their antioxidant activity and bioactive compounds. *Food Chem.* **2017**, *226*, 14–22. [CrossRef] [PubMed]

46. Baldelli, S.; Aiello, G.; De Bruno, A.; Castelli, S.; Lombardo, M.; Stocchi, V.; Tripodi, G. Bioactive compounds and antioxidant potential of truffles: A comprehensive review. *Antioxidants* **2025**, *14*, 1341. [CrossRef] [PubMed]
47. Çayan, F.; Deveci, E.; Tel-Çayan, G.; Duru, M.E. Identification and quantification of phenolic acid compounds of twenty-six mushrooms by HPLC–DAD. *J. Food Meas. Charact.* **2020**, *14*, 1690–1698. [CrossRef]
48. Barros, L.; Dueñas, M.; Ferreira, I.C.; Baptista, P.; Santos-Buelga, C. Phenolic acids determination by HPLC–DAD–ESI/MS in sixteen different Portuguese wild mushrooms species. *Food Chem. Toxicol.* **2009**, *47*, 1076–1079. [CrossRef]
49. Kalač, P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chem.* **2009**, *113*, 9–16. [CrossRef]
50. Reis, F.S.; Barros, L.; Martins, A.; Ferreira, I.C. Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study. *Food Chem. Toxicol.* **2012**, *50*, 191–197. [CrossRef]
51. Tejedor-Calvo, E.; Amara, K.; Reis, F.S.; Barros, L.; Martins, A.; Calhella, R.C.; Venturini, M.E.; Blanco, D.; Redondo, D.; Marco, P.; et al. Chemical composition and evaluation of antioxidant, antimicrobial and antiproliferative activities of *Tuber* and *Terfezia* truffles. *Food Res. Int.* **2021**, *140*, 110071. [CrossRef]
52. Heleno, S.A.; Martins, A.; Queiroz, M.J.R.; Ferreira, I.C. Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chem.* **2015**, *173*, 501–513. [CrossRef]
53. Alves, M.J.; Ferreira, I.C.; Froufe, H.J.; Abreu, R.M.; Martins, A.; Pintado, M. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *J. Appl. Microbiol.* **2013**, *115*, 346–357. [CrossRef]
54. Puia, A.; Pandrea, S.L.; Cruceru, J.; Puia, I.C.; Chedea, V.S.; Ciont, C.; Pop, O.L.; Leopold, L.F.; Ranga, F.; Pop, R.M. Phenolic Compounds with Antimicrobial Properties in Mushrooms Frequently Encountered in Temperate Deciduous Forests. *Life* **2025**, *15*, 1653. [CrossRef]
55. Harir, M.; Bendif, H.; Yahiaoui, M.; Bellahcene, M.; Zohra, F.; Rodríguez-Couto, S. Evaluation of antimicrobial activity of *Terfezia arenaria* extracts collected from Saharan desert against bacteria and filamentous fungi. *3 Biotech* **2019**, *9*, 281. [CrossRef]
56. Gouzi, H.; Belyagoubi, L.; Abdelali, K.N.; Khelifi, A. In vitro antibacterial activities of aqueous extracts from Algerian desert truffles (*Terfezia* and *Tirmania*, Ascomycetes) against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Int. J. Med. Mushrooms* **2011**, *13*, 553–558. [CrossRef]
57. Khojah, H.M.; Abdelhalim, O.B.; Mostafa, M.A.; Habib, E.S.E. Antimicrobial efficacy of extracts of Saudi Arabian desert *Terfezia clavervyi* truffles. *Saudi J. Biol. Sci.* **2022**, *29*, 103462. [CrossRef]
58. Doğan, H.H.; Aydin, S. Determination of antimicrobial effect, antioxidant activity and phenolic contents of desert truffle in Turkey. *Afr. J. Tradit. Complement. Altern. Med.* **2013**, *10*, 52–58. [CrossRef]
59. Petrović, P.; Vunduk, J.; Klaus, A.; Carević, M.; Petković, M.; Vuković, N.; Cvetković, A.; Žižak, Ž.; Bugarski, B. From mycelium to spores: A whole circle of biological potency of mosaic puffball. *S. Afr. J. Bot.* **2019**, *123*, 152–160. [CrossRef]
60. Sevindik, M.; Akata, I.; Saridoğan, B.G.O.; Eraslan, E.C.; Bal, C. Antioxidant, Antimicrobial, and Antiproliferative Activities of Some Edible Wild Mushrooms. *Biol. Bull.* **2023**, *50*, S630–S636. [CrossRef]
61. Asgharpour, F.; Moghadamnia, A.A.; Alizadeh, Y.; Kazemi, S. Chemical Composition and antibacterial activity of hexane extract of *Lycoperdon Pyriforme*. *S. Afr. J. Bot.* **2020**, *131*, 195–199. [CrossRef]
62. White, T.J.; Bruns, T.; Lee, S.J.W.T.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: New York, NY, USA, 1990; pp. 315–322. [CrossRef]
63. Georgé, S.; Brat, P.; Alter, P.; Amiot, M.J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem.* **2005**, *53*, 1370–1373. [CrossRef] [PubMed]
64. Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* **1992**, *40*, 945–948. [CrossRef]
65. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef] [PubMed]
66. Tel, G.; Apaydın, M.; Duru, M.E.; Öztürk, M. Antioxidant and cholinesterase inhibition activities of three *Tricholoma* species with total phenolic and flavonoid contents: The edible mushrooms from Anatolia. *Food Anal. Methods* **2012**, *5*, 495–504. [CrossRef]
67. Mokrani, A.; Krisa, S.; Cluzet, S.; Da Costa, G.; Temsamani, H.; Renouf, E.; Mérillon, J.M.; Madani, K.; Mesnil, M.; Monvoisin, A.; et al. Phenolic contents and bioactive potential of peach fruit extracts. *Food Chem.* **2016**, *202*, 212–220. [CrossRef]
68. Apak, R.; Güçlü, K.; Özyürek, M.; Esin Karademir, S.; Erçağ, E. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *Int. J. Food Sci. Nutr.* **2006**, *57*, 292–304. [CrossRef]
69. Canpolat, Ş.; Canpolat, E.Y.; İşlek, Y.; Akata, I.; İşlek, C. Antioxidative, Antimicrobial Activities and Fatty Acid Compositions of Four Agarics. *Turk. J. Agric.-Food Sci. Technol.* **2024**, *12*, 2257–2265. [CrossRef]
70. Köksal, E.; Tohma, H.; Kılıç, Ö.; Alan, Y.; Aras, A.; Gülçin, İ.; Bursal, E. Assessment of antimicrobial and antioxidant activities of *Nepeta trachonitica*: Analysis of its phenolic compounds using HPLC–MS/MS. *Sci. Pharm.* **2017**, *85*, 24. [CrossRef] [PubMed]

71. Balouiri, M.; Sadiki, M.; Ibensouda, S.K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **2016**, *6*, 71–79. [CrossRef] [PubMed]
72. Benek, A.; Turu, D.; Canli, K. Determination of biological activity and biochemical content of ethanol extract from fruiting body of *Tricholoma bufonium* (Pers.) Gillet. *J. Fungi* **2024**, *10*, 761. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Article

The Role of Phenolic Profile of Salt-Stressed Duckweed (*Lemna minor*) in Synthesis and Biological Activity of Green ZnO Nanoparticles

Nikola Stamenković, Filip Nikolić, Aleksandar Matić, Dragana Antonić Reljin, Marija Milovančević, Danijela Paunović and Olga Radulović *

Institute for Biological Research “Siniša Stanković”—National Institute of the Republic of Serbia, University of Belgrade, 11060 Belgrade, Serbia; nikola.stamenkovic@ibiss.bg.ac.rs (N.S.); filip.nikolic@ibiss.bg.ac.rs (F.N.); aleksandar.matic@ibiss.bg.ac.rs (A.M.); dragana.antonc@ibiss.bg.ac.rs (D.A.R.); marija.djuric@ibiss.bg.ac.rs (M.M.); danijela.paunovic@ibiss.bg.ac.rs (D.P.)

* Correspondence: olga.radulovic@ibiss.bg.ac.rs; Tel.: +381-61177076

Abstract

This study investigated whether salinity during cultivation of the aquatic plant *Lemna minor* (duckweed) influences the phytochemical composition of plant extracts and the properties of green-synthesized zinc oxide nanoparticles (ZnO NPs). Duckweed was cultivated under 0, 10, and 100 mM NaCl, followed by Orbitrap metabolomic profiling, nanoparticle synthesis, physicochemical characterization, and evaluation of antioxidant and antimicrobial activities. Orbitrap analysis revealed pronounced salinity-dependent changes in extract composition, including increased abundance of several flavonoids, glycosylated flavones, and hydroxycinnamic acid derivatives in the order $0 < 10 < 100$ mM. ZnO nanoparticle formation was supported by UV–Vis spectroscopy, which showed characteristic absorption features around 360 nm, and by powder X-ray diffraction (PXRD), which indicated the predominance of the hexagonal wurtzite ZnO phase in all samples. SEM–EDS analysis revealed Zn- and O-rich materials consisting of micron-scale aggregates and finer submicron structures. Raman spectra were dominated by fluorescence, which increased with salinity treatment and may reflect differences in surface-associated phytochemicals rather than substantial changes in the ZnO crystal structure. Nanoparticles synthesized using extracts from salt-stressed duckweed exhibited higher total phenolic content (up to 66.79 ± 0.15 $\mu\text{M GAE g}^{-1}$), antioxidant activity (up to $55.01 \pm 0.21\%$), and antimicrobial activity against *Staphylococcus haemolyticus* D4-2-100/1 (inhibition zone up to 1.55 ± 0.05 cm). Although the mechanisms underlying these differences remain to be fully elucidated, the results suggest that salinity-induced changes in duckweed metabolism may influence the biological properties of the resulting nanomaterials. Overall, this study highlights the potential of manipulating cultivation conditions to modulate plant extract composition and, consequently, influence the characteristics and functionality of green-synthesized ZnO nanoparticles.

Keywords: duckweed; nanoparticles; phenolic compounds; green synthesis; antimicrobial activity

1. Introduction

Green synthesis of nanoparticles using plant extracts has emerged in the last decade as a potentially sustainable, cost-effective, and biologically justifiable alternative to conventional chemical and physical methods [1,2]. This technology relies on the interac-

tion of plants' secondary metabolites with metal ions as stabilizing and reducing agents, which enable the formation of nucleation centers and thus initiate the generation of nanoparticles [3,4]. During this reaction, stabilizing (or capping) agents further adsorb to the nanoparticle surface and prevent uncontrolled aggregation by providing electrostatic repulsion or steric hindrance, ensuring colloidal stability and controlling particle size and morphology [1–3,5,6]. This biologically mediated process not only offers a comparatively greener alternative to conventional methods but also produces nanoparticles with surface-bound biologically active phytochemicals, which can enhance the nanoparticles' biocompatibility and antimicrobial activity [5–7]. The efficiency and characteristics of this synthesis are directly influenced by the phytochemical composition of the extract, which, in turn, is shaped by the plant's physiological state and environmental conditions. In this context, *Lemna minor* L. (duckweed) is gaining recognition as a biofactory for green nanoparticle synthesis due to its rapid vegetative propagation, minimal cultivation requirements, and dynamic phytochemical profile [1,8,9].

The metabolic plasticity of duckweed is well known, especially in responses to salt stress and starvation [10–12]. However, its role in the biosynthesis of duckweed-derived zinc oxide nanoparticles (ZnO NPs) is underexplored [1,8,9]. While multiple studies of green nanomaterial synthesis have focused on optimizing reaction parameters, nanoparticle morphology, and bioactivity, a critical variable, such as the physiological and environmental conditions under which plants are cultivated, remains largely underexplored [1,12,13]. Plant growth conditions are known to influence the concentration and composition of secondary metabolites, which in turn directly affect nanoparticle synthesis efficacy, stability, and functionality [9,14,15]. The effects of microbes are also overlooked in green synthesis studies, where biomass is harvested from decidedly non-sterile conditions [1,8,16]. However, the microbiome of plants is well known to modulate phytochemistry [17,18]. Moreover, non-sterile conditions imply other unexpected factors, e.g., chemical impurities, which may hinder the synthesis. Therefore, in this study, the duckweed biomass was deliberately grown *in vitro*. Addressing these gaps is important for developing standardized and predictable green synthesis protocols, as the phytochemical profile of the extract is not static but dynamically modulated by environmental cues.

Although silver is the most common metal for green nanomaterial synthesis [2,19–21], ZnO NPs offer several potential advantages [5,22–25]. While silver nanoparticles (AgNPs) are known for potent antimicrobial effects, their cytotoxicity, environmental persistence, and higher cost limit their applicability [2,19,20,26]. In contrast, ZnO is classified as “Generally Recognized as Safe” (GRAS) by the U.S. FDA, enabling its use in food, biomedical, and environmental systems without the same regulatory concerns [27]. ZnO NPs exert broad-spectrum antimicrobial activity via Zn^{2+} ion release, reactive oxygen species (ROS) generation, and membrane disruption; all achieved with lower ecotoxicological risks than silver [28–30]. Unlike silver, zinc is also an essential micronutrient, enhancing its biological compatibility and reducing off-target toxicity [27]. Moreover, the biosynthesis of ZnO NPs is more compatible with mild, aqueous extraction systems. Zn^{2+} ions chelate efficiently with polyphenols, flavonoids, and carboxylic acids, facilitating controlled crystallization and surface capping under ambient conditions [1,9]. ZnO also demonstrates superior photocatalytic efficiency, enabling multifunctional use in UV protection, water disinfection, and biofilm disruption [22–24,31]. Together, these properties make ZnO NPs a sustainable and functionally superior alternative to AgNPs, especially when synthesized from plant biomass under defined physiological conditions [9].

Finally, the rise of multidrug resistance and hospital-acquired infections highlights the need for novel antimicrobial agents [3]. The increasing prevalence of multidrug-resistant (MDR) pathogens poses an urgent threat to global public health, with estimates suggesting

that antimicrobial resistance (AMR) could cause up to 10 million deaths annually by 2050 if left unaddressed [32]. Green nanoparticles possibly offer such a solution, as a standalone or supplementary agent with antimicrobial activity.

In this observational and exploratory study, we investigated how NaCl-induced stress (0, 10, and 100 mM) affects the metabolomic composition of duckweed extracts and modulates the synthesis of ZnO NPs. We tested this green synthesis strategy in small-scale, in vitro conditions to test its basic feasibility. To this end, high-resolution Orbitrap mass spectrometry was employed to quantify and identify key stress-responsive phenolic compounds. The optical properties of nanoparticles were monitored via UV–Vis spectroscopy, while X-ray diffraction (XRD) was employed to assess nanoparticle crystallinity and phase purity.

The antimicrobial activity of synthesized ZnO NPs was evaluated using a well diffusion method against two opportunistic bacterial pathogens with potential to acquire MDR: *Staphylococcus haemolyticus* and *Serratia fonticola*. This research integrates plant stress physiology, metabolomic profiling, green chemistry, crystallography, and microbiology to develop an eco-sustainable nanoparticle synthesis approach based on duckweed cultivation. It supports a broader vision of optimizing nanomaterial fabrication through biochemical modulation and biomass valorization and bringing the process closer to the ideals of the circular economy. Ultimately, this study provides insight into the use of aquatic plants as renewable sources of bioactive nanomaterials with antimicrobial potential.

2. Results

2.1. Phytochemical Analysis of the Duckweed Extract

Targeted Orbitrap analysis was employed primarily to characterize the chemical profiles of duckweed extracts exposed to salt stress. In total, 30 naturally occurring compounds were identified by HPLC–Orbitrap–Ms analysis (Table 1). The majority of identified compounds had an aromatic structure, with the exception of jasmonic and geniposidic acid.

Table 1. Qualitative results of Orbitrap analysis ¹.

#	Compound	RT	Formula, [M-H] [−]	Calculated Mass, [M-H] [−]	Exact Mass, [M-H] [−]	ppm	MS2 Fragments	NaCl conc. (mM)/Biological Replicates								
								0			10			100		
								1	2	3	1	2	3	1	2	3
1	Trihydroxybenzoic acid 1	0.51	C ₇ H ₅ O ₅	169.014247	169.014584	−1.99	123.54(12), 125.02(100), 169.01(40)	-	+	-	-	-	-	-	-	-
2	Dihydroxybenzoic acid hexoside	0.71	C ₁₃ H ₁₅ O ₉	315.072156	315.0725586	−1.28	108.02(43), 152.01(100), 153.02(53), 315.071(50), 59.01(4),	+	+	-	+	+	-	+	+	-
3	Caffeic acid hexoside	1.24	C ₁₅ H ₁₇ O ₉	341.087806	341.0876429	0.48	135.04(4), 161.02(100), 179.03 (13)	+	+	+	+	+	+	+	-	+
4	Uralenneoside	1.25	C ₁₂ H ₁₃ O ₈	285.061591	285.0621058	−1.81	108.02(54), 152.01(100), 153.02(58), 285.06 (48)	+	+	+	+	+	+	+	+	-
5	Syringic Acid Hexoside	1.25	C ₁₅ H ₁₉ O ₁₀	359.098371	359.0981958	0.49	59.01(100), 89.02 (90), 101.02(46), 119.03(37), 197.05(95)	+	-	+	-	-	-	+	-	-
6 [33]	p-Coumaroylglucose	4.26	C ₁₅ H ₁₇ O ₈	325.092891	325.0935204	−1.94	59.01(4), 119.05(4), 145.03(100), 163.04 (10)	+	-	-	+	+	-	+	+	-

Table 1. Cont.

#	Compound	RT	Formula, [M-H] [−]	Calculated Mass, [M-H] [−]	Exact Mass, [M-H] [−]	ppm	MS2 Fragments	NaCl conc. (mM)/Biological Replicates
7 [33]	1-O-Sinapoyl-β-D-glucose	4.63	C ₁₇ H ₂₁ O ₁₀	385.114021	385.1138538	0.43	59.01(9), 190.03(17), 205.05 (100), 223.05(15) 93.03(50),	+ - + + + + - + +
8	Hydroxybenzoic acid 1	4.69	C ₇ H ₅ O ₃	137.024418	137.0246764	−1.89	136.86 (100), 137.02(62) 93.03(71),	+ - - + + - + + -
9	Hydroxybenzoic acid 2	4.94	C ₇ H ₅ O ₃	137.024418	137.0247078	−2.11	136.86 (100), 137.02(88) 149.02(85),	- - - - - - - - +
10	Sinapic acid	5.01	C ₁₁ H ₁₁ O ₅	223.061197	223.0614305	−1.05	164.05 (100), 208.04(16) 85.03(30),	+ - + - + + + + +
11	Caffeoylglucaric acid	5.16	C ₁₅ H ₁₅ O ₁₁	371.061985	371.0616524	0.9	111.01 (100), 129.02(12), 154.99(15) 105.03(32),	+ - - + + - + + -
12	Esculetin	5.20	C ₉ H ₅ O ₄	177.019332	177.019521	−1.07	133.02 985(100) 115.00(16),	+ - + + - + - + +
13	Fertraric acid	5.34	C ₁₄ H ₁₃ O ₉	325.056506	325.0567898	−0.87	133.01 (100)	+ + - + - - - + -
14	Cinnamic acid	5.42	C ₉ H ₇ O ₂	147.045153	147.0453699	−1.47	102.95 (100) 134.99(22)	+ + - + + + + + -
15	Caffeic acid	5.47	C ₉ H ₇ O ₄	179.034982	179.0351828	−1.12	135.04 (100), 179.04(23)	- - + - - + + + +
16	Coumaric acid	5.66	C ₉ H ₇ O ₃	163.040068	163.0402948	−1.39	119.05 (100), 163.04(16) 71.01(20), 115.00(11),	+ + + + + + + + -
17	Feruloylmalic acid	5.87	C ₁₄ H ₁₃ O ₈	309.061591	309.0620012	−1.33	134.04(42), 149.06(14), 193.05 (100) 66.00(13),	+ + + + + + + + -
18	Benzoic acid	5.92	C ₇ H ₅ O ₂	121.029503	121.0297292	−1.87	121.03 (100) 93.03(77),	+ + + + + + + + +
19	Hydroxybenzoic acid 3	6.10	C ₇ H ₅ O ₃	137.024418	137.0246535	−1.72	136.86 (100), 137.02(87)	+ + - + + - + + -
20	Ferulic acid	6.33	C ₁₀ H ₉ O ₄	193.050632	193.0508996	−1.39	134.04(36), 193.05 (100)	+ + - + + + + + -
Flavonoids and their glycosides								
21 [34]	Vitexin 2''-O-xyloside	5.19	C ₂₆ H ₂₇ O ₁₄	563.140629	563.1413818	−1.34	353.07 (100), 383.08(71), 563.14(30)	- + + - - + - - +
22 [35]	Schaftoside isomer 1	5.41	C ₂₆ H ₂₇ O ₁₄	563.140629	563.1410354	−0.72	353.07 (100), 383.08(73), 443.10(74), 473.11(54), 563.14(67)	+ + + + + + + + +
23 [36]	Schaftoside isomer 2	5.54	C ₂₆ H ₂₇ O ₁₄	563.140629	563.1409174	−0.51	353.07 (100), 383.08(76), 443.10(57), 473.11(48), 563.14(58)	+ + - + + - + + -
24	Vitexin isomer	5.76	C ₂₁ H ₁₉ O ₁₀	431.098371	431.097928	1.03	59.01(32), 311.06 (100), 341.07(35) 284.03(18),	+ + - + + - + + -
25	Luteolin-7-o-glucoside	5.86	C ₂₁ H ₁₉ O ₁₁	447.093285	447.093126	0.36	285.04 (100), 447.09(25) 59.01(16),	+ + + + + + + + +
26	Apigenin 7-O-hexoside	6.17	C ₂₁ H ₁₉ O ₁₀	431.098371	431.0975957	1.8	268.04(66), 269.04(54), 431.10 (100) 151.00(2),	+ + - + + - + + -
27	Apigenin	6.50	C ₁₅ H ₉ O ₅	269.045547	269.0459727	−1.58	269.05 (100), 270.05(2)	+ + + + + + + + +

Table 1. Cont.

#	Compound	RT	Formula, [M-H] [−]	Calculated Mass, [M-H] [−]	Exact Mass, [M-H] [−]	ppm	MS2 Fragments	NaCl conc. (mM)/Biological Replicates
Iridoid glycosides								
28	Geniposidic acid	6.46	C ₁₆ H ₂₁ O ₁₀	373.114021	373.1141707	−0.4	59.01(38), 85.03(22), 111.01(100)	- - - + - - + + -
Other compounds								
29	Piperonal	5.88	C ₈ H ₅ O ₃	149.024418	149.0245845	−1.12	93.03(12), 121.03(100), 149.02(33) 125.10(26),	+ + - + + - + + -
30	Jasmonic acid	8.98	C ₁₂ H ₁₇ O ₃	209.118318	209.1186386	−1.53	127.90(24), 209.10(19), 209.12(100),	- - - - + - + + -

¹ Compounds are presented with their retention times (RT), molecular formulas, mass accuracy (ppm), and peaks corresponding to masses of molecular fragments in the electric field. Mass accuracy between −5 and +5 was considered acceptable. The presence of a compound in a biological replicate was marked by +; the absence by: −.

Upon exposure to increasing concentrations of NaCl (0, 10, and 100 mM), the duckweed extracts exhibited significant changes in their specialized metabolite profiles (Figure 1).

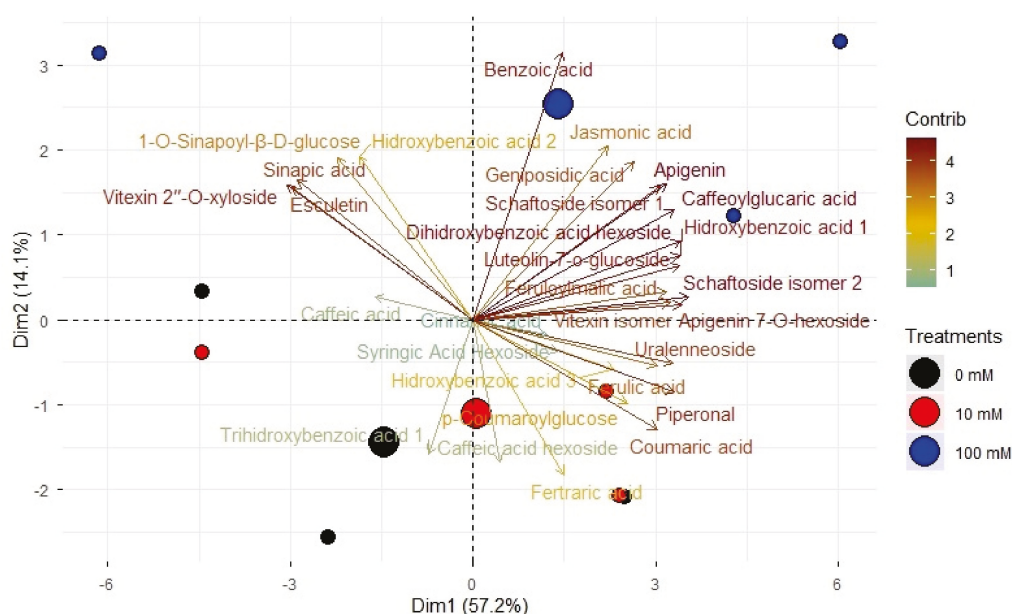


Figure 1. Biplot representing principal component analysis (PCA) plot of 30 compounds isolated from duckweeds exposed to salt stress, combined with duckweed response distribution to salinity stress. The data distribution and vector plots were constructed in R, using FactoExtra and FactoMineR 2.15 packages.

The analysis for all treatments shows that the two principal components (PC1 and PC2) explain 71.3% of total data variability, with PC1 explaining 57.2% and PC2 explaining 14.1% (Figure 1). PC1 was mainly influenced by schaftoside isomers 1 and 2, apigenin and apigenin 7-O-hexoside, hydroxybenzoic acid 1, dihydroxybenzoic acid hexoside, luteolin-7-O-glucoside, piperonal, caffeoylglucaric acid, uralenneoside, feruloylmalic acid, vitexin and vitexin 2''-O-xyloside, coumaric acid, ferulic acid and esculetin. The component PC2 was significantly correlated with benzoic acid, jasmonic acid, hydroxybenzoic acid 2, sinapic acid, 1-O-sinapoyl-β-D-glucose, geniposidic acid, caffeic acid hexoside, fertraric acid, apigenin, vitexin 2''-O-xyloside, trihydroxybenzoic acid 1, schaftoside isomer 1, and esculetin (Supplementary Figure S1A). Data from duckweeds treated with 0 and 10 mM NaCl were mainly grouped between the center and the negative end of the y-axis. The

only difference between these two groups is that data from duckweeds treated with 0 mM were pointed toward negative values of the x-axis, while data from duckweeds treated with 10 mM gravitated toward positive values of the x-axis. Based on the PCA of phenolic profiles of duckweeds, it can be concluded that duckweeds grown at 10 mM NaCl have a similar phenolic profile to duckweeds grown in control conditions due to overlapping. Duckweeds treated with 100 mM NaCl formed a distinctive group located toward the positive end of the y-axis and exhibited a different phenolic profile (Figure 1). Based on the directions of vectors representing phenolic compounds, it can be concluded that benzoic acid, jasmonic acid, hydrohydroxybenzoic acids 1 and 2, geniposidic acid, vitexin 2''-O-xyloside, sinapic acid, 1-O-sinapoyl- β -D-glucose, esculetin, apigenin, schaftoside isomer 1, and caffeoylglucaric acid are associated with salt stress response. On the other hand, piperonal, coumaric acid, fertratic acid, caffeic acid hexoside, trihydroxybenzoic acid 1, ferulic acid, hydrohydroxybenzoic acid 3, syringic acid hexoside, and caffeic acid are correlated with stress-free (0 mM) and low-intensity stress (10 mM) conditions.

The PCA for each treatment further revealed the separation of 0, 10, and 100 mM groups and highlighted the increase in the relative contribution of PC1 to total data variability from 70.4% at 0 mM (Figure 2A) to 82.4% at 10 mM (Figure 2B) and 87.5% at 100 mM (Figure 2C).

For the 0 mM group, the PC1 was determined by ferulic acid, cinnamic acid, luteolin-7-O-glucoside, hydrodihydroxybenzoic acid hexoside, piperonal, schaftoside isomers 1 and 2, feruloylmalic acid, coumaric acid, hydroxybenzoic acid 1, p-coumaroylglucose, caffeoylglucaric acid, uralenneoside, syringic acid hexoside, apigenin and apigenin 7-O-hexoside (Figure 2A). Meanwhile, PC2 was significantly correlated with benzoic acid, hydroxybenzoic acid 3, trihydroxybenzoic acid 1, sinapic acid and 1-O-sinapoyl- β -D-glucose, esculetin, vitexin isomer and vitexin 2''-O-xyloside, and caffeic acid (Supplementary Figure S1B).

For duckweeds grown at 10 mM NaCl, the PC1 and PC2 accounted for 82.4% and 17.6% of the total data variability (Figure 2B). Luteolin-7-O-glucoside, schaftoside isomers 1 and 2, esculetin, caffeic acid and its hexoside, vitexin isomer and its 2''-O-xyloside, sinapic acid and its 1-O- β -D-glucoside, hydrohydroxybenzoic acid 1, apigenin and its 7-O-hexoside, cinnamic acid, dihydroxybenzoic acid hexoside, uralenneoside, coumaric acid and p-coumaroylglucose influenced the PC1. Geniposidic acid, fertratic acid, jasmonic acid, feruloylmalic acid, benzoic acid, caffeic acid hexoside, and hydroxybenzoic acid 3 determined the PC2 (Supplementary Figure S1C).

For duckweeds grown at 100 mM NaCl, PC1 and PC2 accounted for 87.5% and 12.5% of the total data variability (Figure 3). The component PC1 was mainly shaped by ferulic acid, cinnamic acid, caffeoylglucaric acid, coumaric acid and p-coumaroylglucose, apigenin and apigenin 7-O-hexoside, caffeic acid, dihydroxybenzoic acid hexoside, schaftoside isomers 1 and 2, piperonal, geniposidic acid, vitexin isomer and vitexin-2''-O-xyloside, hydroxybenzoic acid 2, sinapic acid and 1-O-sinapoyl- β -D-glucose, hydroxybenzoic acid 1, uralenneoside and luteolin-7-O-glucoside. On the other hand, PC2 was significantly determined by syringic acid hexoside, benzoic acid, fertratic acid, jasmonic acid, and caffeic acid hexoside (Supplementary Figure S1D).

For heatmap analysis, 30 compounds were used, of which 28 were phenolics (Figure 3). Based on the change in $\log_{10}$ values, parameters can be classified into two clusters. Cluster I includes 5 phenolic compounds, which were missing in some treatments. The remaining 25 compounds, which are found in all treatments, form cluster II. Cluster II can be further divided into subclusters II_a and II_b. Subcluster II_a encompasses compounds whose content was not significantly influenced by treatments. Accordingly, phenolic compounds whose content was generally increasing with implemented concentrations of NaCl during treatments were grouped in subcluster II_b. In this group, schaftoside isomers 1 and 2,

luteolin-7-O-glucoside, feruloylmalic acid, vitexin 2''-O-xyloside, uralenneoside, sinapic acid, dihydroxybenzoic acid hexoside, 1-O-sinapoyl- β -D-glucose, caffeic acid hexoside, and coumaric acid were identified.

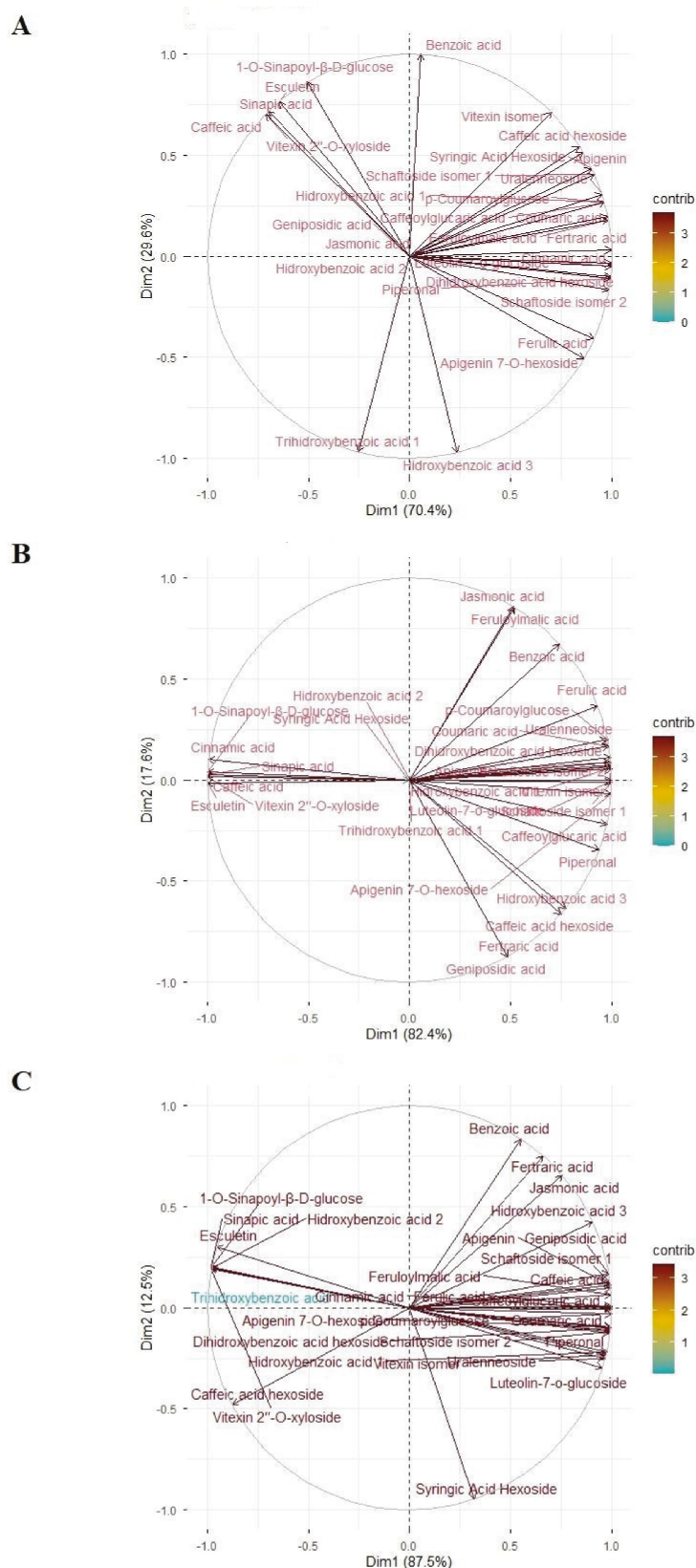


Figure 2. Vector plots of variables corresponding to different salt treatments: (A)—0 mM; (B)—10 mM; (C)—100 mM NaCl. Plots were constructed in R using Factoextra and FactoMineR packages.

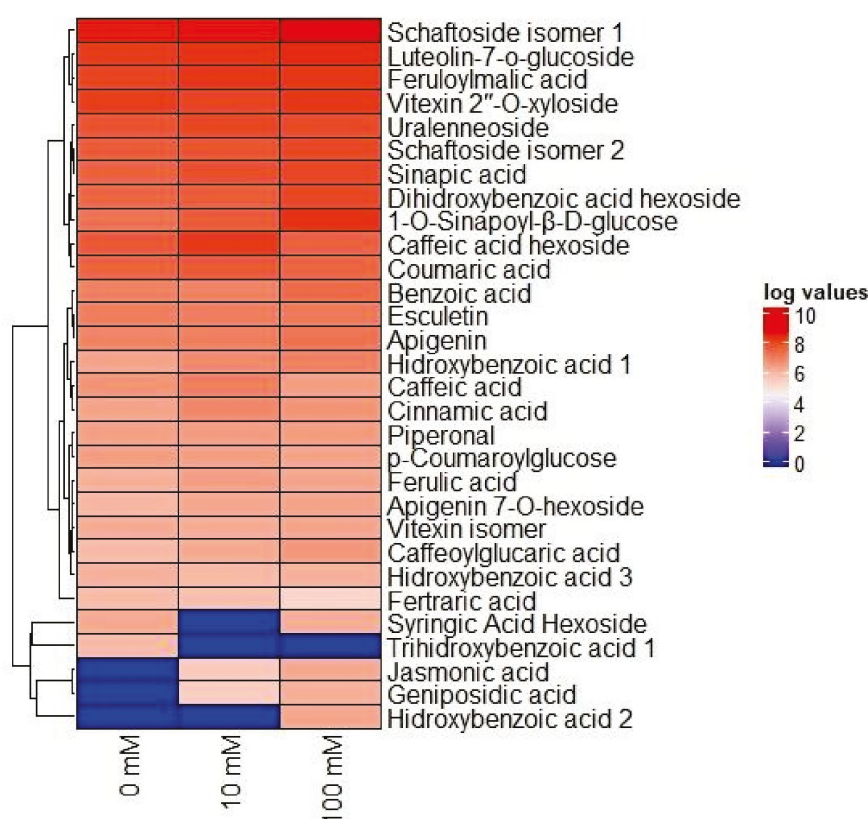


Figure 3. Cluster heatmap analysis representing the changes in 30 compounds isolated from duckweed. Clustering is described by the dendrogram on the left. Red color indicates the increase in different parameters, and blue color indicates the decrease. The heatmap was constructed in R using complexheatmap package.

2.2. Spectroscopic UV–Vis Characterization of ZnO Green Nanoparticles Synthesized from the Duckweed Extract

To assess the optical properties of the solution with ZnO green nanoparticles synthesized from the duckweed extract, and to compare them with the optical properties of the mentioned extract, UV–Vis spectroscopy was performed (Figure 4).

The UV–Vis absorption spectra of the ethanolic extract of duckweed at varying concentrations of the salt treatment (0 mM, 10 mM, and 100 mM) are shown in Figure 4A. All spectra exhibited an absorption maximum in the approximate range of 320–380 nm and a spectral valley with a minimum around 520 nm.

The UV–Vis spectra of the analyzed duckweed-derived nanomaterial had characteristic absorbance peaks (λ_{max}) shifted toward the visible part of the spectrum, most notably around 360 nm (Figure 4B). The absorbance maxima also increased with the salinity increment from 0 to 10 and 100 mM NaCl. The spectral valley around 520 nm was absent.

The Tauc plot deduced from the UV–Vis spectra of ZnO-NPs revealed that the estimated band gap energy (E_g) increased with increasing exposure to salt stress during duckweed cultivation, from approximately 2.5 eV for 0 mM to 2.65 eV for 10 and 100 mM samples (Figure 4C).

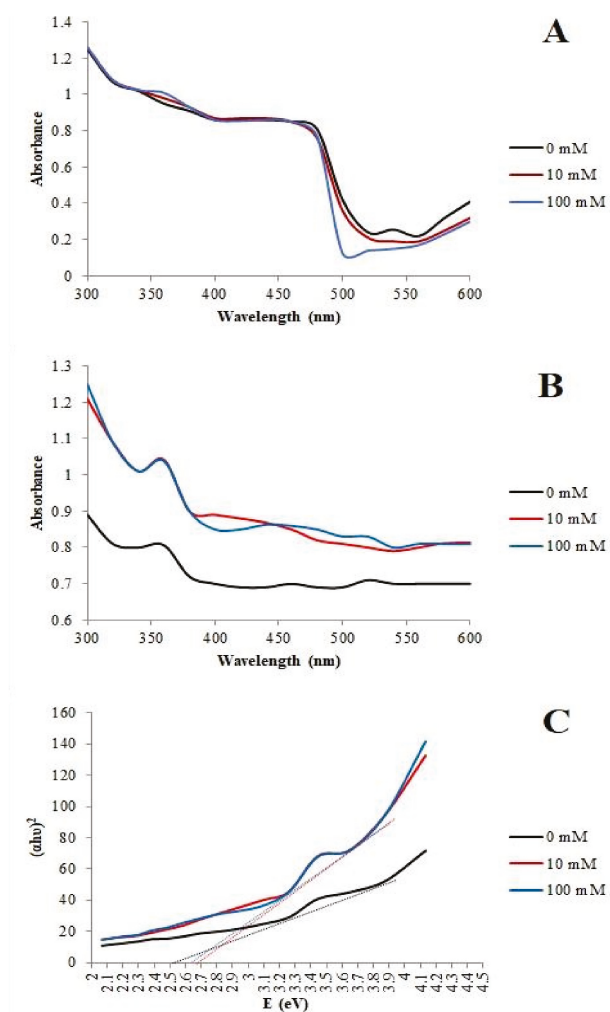


Figure 4. UV-Vis spectra of the duckweed extract (A) and ZnO-NP solution (B) with the corresponding Tauc plot (C).

2.3. Analysis of ZnO NPs with X-Ray Diffraction on Powdered Material (PXRD)

To test the presence of crystalline material in synthesized samples, a PXRD was performed. In all three tested samples, hexagonal ZnO crystals were detected (Figure 5).

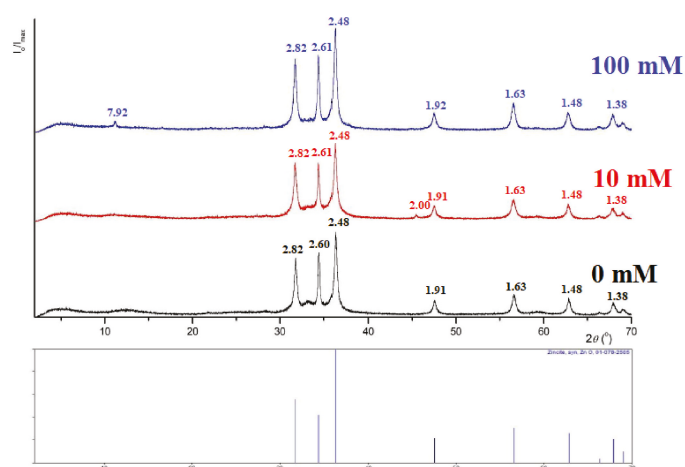


Figure 5. Comparison of diffractograms of three nanomaterial samples synthesized from the extract of duckweed exposed to 0, 10, or 100 mM NaCl, with standard ZnO peaks (diffractogram at the bottom). The d-values (in Å) are shown above each peak.

All three samples (corresponding to 0 mM, 10 mM, and 100 mM NaCl treatments) exhibited diffraction patterns consistent with the hexagonal ZnO (zincite) phase (PDF# 01-078-2585), space group $P6_3mc$. The major reflections matched well with standard ZnO peaks (Figure 5, diffractogram at the bottom). In terms of interplanar distances or spacings (d_{hkl}), the 0 mM diffractogram displayed a pure ZnO phase with only seven characteristic wurtzite peaks, showing the expected d-values (2.82, 2.60, 2.48, 1.91, 1.63, 1.48, and 1.38 Å) and no additional reflections. In the 10 mM diffractogram, in addition to these typical ZnO peaks, a single unidentified reflection was observed at $d = 2.00$ Å. In the 100 mM diffractogram, alongside the characteristic ZnO reflections, an additional non-characteristic and prominent peak was detected at $d = 7.92$ Å. (Figure 5).

2.4. Scanning Electron Microscopy (SEM) and Electron-Dispersive X-Ray Spectroscopy (EDS) of the Green Nanomaterial Derived from Duckweed

The surface morphology of the ZnO particles synthesized using *Lemna minor* extracts was investigated by scanning electron microscopy employing both backscattered electron (BSE) and secondary electron (SE) imaging modes. BSE micrographs revealed the formation of highly agglomerated particulate material consisting of irregular micron-sized aggregates distributed throughout the samples (Figure 6).

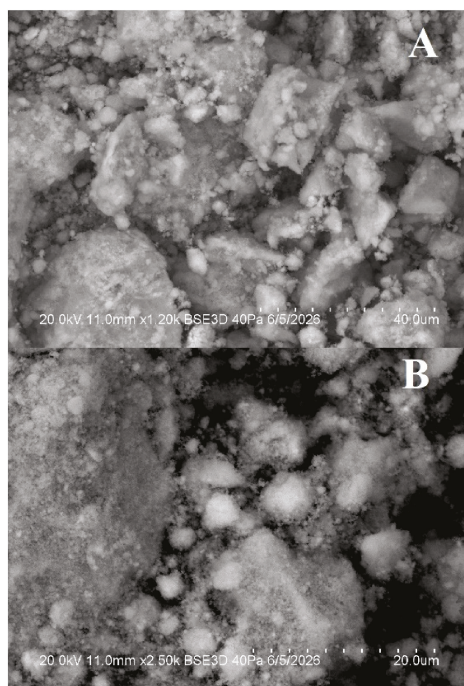


Figure 6. Representative SEM micrographs of ZnO nanoparticles synthesized using *Lemna minor* extracts. The particles formed irregular agglomerates consisting of finer substructures. Images were recorded using a Hitachi S-3700N microscope in variable-pressure mode with a BSE detector at an accelerating voltage of 20 kV. Scale bars: 40 μm (A) and 20 μm (B).

Higher-resolution SE micrographs provided additional information on surface morphology and showed that the larger aggregates were composed of finer submicron particles assembled into densely packed structures (Figure 7). The visible agglomerates ranged from approximately 2 to 30 μm, whereas the finer surface particles were mostly <1 μm. Nevertheless, all samples displayed pronounced aggregation, a characteristic commonly reported for plant-mediated ZnO nanoparticles. Due to the extensive agglomeration and the magnification range employed, individual primary nanoparticles could not be reliably distinguished or measured.

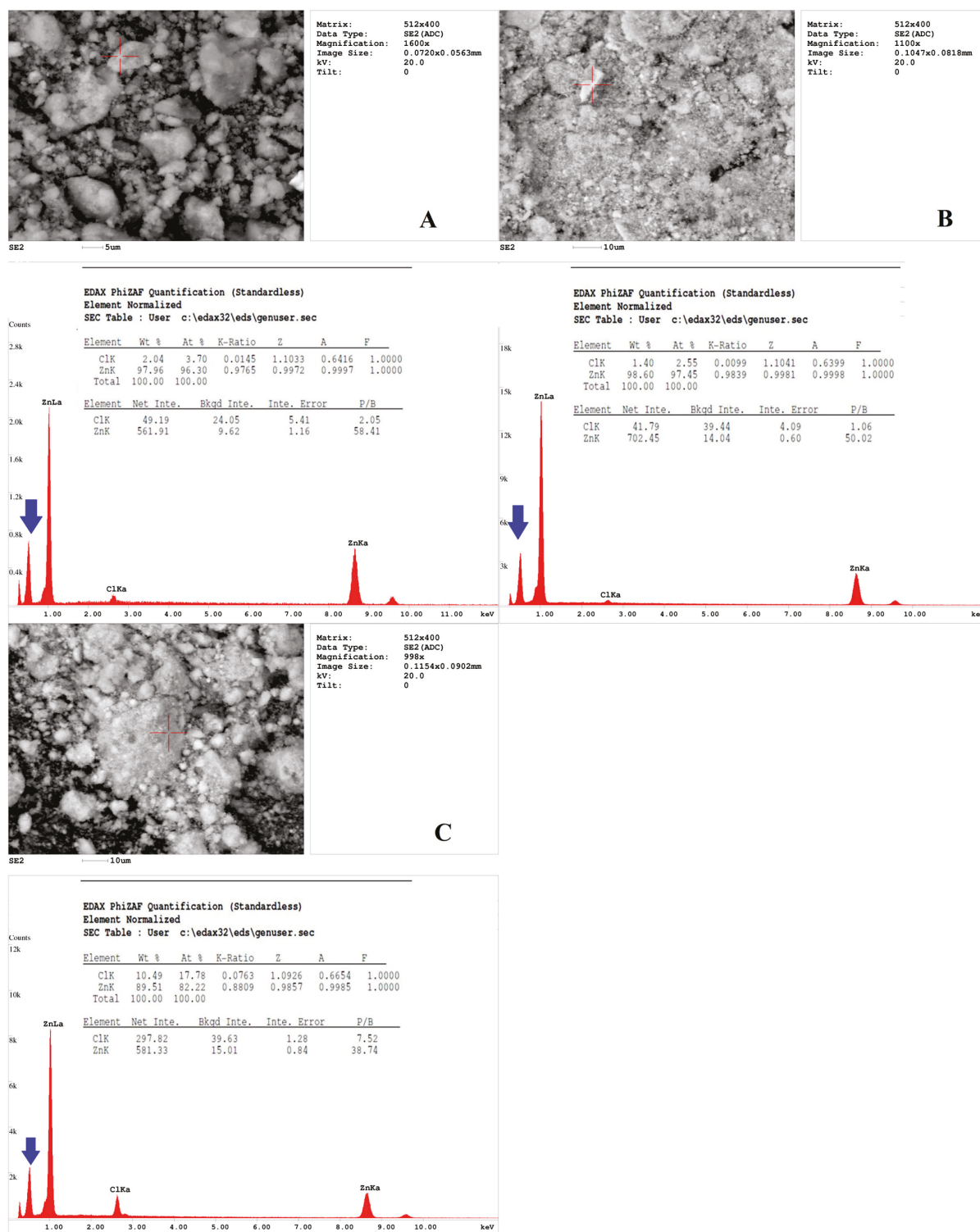


Figure 7. SEM micrographs and EDS spectra of ZnO nanoparticles synthesized using *Lemna minor* extracts obtained from plants grown under different salinity conditions: (A) 0 mM NaCl, (B) 10 mM NaCl, and (C) 100 mM NaCl. Images were acquired using a Hitachi S-3700N SEM operated at 20 kV in variable-pressure mode with a secondary electron (SE) detector, while semi-quantitative analysis was performed with EDS detectors. Blue arrows indicate signals attributable to oxygen. Scale bars: 5 μ m (A) and 10 μ m (B,C).

The elemental composition of the biosynthesized ZnO particles was investigated by energy-dispersive X-ray spectroscopy (EDS). The spectra of all samples were dominated by characteristic Zn signals, confirming zinc as the principal constituent of the

synthesized material (Figure 7A–C). In addition to Zn, minor amounts of chlorine were detected in all samples, indicating the presence of residual chloride-containing species, most likely originating from the ZnCl_2 precursor used during nanoparticle synthesis. Although lower-energy signals attributable to oxygen were consistently observed in the EDS spectra near ~ 0.5 keV, oxygen and carbon were not included in the quantitative analysis because reliable quantification of light elements is limited under the variable-pressure SEM-EDS conditions employed for the analysis of uncoated powder samples. Nevertheless, the combined SEM-EDS and XRD results consistently support the successful formation of ZnO as the predominant phase in all investigated samples.

2.5. (Poly)phenolic Content, DPPH Scavenging and Antimicrobial Activity of Duckweed-Derived ZnO NPs

The Folin–Ciocalteu (FC) test indicated the (poly)phenolic content of duckweed-derived ZnO NPs. This content increased with the concentration of the NP solution (Table 2). The highest phenolic content was measured for ZnO NPs at a 50 mg/mL final concentration. The DPPH scavenging activity also increased with the final concentration of NP suspension. The highest antioxidant activity was measured for ZnO NPs derived from duckweed exposed to 100 mM NaCl at an NP concentration corresponding to 50 mg/mL (Table 2).

Table 2. Total phenolic content (TPC), DPPH scavenging and antimicrobial activity (inhibition zone) of NP solutions.

Salt Treatment, Final Concentration of NPs	TPC (GAE $\mu\text{M/g}$)	Scavenging Activity (%)	Inhibition Zone (cm)
0 mM NaCl, 12.5 mg/mL	0.34 ± 0.01^d	8.47 ± 1.11^c	1.17 ± 0.05^c
0 mM NaCl, 25 mg/mL	38.67 ± 0.12^b	11.51 ± 1.81^{bc}	1.45 ± 0.02^{ab}
0 mM NaCl, 50 mg/mL	61.91 ± 0.36^a	15.31 ± 3.58^{bc}	1.36 ± 1.40^{ab}
10 mM NaCl, 12.5 mg/mL	17.95 ± 0.04^c	9.45 ± 0.39^c	1.30 ± 0.07^{bc}
10 mM NaCl, 25 mg/mL	40.06 ± 0.33^b	11.69 ± 0.57^{bc}	1.55 ± 0.13^a
10 mM NaCl, 50 mg/mL	66.79 ± 0.15^a	11.82 ± 1.63^{bc}	1.44 ± 0.03^{ab}
100 mM NaCl, 12.5 mg/mL	0.48 ± 0.01^d	9.66 ± 0.93^c	1.40 ± 0.10^{ab}
100 mM NaCl, 25 mg/mL	54.38 ± 0.09^b	25.89 ± 13.89^b	1.55 ± 0.05^a
100 mM NaCl, 50 mg/mL	59.97 ± 0.18^a	55.01 ± 0.21^a	1.50 ± 0.05^{ab}

Values represent mean $\pm$ S.E. Different letters within columns indicate significant differences between treatments at $p \leq 0.05$. Maximum values are bold.

To screen and compare the antimicrobial activity of nanoparticles at different concentrations and different duckweed treatments, the well diffusion test was performed (Table 2). There were no measurable halos in the agar plates streaked with *Serratia fonticola* A3-102/3. Also, this strain was absent in plates with 10 mg/mL Marocen as a positive control. Inhibition zones were measurable around wells with NP suspensions in agar plates streaked with *Staphylococcus haemolyticus* D4-2-100/1. These inhibition zones were at least 2.45 times narrower than the inhibition zones around wells with Marocen, which were estimated at (3.80 ± 0.21) cm. The antimicrobial activity of ZnO NP derived from stressed duckweed at 25 mg/mL was approximately 30% higher than that of ZnO NPs derived from mildly stressed or unstressed duckweed at 12.5 mg/mL (Table 2).

3. Discussion

3.1. Phytochemical Composition of Duckweed

Plants dynamically modify their chemical composition in response to different types of stress. In our previous research, we identified specific phenolic compounds such as luteolin 6,8-di-C-hexoside, p-hydroxybenzoic acid, caffeic acid, apigenin 6-C-(2''-pentosyl) hexoside and p-coumaric acid associated with endophytic colonization of duckweed by *Klebsiella oxytoca*, confirming that *L. minor* can remodel its phytochemistry in response to

external (in this case, microbial) stimuli [15,37–39]. In addition, our recent research has shown that *Pseudomonas oryzihabitans* D1-104/3 and *P. gessardii* C31-106/3 differentially modulate the antioxidative response of duckweed to salt stress, leading to elevated levels of phenolic compounds [17]. The metabolic modulation of plants' chemical composition confers multiple benefits to the plants, such as increased resistance to abiotic stress and defense against plant pathogens and predators. These effects may be translated into diverse biotechnological applications, e.g., medicinal use, feed for livestock and food for humans, or raw biomaterial production for bioethanol and biofertilizers, especially in the case of duckweeds [40–44]. Pagliuso et al. (2020) reported 16 flavonoids and their derivatives in the extracts of several duckweed species, most notably derivatives of chlorogenic acid, apigenin, and luteolin [43]. In this study of a single strain of *L. minor*, we identified six: a vitexin isomer, vitexin 2''-O-xyloside, schaftoside isomers 1 and 2, luteolin-7-O-glucoside, apigenin and apigenin 7-O-hexoside. Interestingly, C-glycosides were the most prevalent form of flavones, similarly to our study, where luteolin-O-glucoside and schaftoside isomers were reported. Moreover, the presence of uralenneoside and feruloylmalic acid reported here is a rare and unique finding in *Lemna minor* research. This highlights the metabolic plasticity and differences between phylogenetically close duckweed species and the need to characterize the phenolic content of multiple strains of *L. minor* and of other related duckweed species.

Among the few studies analyzing the connection between salt stress and green nanoparticle synthesis, Gupta et al. (2024) reported that 75 mM NaCl triggered a qualitative shift in the phytochemical composition of the giant duckweed (*Spirodela polyrhiza*), marked by the accumulation of flavonoid subset, namely flavones and their glycosides (orientin, genistin, azelaic acid, sinapic acid, quercetin, apigetrin, vitexin, and naringin) as the key metabolites for the nucleation and stabilization of nanoparticles [9]. In this study, based on the growing contribution of PC1 to total variability, we found that the phenolic profile of *Lemna minor* is strongly associated with salt stress, with distinct metabolic shifts observed across 0, 10, and 100 mM NaCl treatments, which is aligned with our previous work [17]. Moreover, based on the compounds' influence on PC1 and PC2 constructions, it can be concluded that every treatment has a specific phenolic footprint. The following flavonoids and hydroxycinnamic acids responded to increasing salt stress: schaftoside isomers 1 and 2, luteolin-7-o-glucoside, feruloylmalic acid, vitexin 2''-O-xyloside, uralenneoside, sinapic acid, 1-O-sinapoyl- β -D-glucose, dihydroxybenzoic acid hexoside, caffeic acid hexoside, and coumaric acid. Similarly, as in Gupta et al., glycosylated flavones (schaftoside isomers, luteolin, vitexin, and uralenneoside derivatives) also accumulated with increasing salt stress [9].

Therefore, it would be of research interest to test multiple duckweed strains and species, as duckweeds (family Lemnaceae) exhibit extraordinary metabolic plasticity and variety, with the added advantage of rapid biomass doubling and minimal growth requirements compared to some other conventionally used plant species. In the future, special attention should be given to phenylpropanoid and flavonoid pathways, as they are key determinants of the physicochemical properties of biosynthesized nanoparticles.

3.2. UV–Vis Spectroscopic Properties of Duckweed-Derived ZnO Nanoparticles

The UV–Vis absorption spectra of nanoparticles synthesized from *Lemna minor* extracts grown under different NaCl concentrations provide further insight into the influence of metabolic plasticity on nanoparticle formation.

All samples exhibited distinct peaks around 360 nm, indicative of small, uniformly distributed nanoparticles, as supported by relevant literature [1,45,46]. This also corresponds well with the Orbitrap analysis, which revealed elevated levels of key reducing

and stabilizing phenolics such as caffeic acid hexoside and ferulic acid, both known to accelerate metal ion reduction and promote controlled nucleation [3,47].

The almost fixed position of the characteristic 360 nm absorbance maximum across all spectra confirmed the presence of the same type of ZnO nanoparticles in all samples, while the differences in intensity hinted at different reaction yields and colloidal effects (most probably scattering and aggregation), as well as interactions between nanoparticles and other components in the suspension (e.g., unbound phytochemicals). This agrees with Gupta et al. (2024), who observed diverse, polydispersed, and rosette-like zinc nanostructures synthesized from the extract of duckweed grown at 75 mM NaCl compared to control plants [9]. However, whether similar structures are indeed present in the ZnO NP samples in this study remains to be seen.

The apparent E_g values were consistent with those reported for similar green-synthesized ZnO nanomaterials [48]. Interestingly, Rafique et al. also reported band gaps in the 2.20–2.60 eV range, where band gaps decreased as extract volume increased [1]. The E_g differences between samples might also reflect this effect. Moreover, the apparent band gap shrinking compared to bulk ZnO (3.3 eV) may be associated with differences in defect concentration, lattice disorder, surface states, and/or the presence of phytochemical species adsorbed on the nanoparticle surface. This interpretation is indirectly supported by the fluorescence-dominated Raman spectra, which hint at the presence of surface-associated organic compounds, as well as by SEM–EDS analysis confirming Zn-rich particulate materials with prominent peaks corresponding to O K α emissions and minor variations in residual chloride content among the samples. Further investigations employing photoluminescence spectroscopy, X-ray photoelectron spectroscopy, or advanced defect characterization techniques would be required to elucidate the origin of the apparent band gap reduction compared to bulk ZnO.

3.3. The Polycrystalline Nature of Duckweed-Derived ZnO Green Nanoparticles

The X-ray diffraction (XRD) results provide compelling evidence for the successful synthesis of ZnO nanoparticles under all tested salinity conditions, as confirmed by the presence of characteristic ZnO diffraction peaks. However, variations in the diffraction patterns across different salinity levels also suggest that the salinity exposure of the duckweed extract had an influence on the phase purity and possible secondary phase formation during nanoparticle synthesis.

At 0 mM NaCl, the XRD profile exhibited a clean and sharp pattern corresponding exclusively to the hexagonal wurtzite ZnO structure, with no detectable secondary reflections. This indicates that the extract obtained from unstressed duckweed acts as an effective biological reducing and stabilizing agent, capable of facilitating the formation of pure ZnO nanoparticles. The absence of any impurity peaks suggested minimal interference from plant metabolites, precursor residues or decomposition products in the synthesis process.

In contrast, the XRD pattern of the 10 mM NaCl sample showed a minor, yet notable, unidentified diffraction peak at $d = 2.00$ Å. While the dominant peaks still matched the ZnO phase, the emergence of this additional reflection implies the presence of a secondary crystalline phase, possibly a minor chloride crystalline residue stemming from either the precursor or NaCl treatment. This could tentatively be attributed to subtle alterations in the biochemical composition of the duckweed extract induced by moderate salt stress.

However, the most pronounced deviation from the expected diffraction pattern was observed in the 100 mM NaCl sample, where, in addition to typical ZnO peaks, a strong reflection at $d = 7.92$ Å was recorded. The intense abiotic stress at 100 mM NaCl likely prompted a biochemical shift in the duckweed extract, enhancing the complexity of interactions between Zn^{2+} ions and organic constituents during nanoparticle formation. In other

studies, similar additional signals corresponded to layered zinc hydroxide complexes, the by-products of nanoparticle synthesis [13,49]. The isolated non-ZnO reflections differed among the samples, suggesting that the minor secondary crystalline components were not identical under all synthesis conditions. The absence of the $d \approx 2.00 \text{ \AA}$ reflection in the 100 mM sample indicates that this phase was either absent, below the XRD detection limit, or replaced by another minor phase responsible for the low-angle $d \approx 7.92 \text{ \AA}$ reflection.

Raman spectroscopy was used as a complementary technique to probe vibrational properties and structural disorder of the synthesized ZnO nanoparticles. However, due to the intense fluorescence of ZnO NPs, Raman spectra remained broad and featureless, without clearly resolved ZnO Raman bands (Supplementary Figure S2). This behavior is consistent with the observation that Raman spectra of plant-extract-derived nanomaterials are sensitive to fluorescence originating from surface-bound phytochemicals [2]. For wurtzite ZnO, the $E_2(\text{high})$ mode near $437\text{--}440 \text{ cm}^{-1}$ is generally associated with crystalline lattice order, whereas LO-region bands around $590\text{--}660 \text{ cm}^{-1}$ are commonly linked to intrinsic defects such as oxygen vacancies and zinc interstitials [50,51].

In our study, Raman spectra were dominated by a strong fluorescence background, resulting in broad and poorly resolved spectral features (Supplementary Figure S2.1). Weak contributions were observed within the spectral regions commonly associated with the $E_2(\text{high})$ and LO modes of wurtzite ZnO; however, their assignment remains tentative due to the low signal-to-background ratio and extensive band broadening. Such broadening may arise from nanoscale disorder, defect-related states, and interactions between the ZnO surface and phytochemical capping agents. The progressive increase in fluorescence intensity from 0 to 10 and 100 mM samples further supports an increasing contribution of surface-associated phytochemicals, in agreement with the Orbitrap metabolomic analysis.

Therefore, XRD and SEM-EDS were used as the primary evidence for ZnO phase formation, while Raman spectroscopy was considered complementary and mainly informative regarding the surface-modified, defect-rich nature of the green-synthesized particles. Collectively, the Orbitrap, UV-Vis, XRD, SEM-EDS, and Raman results suggest that salinity predominantly influenced the surface chemistry of the green-synthesized ZnO nanoparticles through modulation of phytochemical composition, while the underlying ZnO crystalline structure remained largely preserved.

Overall, these observations highlight the dual role of the duckweed extract as both a reducing/stabilizing agent and a modulator of the final nanoparticle structure, depending on its physiological state under salinity stress. The emergence of secondary reflections in stressed samples suggests that stress-induced phytochemicals can alter the crystallization pathway, leading to hybrid or composite nanostructures, which may explain the additional signals in diffractograms. The differences between diffractograms can, hypothetically, be the result of distinctive ratios of phenolic compounds during treatments, a phenomenon underlined by PCA. The positive FC test hinted at the presence of (poly)phenolic compounds in green ZnO NPs. Interestingly, Gupta et al. (2024) studied the structure of nanoparticles derived from stressed giant duckweed, grown at 75 mM NaCl and reported broadly similar observations to those in our paper: accumulation of flavonoids, wurtzite ZnO resulting from green synthesis, and micronic agglomerates of nanomaterial with finer submicronic structures [9]. To the best of our knowledge, this study is the first to compare diffraction patterns, EDS-SEM, and Raman spectra of nanoparticles derived from the extract of duckweed grown under different physiological conditions. These observations highlight the potential of manipulating plant stress responses to fine-tune the properties of green-synthesized nanomaterials. Although the combined Orbitrap, UV-Vis, XRD, SEM-EDS, and Raman analyses provide valuable insight into the properties of the green-synthesized ZnO nanoparticles, further characterization by TEM, XPS, FTIR, and BET analysis would be beneficial for a more

comprehensive understanding of nanoparticle morphology, surface chemistry, porosity, and capping mechanisms. These investigations will be the subject of future studies.

3.4. Biological Activity of Duckweed-Derived ZnO Nanoparticles

The antioxidant and antimicrobial performance of duckweed-derived ZnO nanoparticles was significantly influenced by both salt treatment and nanoparticle concentration. Total phenolic content (TPC) increased notably with higher NP concentrations, particularly under moderate (10 mM) and high (100 mM) NaCl stress, hinting at the role of salt stress in enhancing phenolic components in the structure of duckweed-derived ZnO NPs. The highest TPC (66.79 μM GAE/g) was observed at 50 mg/mL ZnO NPs in duckweed exposed to 10 mM NaCl, suggesting this condition effectively promotes (poly)phenol incorporation during synthesis, which is aligned with the shifts in phenolic profiles revealed by Orbitrap analysis. Similar results were reported in other studies [1,2,46]. Overall, these results hint at the important role of phenolics in the structural organization and biofunctionality of duckweed-derived ZnO nanoparticles.

Antioxidant activity, as measured by DPPH scavenging, showed a sharp increase under 100 mM NaCl at 50 mg/mL, reaching 55.01% and far surpassing other treatments. This was to be expected from our previous antioxidant activity study of pure duckweed extract [17]. Again, this indicated that severe salt stress may trigger the accumulation of potent antioxidant compounds that are retained in the nanoparticle matrix.

Antimicrobial activity, as reflected by inhibition zones, also improved, albeit modestly, with both NP concentration and salt stress, with the largest zones (1.55 cm) observed for NPs synthesized under 10 and 100 mM NaCl at 25 mg/mL. An interesting observation was made regarding the biological specificity of ZnO NPs in this study, where the ZnO NPs exerted inhibitory effects only against the Gram-positive strain and apparently had no effect on the Gram-negative one. In similar studies, green NPs exerted antimicrobial effects against both Gram-positive and -negative bacteria equally, or more against Gram-positive bacteria while Gram-negative appeared more resistant [2,9,16,19,52]. For instance, Shukla et al. (2025) report equal inhibitory zones of ZnO NPs against *Bacillus subtilis* (a Gram-positive bacterium) and *Salmonella typhi* (a Gram-negative bacterium) [1]. The inhibitory zones in this and similar studies were comparable to those measured in this study, in the range of 0.9–1.5 cm [1]. This underlines the biological specificity of ZnO NPs, which can at least in part be explained by the universal differences in the structure of the bacterial cell wall of Gram-positive versus Gram-negative bacteria, as well as by the resistance mechanisms of individual bacterial strains tested herein [2,5]. The effect of NP concentration was also reported and hinted that higher NP concentrations may effectively reduce their mobility and diffusion through the agar, mostly due to aggregation [29]. At higher concentrations, NPs tend to form clusters that may hinder their mobility through the porous structure of agar, which reduces their effective antimicrobial potency even though their biological activity remains unchanged.

Together, these results suggest that salt stress enhances the biofunctionalization of ZnO NPs by increasing the availability and activity of phenolic compounds, particularly at higher NP concentrations, thus improving their antioxidant and antimicrobial efficacy. The green ZnO NPs synthesized in this study may have some practical applications, such as, for instance, topical antimicrobial ointments against infections caused by Gram-positive bacteria or as a treatment against Gram-positive bacterial phytopathogens. Moreover, the generally low toxicity and environmental impact of zinc in this hypothetical formulation sets it apart from other, more commonly used silver-based nanomaterials [27,29]. However, stability testing of the nanoparticle suspensions, including shelf-life, dispersion stability, and retention of functional properties over time, was not conducted in this study due to

the preliminary nature of the work. Given the importance of these parameters for assessing real-world applicability, their evaluation remains a priority for future research.

Finally, as duckweed biomass is rapidly doubled, the green synthesis of nanomaterials in this case would be a particularly affordable and self-sustainable process. Thus, the green synthesis of antimicrobial ZnO NPs based on duckweed biomass connects circular economy with sustainable biomedicine, revalorizing duckweed biomass which is, as it currently stands, globally underutilized.

4. Materials and Methods

4.1. Duckweed (*L. minor*) Growth Conditions

Duckweed was grown under the same conditions as described before [17]. In brief, surface-sterilized duckweed was maintained in stock cultures with complete Murashige–Skoog medium supplemented with 3% sucrose at 24 ± 2 °C under fluorescent light of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 16 h light/8 h dark photoperiod. For the salt exposure experiments, 150–300 individual fronds were picked and transferred to MS medium without vitamins or sucrose (in further text: MS medium). Fronds were acclimatized and then transferred to fresh sterile MS medium in three groups based on supplementation with sodium chloride (NaCl)—control (0 mM), 10 mM, and 100 mM—for 14 days.

4.2. Phytochemical Characterization of Phenolic Compounds in Duckweed Exposed to Salt Stress

4.2.1. Sample Preparation

To identify the presence of different metabolites in the extracts of duckweed exposed to increasing salt stress, the fresh weight of duckweed was collected after 14 days of cultivation and immediately ground to fine powder in liquid nitrogen as described in our previous work [17]. The ensuing extracts were sonicated for 1 h with cooling in ice and centrifuged, and the supernatants were filtered through a syringe with a filter pore size of $0.2 \mu\text{m}$ immediately before HPLC–Orbitrap-MS analysis.

4.2.2. HPLC Parameters

An analytical Hypersil GOLD™ C18 column (Thermo Fisher Scientific, Waltham, MA, USA; 50×2.1 mm, $1.9 \mu\text{m}$ particle size) was used. Injection volume was $5 \mu\text{L}$ with a constant flow rate of $300 \mu\text{L}/\text{min}$. Mobile phases: ultra-pure water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were eluted in the following ratios: 5% B at minute 1; 5–95% B from minute 1 to 10; 95% B from minute 10 to 12; and 5% B until minute 15.

4.2.3. Orbitrap Parameters

The Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific) was equipped with an electrospray ionization (ESI) source in negative ionization mode. Full MS scans were performed in the 100–1500 m/z range with a resolution of 60,000 FWHM. Data-dependent acquisition (DDA) MS2 experiments were conducted at a resolution of 15,000 FWHM with a normalized collision energy (CID) of 35%. Dynamic exclusion was set to 10 s, with exclusion after two scans, and the intensity threshold was set at 1×10^5 .

4.3. Green Synthesis of ZnO Duckweed-Derived Nanoparticles

Duckweed was collected and oven-dried at 60 °C until a constant dry weight (DW) was achieved. The DW was ground in liquid nitrogen with a mortar and pestle. Ethanol solution (80%) was added as an extractant in a 1:20 ratio ($v:v$). The ensuing extract was heated at 60 °C for 20 min and gently mixed. After cooling, the extract was used in green synthesis according to [1]. Briefly, a 0.5 M solution of zinc chloride (ZnCl_2) was prepared. The plant extract was added dropwise to the ZnCl_2 solution at a 1:2.5 ratio ($v:v$). The pH of the mixture was adjusted

to 9.0 by the gradual addition of 1 M NaOH solution. The flask was heated to 70 °C on a magnetic stirrer with a constant stirring and heating option. The appearance of a pale yellow to pale yellow-green precipitate indicated the formation of ZnO nanoparticles.

Post-Processing

The precipitate from the previous step was sequentially centrifuged and washed 3 times and then sonicated for 20 min at 40% frequency in an ultrasonic water bath (Bandelin Sonorex Digiplus, Bandelin GmbH, Berlin, Germany). The resulting sediment was oven-dried at +60 °C for 15 h in glass Petri dishes. After the sediment was dried, thermal processing was performed at 150 °C for 2 h in a drying oven (Memmert GmbH, Schwabach, Germany). The dry sediment was then ground to a fine powder with a mortar and pestle and kept in airtight containers in the dark for further analysis.

4.4. X-Ray Diffraction (XRD) of Powdered ZnO Duckweed-Derived Nanoparticles

To determine the phase purity and crystallinity of synthesized nanoparticles, X-ray diffraction (XRD) on powdered material was performed on a Rigaku SmartLab diffractometer (Rigaku Holdings Corporation, Tokyo, Japan) at the Laboratory for Crystallography of the Faculty of Mining and Geology, University of Belgrade. Zinc oxide nanoparticles were synthesized using extracts of duckweed (*Lemna minor*) cultivated under varying NaCl concentrations: 0 mM, 10 mM, and 100 mM. The crystalline phases of the resulting powders were analyzed by powder X-ray diffraction (PXRD) using CuK α roentgen radiation ($\lambda = 1.54178 \text{ \AA}$) emitted from a copper anticathode over a 2θ range of 2–70°, with a step size of 0.01° and a scan rate of 5°/min. The working voltage (U) was 40 kV, with the current intensity (I) of 30 mA. The obtained data of diffraction maxima 2θ (°), the interplanar distances d_{hkl} (Å) for all three hkl reflections, and the appropriate relative intensities I/I_{max} were presented graphically. The crystalline phases were identified based on the acquired values of I/I_{max} , the interplanar distances d and the comparison with literature data and the ICDD database.

4.5. Scanning Electron Microscopy and Energy-Dispersive X-Ray Spectroscopy (SEM–EDS)

The morphology and elemental composition of the synthesized ZnO samples were examined using a Hitachi S-3700N scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan) equipped with a secondary electron (SE), a backscattered electron (BSE), and energy-dispersive X-ray (EDS) detectors. Elemental analysis was performed using a Quantax 200 EDS system (Bruker, Mannheim, Germany) with an energy resolution of 123 eV at Mn K α . SEM observations were conducted under variable-pressure conditions to minimize the charging effects of the non-conductive powder samples. The EDS measurements were used for the semi-quantitative determination of the elemental composition and distribution within the analyzed samples.

4.6. UV–Vis Characterization of ZnO Duckweed-Derived Nanoparticles

The nanomaterial was resuspended in double-distilled water. Absorbance was measured across the wavelength range of 300–600 nm with a UV–Vis Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in a 1400 μL Suprasil quartz cuvette (Hellma GmbH & Co. KG, Müllheim, Germany). Multiple spectra (at least 3 per sample) were read, and optical characteristics were monitored. From the UV–Vis spectra, the Tauc plots were deduced. The Tauc method is based on the equation [51]:

$$(\alpha \times h\nu)^{\frac{1}{n}} = B \times (h\nu - E_g) \quad (1)$$

where h is the Planck constant, ν is the photon's frequency, E_g is the apparent band gap energy, and B is a constant. The γ factor depends on the nature of the electron transition and is equal to 1/2 or 2 for the direct and indirect transition band gaps, respectively.

4.7. Total Polyphenol Content (TPC) and DPPH Method (Antioxidant Capacity) on ZnO Duckweed-Derived Nanoparticles

The methods outlined in our previous work were used to determine the total polyphenol content (TPC) and antioxidant capacity of ZnO nanoparticles [17]. Briefly, the quantification of TPC was conducted based on Folin–Ciocalteu principles, whereas antioxidant capacity was determined with the use of the DPPH (1,1-diphenyl-2-picrylhydrazyl) technique. For TPC, the absorbance was measured at 765 nm. Gallic acid was used as a standard. For DPPH, the same nanoparticle solutions used for the FC test were mixed with DPPH reagent and dissolved in methanol. The samples were incubated at room temperature in the dark alongside controls (reaction mixture without DPPH, to account for the relatively high absorbance of nanoparticles around 520 nm), and absorbance was measured at 520 nm. The scavenging ability of DPPH radical was calculated as:

$$\text{Inhibition (\%)} = 1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}) \times 100 \quad (2)$$

4.8. Antimicrobial Activity of ZnO Duckweed-Derived Nanoparticles (Well Diffusion Test)

For the well diffusion test, fresh overnight cultures of bacterial strains *Staphylococcus haemolyticus* D4-2-100/1 and *Serratia fonticola* A3-102/3 from our previous work were used for the test and cultivated as reported before [17,53]. The wells were created with sterile 1 mL pipette tips and filled with 50 μ L of gradually increasing concentrations of ZnO NP solutions (12.5, 25, and 50 mg/mL). Sterile double-distilled water was used as a negative control. Ciprofloxacin (Marocen, Hemofarm, Vršac, Serbia) was used as a positive control. The plates were incubated at +37 °C for 24 h. The halo zones were measured in *ImageJ* Version 1.53K [54] as an indication of the inhibitory effect of the antibiotic and the nanomaterial.

4.9. Statistical Analysis and Graphic Presentation of Results

A one-way ANOVA test ($p < 0.05$) was performed in STATISTICA software, Version 7, with a post hoc Fisher LSD test for antimicrobial and DPPH activity and the FC test. For the principal component analysis (PCA) and the heatmap, the open-source statistical software R, version 4.5.1 [55], was used. PCA and construction of a vector plot of variables were performed using the FactoMineR package [56]. Additionally, the Factoextra package was used for data clustering and biplot representation of data [57,58]. Heatmap analysis was performed with the ComplexHeatmap package, as log10 values of means from each treatment and result were clustered based on change in parameter values [59]. For distance clustering, the “maximum distance” method was used, in which the distance between two clusters is determined by finding the single pair of points (one from each cluster) that are farthest apart and using that distance as the measure.

5. Conclusions

This observational study provides preliminary evidence that the physiological state of *Lemna minor* during cultivation may influence both the phytochemical composition of plant extracts and certain properties of green-synthesized ZnO nanoparticles. Orbitrap analysis revealed salinity-dependent shifts in the metabolite profile of duckweed, including increased abundance of several flavonoids, glycosylated flavones, and hydroxycinnamic acid derivatives under salt stress. These changes were accompanied by differences in the characteristics of the resulting nanomaterials.

UV–Vis spectroscopy, PXRD, SEM–EDS, and Raman spectroscopy collectively support the formation of ZnO nanoparticles using duckweed extracts cultivated under 0, 10, and 100 mM NaCl. PXRD analysis indicated the predominance of crystalline hexagonal wurtzite ZnO in all samples, while SEM observations revealed micron-scale agglomerates composed of finer submicron structures. EDS spectra confirmed Zn-rich materials with oxygen-associated signals consistent with ZnO formation. Raman spectra were dominated by fluorescence, which increased with salinity treatment and may reflect differences in surface-associated phytochemical species among the samples. Taken together, the available data tentatively suggest that salinity had a limited effect on the crystalline structure of the nanoparticles but may have influenced their surface chemistry through changes in extract composition. However, the extent to which these phytochemical differences contribute to the observed antioxidant and antimicrobial activities remains unclear. Additional studies are needed to distinguish between the effects of nanoparticle structure, surface-bound phytochemicals, and potential synergistic interactions between them.

The biological properties of the nanoparticles also appeared to vary with the cultivation conditions of the source biomass. Nanoparticles synthesized using extracts from salt-stressed duckweed exhibited higher total phenolic content, antioxidant capacity, and antimicrobial activity against *Staphylococcus haemolyticus*. While the underlying mechanisms remain to be fully elucidated, these observations are consistent with the possibility that salinity-induced metabolic changes in duckweed contributed to the biological properties of the resulting nanomaterials.

Overall, the results suggest that environmental manipulation of plant biomass may represent a promising approach for influencing green nanoparticle synthesis. By integrating plant stress physiology, metabolomic profiling, and nanomaterial characterization, this study highlights potential links between cultivation conditions and nanoparticle properties, while also emphasizing the need for further investigations to establish the mechanistic basis of these relationships.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules31132326/s1>, Figure S1A–D: Histograms representing influence of phenolic compounds on PC1 and PC2, which form coordinates for principal component analysis (PCA) plot of 30 phenolic compounds isolated from duckweed grown at 0 mM NaCl, 10 mM NaCl, and 100 mM NaCl (A); at 0 mM (B); at 10 mM (C); at 100 mM (D); Figure S2: Raman spectroscopy of three green ZnO nanomaterials.

Author Contributions: Conceptualization, O.R. and N.S.; methodology, F.N., O.R., N.S., and A.M.; software, N.S. and F.N.; validation, D.A.R., D.P. and M.M.; formal analysis, N.S. and O.R.; investigation, A.M., D.A.R., D.P. and M.M.; resources, O.R.; data curation, D.A.R., D.P. and M.M.; writing—original draft preparation, O.R., N.S. and F.N.; writing—review and editing, N.S., D.A.R., D.P. and M.M.; visualization, O.R. and N.S.; supervision, O.R.; project administration, D.A.R., D.P., M.M., and O.R.; funding acquisition, O.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia. Contract No.: 451-03-33/2026-03/200007 (valid from 11 February 2026). The APC was funded by the Institute for Biological Research “Siniša Stanković”—the National Institute of the Republic of Serbia.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author due to technical reasons.

Acknowledgments: The authors thank Sabina Kovač for performing the X-ray diffraction measurements and for her valuable technical assistance; Joy Desor and Mineralanalytik for SEM-EDS and Raman, Angelina Subotić for her unwavering support during the preparation of the manuscript, and Milena Dojčinović for critically reviewing the manuscript and providing constructive feedback.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

ZnO NPs	Zinc-oxide nanoparticles
FTIR	Fourier transform infrared spectroscopy
SEM/TEM	Scanning electron microscopy / transmission electron microscopy
EDS	Energy-dispersive X-ray spectroscopy
XPS	X-ray photoelectron spectroscopy
BET	Brunauer–Emmett–Teller analysis

References

- Shukla, Y.K.; Pandey, P.; Pandey, J.P.; Shukla, A.; Kumar, J. Duckweed Extract-Mediated Green Synthesis of ZnO Nanoparticles and Its Antibacterial, Antioxidant, and Photocatalytic Properties. *Biomass Convers. Biorefin.* **2025**, *15*, 21477–21491. [CrossRef]
- De Mel, S.; Gruenler, J.; Khoury, L.; Heynes, A.; Fazekas, J.; Damaske, K.; Galbadage, T.; Gunasekera, R.S.; Anderson, R.S. Green Synthesis of Silver Nanoparticles Using Magnolia Alba Leaf Extracts and Evaluating Their Antimicrobial, Anticancer, Antioxidant, and Photocatalytic Properties. *Sci. Rep.* **2025**, *15*, 23709. [CrossRef] [PubMed]
- Jaison, J.P.; Balasubramanian, B.; Gangwar, J.; James, N.; Pappuswamy, M.; Anand, A.V.; Al-Dhabi, N.A.; Valan Arasu, M.; Liu, W.C.; Sebastian, J.K. Green Synthesis of Bioinspired Nanoparticles Mediated from Plant Extracts of Asteraceae Family for Potential Biological Applications. *Antibiotics* **2023**, *12*, 543. [CrossRef] [PubMed]
- Suresh, D.; Nethravathi, P.C.; Udayabhanu; Rajanaika, H.; Nagabhushana, H.; Sharma, S.C. Green Synthesis of Multifunctional Zinc Oxide (ZnO) Nanoparticles Using *Cassia fistula* Plant Extract and Their Photodegradative, Antioxidant and Antibacterial Activities. *Mater. Sci. Semicond. Process* **2015**, *31*, 446–454. [CrossRef]
- Alyamani, A.A.; Albukhaty, S.; Aloufi, S.; Almalki, F.A.; Al-Karagoly, H.; Sulaiman, G.M. Green Fabrication of Zinc Oxide Nanoparticles Using *Phlomis* Leaf Extract: Characterization and in vitro Evaluation of Cytotoxicity and Antibacterial Properties. *Molecules* **2021**, *26*, 6140. [CrossRef] [PubMed]
- Al-Dhabi, N.A.; Arasu, M.V. Environmentally-Friendly Green Approach for the Production of Zinc Oxide Nanoparticles and Their Anti-Fungal, Ovicidal, and Larvicidal Properties. *Nanomaterials* **2018**, *8*, 500. [CrossRef] [PubMed]
- Sivalingam, A.M. Green Synthesis of *Avicennia marina*-Derived ZnO Nanoparticles in vitro Antioxidant and Antimicrobial Activity, Drug Genotoxic Effects on MCF-7 Cells. *Inorg. Chem. Commun.* **2026**, *183*, 115767. [CrossRef]
- Abdel-Megeed, A.; Abdel-Rheim, K.H.M.; Kordy, A.M.A.; El-Ghannam, S.A.; Salem, M.Z.M.; Adel, M.M. Duckweed (*Lemna minor*) Biomass Aqueous Extract-Mediated Synthesis of Silver Nanoparticles as a Novel Formulation Era for Long-Term Efficacy against *Tribolium castaneum* and *Sitophilus oryzae* Adults on Wheat Seeds. *Biomass Convers. Biorefin.* **2024**, *15*, 5429–5446. [CrossRef]
- Gupta, S.; Das, A.; Ganguli, K.; Chakraborty, N.; Fayeizadeh, M.R.; Sil, S.K.; Adak, M.K.; Hasanuzzaman, M. Salinity-Responsive Hyperaccumulation of Flavonoids in *Spirodela polyrrhiza*, Resultant Maneuvering in the Structure and Antimicrobial as Well as Azo Dye Decontamination Profile of Biofabricated Zinc Oxide Nanoentities. *Sci. Rep.* **2024**, *14*, 24554. [CrossRef] [PubMed]
- de Moraes, M.B.; Barbosa-Neto, A.G.; Willadino, L.; Ulisses, C.; Calsa Junior, T. Salt Stress Induces Increase in Starch Accumulation in Duckweed (*Lemna aequinoctialis*, Lemnaceae): Biochemical and Physiological Aspects. *J. Plant Growth Regul.* **2019**, *38*, 683–700. [CrossRef]
- Appenroth, K.J.; Ziegler, P.; Sree, K.S. Accumulation of Starch in Duckweeds (*Lemnaceae*), Potential Energy Plants. *Physiol. Mol. Biol. Plants* **2021**, *27*, 2621–2633. [CrossRef] [PubMed]
- Ziegler, P.; Appenroth, K.J.; Sree, K.S. Survival Strategies of Duckweeds, the World's Smallest Angiosperms. *Plants* **2023**, *12*, 2215. [CrossRef] [PubMed]
- Valtsifer, V.A.; Sivtseva, A.V.; Kondrashova, N.B.; Shamsutdinov, A.S.; Averkina, A.S.; Valtsifer, I.V.; Feklistova, I.N.; Strelnikov, V.N. Influence of Synthesis Conditions on the Properties of Zinc Oxide Obtained in the Presence of Nonionic Structure-Forming Compounds. *Nanomaterials* **2023**, *13*, 2537. [CrossRef] [PubMed]
- Acosta, K.; Sree, K.S.; Okamoto, N.; Koseki, K.; Sorrels, S.; Jahreis, G.; Watanabe, F.; Appenroth, K.J.; Lam, E. Source of Vitamin B12 in Plants of the Lemnaceae Family and Its Production by Duckweed-Associated Bacteria. *J. Food Compos. Anal.* **2024**, *135*, 106603. [CrossRef]
- Ullah, H.; Gul, B.; Khan, H.; Zeb, U. Effect of Salt Stress on Proximate Composition of Duckweed (*Lemna minor* L.). *Heliyon* **2021**, *7*, e07399. [CrossRef] [PubMed]

16. Moalwi, A.; Kamat, K.; Muddapur, U.M.; Aldoah, B.; AlWadai, H.H.; Alamri, A.M.; Alrashid, F.F.; Alsareii, S.A.; Mahnashi, M.H.; Shaikh, I.A.; et al. Green Synthesis of Zinc Oxide Nanoparticles from *Wodyetia bifurcata* Fruit Peel Extract: Multifaceted Potential in Wound Healing, Antimicrobial, Antioxidant, and Anticancer Applications. *Front. Pharmacol.* **2024**, *15*, 1435222. [CrossRef] [PubMed]
17. Popržen, T.; Antonić Reljin, D.; Uzelac, B.; Milovančević, M.; Paunović, D.; Trifunović-Momčilov, M.; Marković, M.; Raspor, M.; Nikolić, I.; Stanković, S.; et al. *Pseudomonas oryzae* D1-104/3 and *P. gessardii* C31-106/3 Differentially Modulate the Antioxidative Response of Duckweed (*Lemna minor* L.) to Salt Stress. *Front. Microbiol.* **2024**, *15*, 1481437. [CrossRef] [PubMed]
18. Mandal, S.M.; Chakraborty, D.; Dey, S.; Mandal, S.M.; Chakraborty, D.; Dey, S. Phenolic Acids Act as Signaling Molecules in Plant-Microbe Symbioses. *Plant Signal. Behav.* **2017**, *2324*, 359–368. [CrossRef] [PubMed]
19. Tekin, V.; Kozgus Guldu, O.; Dervis, E.; Yurt Kilcar, A.; Uygur, E.; Biber Muftuler, F.Z. Green Synthesis of Silver Nanoparticles by Using Eugenol and Evaluation of Antimicrobial Potential. *Appl. Organomet. Chem.* **2019**, *33*, e4969. [CrossRef]
20. Faisal, S.; Shah, S.A.; Shah, S.; Akbar, M.T.; Jan, F.; Haq, I.; Baber, M.E.; Aman, K.; Zahir, F.; Bibi, F.; et al. In vitro Biomedical and Photo-Catalytic Application of Bio-Inspired *Zingiber officinale* Mediated Silver Nanoparticles. *J. Biomed. Nanotechnol.* **2020**, *16*, 492–504. [CrossRef] [PubMed]
21. Kesbi, B.; Salhi, N.; Khane, Y.; Albukhaty, S.; Addad, A.; Abideen, Z.; Alsufyani, H.; AlMalki, F.A. Potential Effect of Phyto-Synthesized Silver Nanoparticles Using *Cotula cinerea* Raw Extract on Salt Tolerance of Wheat Seeds (*Triticum durum* Desf, Boussellam Variety) Germination. *Sci. Rep.* **2025**, *15*, 28061. [CrossRef] [PubMed]
22. Nhu, V.T.T.; Dat, N.D.; Tam, L.M.; Phuong, N.H. Green Synthesis of Zinc Oxide Nanoparticles toward Highly Efficient Photocatalysis and Antibacterial Application. *Beilstein J. Nanotechnol.* **2022**, *13*, 1108–1119. [CrossRef] [PubMed]
23. Wang, Q.; Xu, S.; Zhong, L.; Zhao, X.; Wang, L. Effects of Zinc Oxide Nanoparticles on Growth, Development, and Flavonoid Synthesis in *Ginkgo biloba*. *Int. J. Mol. Sci.* **2023**, *24*, 15775. [CrossRef] [PubMed]
24. Lail, N.U.; Sattar, A.; Omer, M.O.; Hafeez, M.A.; Khalid, A.R.; Mahmood, S.; Shabbir, M.A.; Ahmed, W.; Aleem, M.T.; Alouffi, A.; et al. Biosynthesis and Characterization of Zinc Oxide Nanoparticles Using *Nigella sativa* against Coccidiosis in Commercial Poultry. *Sci. Rep.* **2023**, *13*, 6568. [CrossRef] [PubMed]
25. Abomuti, M.A.; Danish, E.Y.; Firoz, A.; Hasan, N.; Malik, M.A. Green Synthesis of Zinc Oxide Nanoparticles Using *Salvia officinalis* Leaf Extract and Their Photocatalytic and Antifungal Activities. *Biology* **2021**, *10*, 1075. [CrossRef] [PubMed]
26. Tran, I.T.; Heiman, J.A.; Lydy, V.R.; Kisson, L.T. Silver Inhibits *Lemna minor* Growth at High Initial Frond Densities. *Plants* **2023**, *12*, 1104. [CrossRef] [PubMed]
27. Schoofs, H.; Schmit, J.; Rink, L. Zinc Toxicity: Understanding the Limits. *Molecules* **2024**, *29*, 3130. [CrossRef] [PubMed]
28. Singh, D.K.; Pandey, D.K.; Yadav, R.R.; Singh, D. A Study of Nanosized Zinc Oxide and Its Nanofluid. *Pramana J. Phys.* **2012**, *78*, 759–766. [CrossRef]
29. Rani, S.; Kumar, P.; Dahiya, P.; Dang, A.S.; Suneja, P. Biogenic Synthesis of Zinc Nanoparticles, Their Applications, and Toxicity Prospects. *Front. Microbiol.* **2022**, *13*, 824427. [CrossRef] [PubMed]
30. Panda, K.K.; Golari, D.; Venugopal, A.; Achary, V.M.M.; Phaomei, G.; Parinandi, N.L.; Sahu, H.K.; Panda, B.B. Green Synthesized Zinc Oxide (ZnO) Nanoparticles Induce Oxidative Stress and DNA Damage in *Lathyrus sativus* L. Root Bioassay System. *Antioxidants* **2017**, *6*, 45. [CrossRef] [PubMed]
31. Khalafi, T.; Buazar, F.; Ghanemi, K. Phycosynthesis and Enhanced Photocatalytic Activity of Zinc Oxide Nanoparticles Toward Organosulfur Pollutants. *Sci. Rep.* **2019**, *9*, 6866. [CrossRef] [PubMed]
32. Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D.L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y.; et al. Discovery, Research, and Development of New Antibiotics: The WHO Priority List of Antibiotic-Resistant Bacteria and Tuberculosis. *Lancet Infect. Dis.* **2018**, *18*, 318–327. [CrossRef] [PubMed]
33. Krause, J.; Strack, D. High-Performance Liquid Chromatographic Separation of Glucose Esters and Quinic Acid Esters of Hydroxycinnamic Acids. *J. Chromatogr.* **1979**, *176*, 465–469. [CrossRef]
34. Franz, G.; Grün, M. Chemistry, Occurrence and Biosynthesis of C-Glycosyl Compounds in Plants. *J. Med. Plant Res.* **1983**, *47*, 131–140. [CrossRef] [PubMed]
35. Wahman, R.; Moser, S.; Bieber, S.; Cruzeiro, C.; Schröder, P.; Gilg, A.; Lesske, F.; Letzel, T. Untargeted Analysis of *Lemna minor* Metabolites: Workflow and Prioritization Strategy Comparing Highly Confident Features between Different Mass Spectrometers. *Metabolites* **2021**, *11*, 832. [CrossRef] [PubMed]
36. Wahman, R.; Graßmann, J.; Sauvêtre, A.; Schröder, P.; Letzel, T. *Lemna minor* Studies under Various Storage Periods Using Extended-Polarity Extraction and Metabolite Non-Target Screening Analysis. *J. Pharm. Biomed. Anal.* **2020**, *188*, 113362. [CrossRef] [PubMed]
37. Guo, L.; Fang, Y.; Wang, S.; Xiao, Y.; Ding, Y.; Jin, Y.; Tian, X.; Du, A.; Liao, Z.; He, K.; et al. Duckweed: A Starch-Hyperaccumulating Plant under Cultivation with a Combination of Nutrient Limitation and Elevated CO₂. *Front. Plant Sci.* **2025**, *16*, 1531849. [CrossRef] [PubMed]

38. Sree, K.S.; Appenroth, K.J. Starch Accumulation in Duckweeds (*Lemnaceae*) Induced by Nutrient Deficiency. *Emir. J. Food Agric.* **2022**, *34*, 204–212. [CrossRef]
39. Radulović, O.; Gašić, U.; Marković, M. Phenolic Compounds as Biomarkers of Interactions between the Endophyte *Klebsiella oxytoca* and the Common Duckweed, *Lemna minor* L. *Biol. Life Sci. Forum* **2022**, *11*, 52. [CrossRef]
40. Gebrehiwot, G.; Gebremichael, T. An Insight into the Production of Bioethanol from the Duckweed. *J. Catal. Catal.* **2020**, *7*, 48–56.
41. Xu, J.; Cui, W.; Cheng, J.J.; Stomp, A.M. Production of High-Starch Duckweed and Its Conversion to Bioethanol. *Biosyst. Eng.* **2011**, *110*, 67–72. [CrossRef]
42. Appenroth, K.J.; Sree, K.S.; Böhm, V.; Hammann, S.; Vetter, W.; Leiterer, M.; Jahreis, G. Nutritional Value of Duckweeds (*Lemnaceae*) as Human Food. *Food Chem.* **2017**, *217*, 266–273. [CrossRef] [PubMed]
43. Pagliuso, D.; Palacios Jara, C.E.; Grandis, A.; Lam, E.; Pena Ferreira, M.J.; Buckeridge, M.S. Flavonoids from Duckweeds: Potential Applications in the Human Diet. *RSC Adv.* **2020**, *10*, 44981–44988. [CrossRef] [PubMed]
44. Baek, G.Y.; Saeed, M.; Choi, H.K. Duckweeds: Their Utilization, Metabolites and Cultivation. *Appl. Biol. Chem.* **2021**, *64*, 73. [CrossRef] [PubMed]
45. Nabil, M.; Perez-Quintana, I.V.; Acosta, M.; Mendez-Gamboa, J.A.; Castro-Rodriguez, R. Morphological, Structural, and Optical Bandgap Characterization of Extracted ZnO Nanoparticles from Commercial Paste. *Adv. Mat. Sci. Eng.* **2021**, *2021*, 9926544. [CrossRef]
46. Matinise, N.; Fuku, X.G.; Kaviyarasu, K.; Mayedwa, N.; Maaza, M. ZnO Nanoparticles via *Moringa oleifera* Green Synthesis: Physical Properties & Mechanism of Formation. *Appl. Surf. Sci.* **2017**, *406*, 339–347. [CrossRef]
47. Edo, G.I.; Mafe, A.N.; Ali, A.B.M.; Akpogheli, P.O.; Yousif, E.; Isoje, E.F.; Igbuku, U.A.; Zainulabdeen, K.; Owhero, J.O.; Essaghah, A.E.A.; et al. Eco-Friendly Nanoparticle Phytosynthesis via Plant Extracts: Mechanistic Insights, Recent Advances, and Multifaceted Uses. *Nano TransMed* **2025**, *4*, 100080. [CrossRef]
48. Ekennia, A.; Uduagwu, D.; Olowu, O.; Nwanji, O.; Oje, O.; Daniel, B.; Mgbii, S.; Emma-Uba, C. Biosynthesis of Zinc Oxide Nanoparticles Using Leaf Extracts of *Alchornea laxiflora* and Its Tyrosinase Inhibition and Catalytic Studies. *Micron* **2021**, *141*, 102964. [CrossRef] [PubMed]
49. Dominiak, B.; Rosowska, J.; Wal, A.; Majewska, A.; Witkowski, B.S.; Wachnicki, Ł.; Kaszewski, J.; Słowska, A.; Cymerys, J.; Gralak, M.A.; et al. Zinc Oxide Nanoparticles Affect the Genomic and Redox Status of Chicken Embryo—Influence of Shape. *Nanomaterials* **2025**, *15*, 1412. [CrossRef] [PubMed]
50. Marković, S.; Stanković, A.; Dostanić, J.; Veselinović, L.; Mančić, L.; Škapin, S.D.; Dražić, G.; Janković-Častvan, I.; Uskoković, D. Simultaneous enhancement of natural sunlight and artificial UV-driven photocatalytic activity of a mechanically activated ZnO/SnO₂ composite. *RSC Adv.* **2017**, *7*, 42725–42737. [CrossRef]
51. Marković, S.; Stojković Simatović, I.; Ahmetović, S.; Veselinović, L.; Stojadinović, S.; Rac, V.; Škapin, S.D.; Bogdanović, D.B.; Častvan, I.J.; Uskoković, D. Surfactant-assisted microwave processing of ZnO particles: A simple way for designing the surface-to-bulk defect ratio and improving photo(electro)catalytic properties. *RSC Adv.* **2019**, *9*, 17165–17178. [CrossRef] [PubMed]
52. Takó, M.; Kerekes, E.B.; Zambrano, C.; Kotogán, A.; Papp, T.; Krisch, J.; Vágvölgyi, C. Plant Phenolics and Phenolic-Enriched Extracts as Antimicrobial Agents against Food-Contaminating Microorganisms. *Antioxidants* **2020**, *9*, 165. [CrossRef] [PubMed]
53. Makuła, P.; Pacia, M.; Macyk, W. How To Correctly Determine the Band Gap Energy of Modified Semiconductor Photocatalysts Based on UV-Vis Spectra. *J. Phys. Chem. Lett.* **2018**, *9*, 6814–6817. [CrossRef] [PubMed]
54. Radulović, O.; Petrić, M.; Raspor, M.; Stanojević, O.; Janakiev, T.; Tadić, V.; Stanković, S. Culture-Dependent Analysis of 16S rRNA Sequences Associated with the Rhizosphere of *Lemna minor* and Assessment of Bacterial Phenol-Resistance: Plant/Bacteria System for Potential Bioremediation—Part II. *Pol. J. Environ. Stud.* **2019**, *28*, 811–822. [CrossRef]
55. Rueden, C.T.; Schindelin, J.; Hiner, M.C.; DeZonia, B.E.; Walter, A.E.; Arena, E.T.; Eliceiri, K.W. ImageJ2: ImageJ for the next Generation of Scientific Image Data. *BMC Bioinform.* **2017**, *18*, 529. [CrossRef] [PubMed]
56. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2025; Available online: <https://www.R-project.org/> (accessed on 24 November 2025).
57. Lê, S.; Josse, J.; Rennes, A.; Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [CrossRef]
58. Kassambara, A.; Mundt, F. *Factoextra: Extract and Visualize the Results of Multivariate Data Analyses*, version 1.0.7; GESIS: Mannheim, Germany, 2020. Available online: <https://CRAN.R-project.org/package=factoextra> (accessed on 24 November 2025).
59. Gu, Z. Complex Heatmap Visualization. *iMeta* **2022**, *1*, e43. [CrossRef] [PubMed]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

MDPI AG
Grosspeteranlage 5
4052 Basel
Switzerland
Tel.: +41 61 683 77 34

Molecules Editorial Office
E-mail: molecules@mdpi.com
www.mdpi.com/journal/molecules



Disclaimer/Publisher's Note: The title and front matter of this reprint are at the discretion of the Guest Editor. The publisher is not responsible for their content or any associated concerns. The statements, opinions and data contained in all individual articles are solely those of the individual Editor and contributors and not of MDPI. MDPI disclaims responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



Academic Open
Access Publishing

mdpi.com

ISBN 978-3-7258-8720-0