

Special Issue Reprint

Organic Compounds with Biological Activity

Edited by
Enrique Domínguez-Álvarez and Małgorzata Anna Marć

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Organic Compounds with Biological Activity

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Guest Editors

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Guest Editors

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4052 Basel, Switzerland

This is a reprint of the Special Issue, published open access by the journal *Compounds* (ISSN 2673-6918), freely accessible at: https://www.mdpi.com/journal/compounds/special_issues/V489KW2208.

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.
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ISBN 978-3-7258-8847-4 (Hbk)

ISBN 978-3-7258-8848-1 (PDF)

<https://doi.org/10.3390/books978-3-7258-8848-1>

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About the Editors

Enrique Domínguez-Álvarez

Enrique Domínguez-Álvarez has been a CSIC Tenured Scientist at the Institute of General Organic Chemistry (IQOG) of the Spanish National Research Council (CSIC) in Madrid (Spain) since 2017, where he leads his own research group. His main interests are the synthesis, characterization and biological evaluation of selenocompounds as potential novel anticancer and antibacterial derivatives, preferably against resistant cancers and bacterial strains. As secondary research lines, he has participated in obtaining antifungal and antiparasitic compounds and has gained expertise in other fields such as nanoparticles, Alzheimer's disease, inhibition of multidrug resistant mechanisms, ADMETox studies, pharmacology, biochemical assays, voltammetry and nutraceuticals through his collaborations, PhD and previous postdocs or research stays in universities of Poland, Spain, Hungary, Germany and Russia. Currently, he is also serving as reviewer in more than 50 scientific journals and as Editorial Board Member in the journals *Compounds*, *Antibiotics* and *Heterocyclic Communications*.

Małgorzata Anna Marć

Małgorzata Anna Marć is currently doing an EU-funded Marie Skłodowska-Curie Postdoctoral Fellowship at the Cell Engineering Laboratory of La Paz University Hospital Health Research Institute (IdiPAZ) in Madrid (Spain). Her main interests are the study of the microbiota and the pharmacological ADMETox evaluation (Absorption, Distribution, Metabolism, Excretion and Toxicity) of strongly bioactive compounds. Her trajectory combines experience in both industry (Olimp Laboratories, Poland) and academy, where she has worked in leading research centres in Poland, Spain, Italy and Hungary. She has acquired a wide expertise in pharmacology, molecular biology, nutrition, bioengineering, drug interactions, biochemical assays, instrumental techniques and the assessment of physico-chemical properties of drug candidates. Regarding her Editorial experience, she has been a reviewer in more than 20 journals; she is serving as Early Career Editorial Board Member in the journal *Pharmaceutics* and as Guest Editor in the journals *Compounds* and *Antibiotics*.

Organic Compounds with Biological Activity

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1. Introduction

The Special Issue with the title ‘Organic Compounds with Biological Activity’ [1] was launched at the end 2022. It aimed to collect scientific articles submitted to the journal ‘Compounds’ that either explore the search of organic compounds with biological activity that can have applications in Medicine, Veterinary, Agriculture, and in the disinfection of tools, spaces, and surfaces, or review the biological activities or applications of chemical compounds in these fields.

2. Contributions

Researchers from Europe and from America have published articles in this Special Issue. Together, seven research articles and two reviews have been published, and this editorial is the tenth and last publication in this Special Issue. Of these ten publications, four articles were published by Brazilian researchers, and three by scientists from the United States. Finally, Argentina and Greece are also represented with one publication each (Spain as well if counting this editorial). These publications are summarized and grouped by activities and compound origin in Table 1.

Out of the seven research papers published in this Special Issue, three papers evaluate the biological activity of a series of synthetic compounds [2–4], and four works [5–8] evaluate the biological activity of natural products obtained from different plants. Among the synthetic compounds, two papers focus on the evaluation of specific compounds as anticancer agents, and the third, on the *in silico* determination of properties of previously reported antibacterial agents.

The first anticancer paper [2], written by O’Brien et al. (from the University of Alaska Fairbanks and Spokane University in USA), reports the synthesis, characterization, structure, and anticancer activity of a platinum organic complex stabilized with the nucleotide adenine, which was evaluated in the panel of 60 human tumor cell lines of the US National Cancer’s Institute Developmental Therapeutics Program.

The second article focuses on anticancer activities and describes the synthesis, characterization, and anticancer activity against the HepG2 cell line exerted by a quinaldehyde *o*-nitrobenzoyl hydrazone. The paper was prepared by Mossine et al. from the University of Missouri, USA [3].

The last contribution on synthetic compounds was prepared by Pooladian et al. [4] from the Southeast Missouri State University (USA). It reports *in silico* calculations of the reduction potential of a series of 1,4-di-*N*-oxide quinoxaline-2-carboxamide derivatives that were reported to have anti-tuberculosis activity in a previous work.

Table 1. List of the contributions in the Special Issue ‘Organic Compounds with Biological Activity’, grouped by the type of compound or article.

Type of Compound/Article	Title	Activity	Authors	Ref.
Synthetic compounds	Synthesis, Structure, and Anticancer Activity of a Dinuclear Organoplatinum(IV) Complex Stabilized by Adenine	Anticancer	A.M. O'Brien et al.	[2]
	Quinaldehyde o-Nitrobenzoylhydrazone: Structure and Sensitization of HepG2 Cells to Anti-Cancer Drugs	Anticancer	V.V. Mossine et al.	[3]
	Reduction Potential Predictions for Thirty-Seven 1,4-di-N-Oxide Quinoxaline-2-Carboxamide Derivatives with Anti-Tuberculosis Activity	Antibacterial	F. Pooladian et al.	[4]
Natural compounds	Identification of Constituents and Evaluation of Biological Activity of <i>Piptadenia stipulacea</i> (Benth.) Ducke Ethanol Extract	Anticancer and antibacterial	S.A.D.N.M. Silva et al.	[5]
	Protective Effect of Biflavones from <i>Platonia insignis</i> Mart. against Ethidium Bromide-Induced Lethality in <i>Staphylococcus aureus</i>	Cytoprotective	A.S.G. Moura et al.	[6]
	In Vitro Schistosomicidal Activity and Molecular Modeling of Quercitrin and Afzelin Isolated from the Leaves of <i>Copaifera oblongifolia</i>	Antiparasitic	R.C. Ramos et al.	[7]
	Novel Butyrylcholinesterase Inhibitor Alkaloids from <i>Cannabis sativa</i> Roots: Bioguided Isolation and In Silico Study	Anti Alzheimer disease	J.E. Ortiz et al.	[8]
Review articles	Therapeutic Capabilities of Triterpenes and Triterpenoids in Immune and Inflammatory Processes: A Review	Antiinflammatory	M. Mantiniotou et al.	[9]
	Formation and Characterization of Aroma Compounds in Camembert Cheese	Aroma compounds	B.F.D. Ramirez et al.	[10]

Compounds obtained from plants are the subject of the next four contributions to this Special Issue [5–8], and interestingly, all of them were developed in South America, in the countries of Brazil [5–7] and Argentina [8].

Of these works, the first was written by Silva and coauthors [5] from two Brazilian universities (Universidade Federal do Delta do Parnaíba and Universidade Federal do Piauí). This work intends to determine the flavonoid bioactive compounds extracted with ethanol from *Piptadenia stipulacea* and reports the anticancer, antioxidant, and antibacterial activity of these compounds.

Compounds from Brazilian flora were also studied in the work of Moura et al. [6] and colleagues from the Federal University of Piauí and Regional University of Cariri. They evaluate the cytoprotective effect exerted by biflavones obtained from *Platonia insignis*. In particular, these biflavones mitigate the cell damage exerted by ethidium bromide in a *Staphylococcus aureus* model.

A third study on compounds from Brazilian plants was prepared by Ramos et al. [7] from a team comprising researchers of the Universidade da Franca, Universidade de São Paulo, and the Universidade Federal do Espírito Santo. The authors evaluate the activity *in vitro* against *Schistosoma mansori* exerted by quercitrin and afzelin, two compounds obtained from the crude extract of *Copaifera oblongifolia*.

The last study based on natural compounds was performed by Ortiz et al. [8] and his colleagues from different Argentinian research centers (Universidad Nacional de San Juan, CONICET and Instituto Tecnología de Alimentos, Centro de Investigación de Agroindustria CIA-INTA). This work explores the inhibitory activity of spermidine alkaloids obtained from *Cannabis sativa* towards enzymes involved in Alzheimer’s disease development, such as the enzymes acetylcholinesterase and butyrylcholinesterase.

Finally, two reviews were published in this Special Issue. The first was written by Mantiniotou et al. [9] from the University of Thessaly in Greece. It revises the potential applications of triterpenes and triterpenoids, found in plant extracts, in the modulation of inflammatory and immune responses. The second review, prepared by Ramirez et al. [10],

from the University of Campinas in Brazil, focuses on the characterization of the volatile compounds responsible for the aroma of camembert cheese. It aims to explain how these compounds are formed during cheese production and how this formation can vary depending on certain conditions.

3. Conclusions and Final Remarks

These contributions provide a wide overview of the potential applications of bioactive synthetic compounds and natural compounds. Therefore, we thank all authors who have contributed to this Special Issue ‘Organic Compounds with Biological Activity’, for making this publication in *Compounds* possible, adding to the rich, interesting, interdisciplinary, and diverse compilation of works at the interface of Chemistry, Physics, Biology, and Pharmacology.

As a final point, we will take this opportunity to invite the entire scientific community to participate in the second edition of this Special Issue, titled ‘Organic Compounds with Biological Activity (2nd Edition)’ [11], through the submission of regular articles, communications, or review articles that fit its scope. We expect this new Special Issue to also be an interesting compilation of valuable and stimulating works that would be of interest for readers of *Compounds*.

Author Contributions: Writing—original draft preparation, E.D.-Á. and M.A.M.; writing—review and editing, E.D.-Á. and M.A.M. All authors have read and agreed to the published version of the manuscript.

Funding: Authors want to express their gratitude to the funding agencies Agencia Estatal de Investigación (MCIN/AEI/10.13039/501100011033, E.D.-Á.; and PTA2022-022277-I, M.A.M) and to “ERDF A way of making Europe”, by the European Union, grant PID2022-136438OB-I00 (E.D.A.).

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: We acknowledge the invaluable work of the reviewers who participated in the peer-review process of this Special Issue.

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

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Article

Synthesis, Structure, and Anticancer Activity of a Dinuclear Organoplatinum(IV) Complex Stabilized by Adenine

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Abstract: The dinuclear platinum(IV) compound $\{\text{Pt}(\text{CH}_3)_3\}_2(\mu\text{-I})_2(\mu\text{-adenine})$ (abbreviated Pt_2ad), obtained by treating cubic $[\text{Pt}^{\text{IV}}(\text{CH}_3)_3(\mu_3\text{-I})]_4$ with two equivalents of adenine, was isolated and structurally characterized by single crystal X-ray diffraction. The National Cancer Institute Developmental Therapeutics Program's in vitro sulforhodamine B assays showed Pt_2ad to be particularly cytotoxic against the central nervous system cancer cell line SF-539, and the human renal carcinoma cell line RXF-393. Furthermore, Pt_2ad displayed some degree of cytotoxicity against non-small cell lung cancer (NCI-H522), colon cancer (HCC-2998, HCT-116, HT29, and SW-620), melanoma (LOX-IMVI, Malme-3M, M14, MDA-MB-435, SK-MEL-28, and UACC-62), ovarian cancer (OVCAR-5), renal carcinoma (A498), and triple negative breast cancer (BT-549, MDA-MB-231, and MDA-MB-468) cells. Although anticancer studies involving some adenine platinum(II) compounds have been reported, this study marks the first assessment of the anticancer activity of an adenine platinum(IV) complex.

Keywords: cancer; platinum; chemotherapy; X-ray structures

1. Introduction

FDA-approved platinum-based chemotherapy agents such as cisplatin, carboplatin, and oxaliplatin are the clinical first-line standard of care for epithelial ovarian cancer [1] and non-small cell lung cancer [2,3] and often also play significant roles in treating other types of cancer [4]. The anticancer effectiveness arises from the interaction between the platinum compound and the cancerous DNA [5,6], although alternative mechanisms involving platinum–protein interactions also likely contribute to the cytotoxicity [7]. To facilitate interactions with DNA, some experimental platinum-based anticancer drugs have been fitted with flat aromatic ancillary ligands capable of engaging in π – π interactions with nucleobases [8–11]. These π – π interactions drive the intercalation of the platinum compound into the major or minor grooves of the DNA and bring the platinum center within the bonding proximity of the nucleobases.

In light of the significance of such π – π interactions, platinum compounds with nucleobase ligands would seem to be ideal anticancer agents, since the nucleobase ligand would naturally intercalate between base pair layers in the DNA. Indeed, various platinum(II) complexes of nucleotides [12], nucleosides [13–15], and nucleobase derivatives [16] have been reported to be highly lethal to different kinds of cancers. Several platinum(II) compounds containing adenosine [17,18] or an adenine derivative [19–30] as ancillary ligands have also been reported to be highly cytotoxic toward various cancer cell lines.

However, electronically and coordinatively unsaturated platinum(II) complexes undergo unwanted side reactions with the many Lewis base biomolecules in living systems, rendering such compounds unsafe for chemotherapy drugs. One innovative solution is to use electronically and coordinatively saturated platinum(IV) complexes, which tend to undergo fewer unwanted side reactions in living systems. Our group recently reported the cytotoxicity of octahedral $\text{Pt}^{\text{IV}}(\text{CH}_3)_2\text{I}_2\{2,2'\text{-bipyridine}\}$ toward the human breast cancer cell line ZR-75-1 [31]. This compound is easily prepared by treating $[\text{Pt}(\text{CH}_3)_2\text{I}_2]_x$ with 2,2'-bipyridine. Indeed, the Lewis acids $[\text{Pt}(\text{CH}_3)_2\text{X}_2]_x$ ($\text{X} = \text{Br}, \text{I}$) and cubic $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})_4]$ [32] can be treated with diverse Lewis base ligands to produce a variety of organoplatinum(IV) derivatives, which could display a broad spectrum of very different anticancer properties.

In this work, we report the synthesis, structural characterization, and in vitro anticancer activity of $[\text{Pt}(\text{CH}_3)_3]_2(\mu\text{-I})_2(\mu\text{-adenine})$, a compound prepared by treating $[\text{Pt}^{\text{IV}}(\text{CH}_3)_3(\mu_3\text{-I})_4]$ with adenine. Although several platinum(IV) complexes with adenine derivatives as ligands have been reported in the literature [33], none have been tested as anticancer drugs. This present study is the first assessment of the anticancer properties of an adenine platinum(IV) complex.

2. Materials and Methods

General Considerations. All ^1H , ^{13}C , and ^{195}Pt NMR spectra were obtained at room temperature on a Bruker Ascend 600 MHz FTNMR spectrometer running Topspin 3.6 at frequencies of 600.16 MHz, 150.91 MHz, and 129.015 MHz, respectively. ^1H and ^{13}C chemical shifts were reported in parts per million relative to SiMe_4 ($\delta = 0$) and were referenced internally with respect to the protic solvent impurity ($\delta = 3.31$ ppm for HD_2COD) or the ^{13}C resonances ($\delta = 49.15$ ppm for CD_3OD), respectively. ^{195}Pt NMR spectra were referenced externally to a solution of K_2PtCl_4 in D_2O ($\delta = -1620$ ppm) [34]. Infrared spectra were recorded as KBr pellets on a Nicolet Magna-IR 560 spectrometer. Elemental analyses were carried out by Atlantic Microlab, Inc. (Norcross, GA, USA). Unless otherwise noted, all reactions and manipulations were carried out in the presence of air. All reagents and solvents were obtained from commercial suppliers and were used without further purification. $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})_4]$ was prepared by a procedure (vide infra) modified from that of Clark and Manzer [32].

Synthesis of $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})_4]$. A solution consisting of $[1,5\text{-cyclooctadiene}]\text{Pt}(\text{CH}_3)_2$ (0.653 g, 1.96 mmol) in CH_3I (6 mL) in a glass bomb was stirred magnetically at 70°C for 4.5 h. The pale yellow homogeneous solution was cooled to room temperature, and pale yellow crystals precipitated. Yield = 0.370 g (51%). The crystals were spectroscopically identical to $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})_4]$ and were used without any further purification.

Synthesis of $[\text{fac-Pt}(\text{CH}_3)_3]_2(\mu\text{-I})_2(\mu\text{-adenine})$. A mixture of $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})_4]$ (0.311 g, 0.212 mmol) and adenine (0.058 g, 0.429 mmol) in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1 by volume, 10 mL) was stirred at 75°C for 4 h. The volatile components were removed in vacuo, and the residue was washed with CHCl_3 (2×5 mL), extracted into CH_3OH (10 mL), and crystallized from the CH_3OH solution at room temperature as a white powder (yield = 0.135 g). A second batch of the product was crystallized from CHCl_3 washes (yield = 0.156 g). Yield = 0.291 g (79%). Anal. Calc. for $\text{C}_{11}\text{H}_{23}\text{I}_2\text{N}_5\text{Pt}_2$: C, 15.20%; H, 2.67%; N, 8.06%. Found: C, 15.30%; H, 2.86%; N, 7.94%. ^1H NMR (CD_3OD , δ ppm): 1.396 ppm (s, 6 H, $^2J_{\text{Pt-H}} = 75$ Hz), 1.399 ppm (s, 6 H, $^2J_{\text{Pt-H}} = 75$ Hz), 1.68 ppm (s, 3 H, $^2J_{\text{Pt-H}} = 74$ Hz), 1.76 ppm (s, 3 H, $^2J_{\text{Pt-H}} = 74$ Hz), 8.22 ppm (s, 1 H, $^3J_{\text{Pt-H}} = 13$ Hz), 8.51 ppm (s, 1 H, $^3J_{\text{Pt-H}} = 10$ Hz). $^{13}\text{C}\{^1\text{H}\}$ NMR (CD_3OD , δ ppm): -8.04 ppm ($^1J_{\text{Pt-C}} = 659$ Hz), -7.16 ppm ($^1J_{\text{Pt-C}} = 661$ Hz), 10.19 ppm ($^1J_{\text{Pt-C}} = 708$ Hz), 11.68 ppm ($^1J_{\text{Pt-C}} = 718$ Hz), 112.4 ppm, 144.0 ppm, 154.3 ppm, 155.8 ppm, 157.0 ppm. $^{195}\text{Pt}\{^1\text{H}\}$ NMR (CD_3OD , δ ppm): -2863 ppm, -2929 ppm. IR

(KBr): 3442 (vs), 2963 (m), 2911 (m), 2898 (s), 2853 (m), 2816 (m), 1653 (s), 1560 (m), 1541 (m), 1509 (w), 1507 (w), 1472 (m), 1457 (m), 1397 (m), 1322 (w), 1266 (w), 1224 (m), 1109 (m), 1082 (m), 1028 (w), 911 (w), 789 (m), 732 (m), 668 (m), 611 (s), 560 (s), 467 (m).

X-ray Diffraction Studies. Pale yellow plates of $\{fac\text{-Pt}(\text{CH}_3)_3\}_2(\mu\text{-I})_2(\mu\text{-adenine})$ were crystallized by the slow addition of CHCl_3 (g) to a $\text{C}_2\text{H}_5\text{OH}$ solution at room temperature. X-ray intensity data were collected using a Bruker D8 Venture diffractometer equipped with a graphite monochromator and a Mo $\text{K}\alpha$ microfocus INCOATEC I μ s 3.0 sealed tube at 0.71073 Å. Data sets were corrected for Lorentz and polarization effects and absorption. The criterion for the observed reflections is $I > 2\sigma(I)$. Lattice parameters were determined from least squares analysis and reflection data. Empirical absorption corrections were applied using SADABS [35]. The structure was solved by direct methods and refined by full-matrix least squares analysis of F^2 using X-Seed [36] equipped with SHELXT [37]. All nonhydrogen atoms were refined anisotropically by full-matrix least squares on F^2 using the SHELXL [37] program. Hydrogen atoms were included in idealized geometric positions with $U_{\text{iso}} = 1.2 U_{\text{eq}}$ of the atom to which they are attached ($U_{\text{iso}} = 1.5 U_{\text{eq}}$ for methyl groups). The hydrogen atoms attached to nitrogen or oxygen were located in difference maps and assigned $1.2 \times U_{\text{eq}}$. The frames were integrated with the Bruker SAINT software package version 8.40 using a narrow-frame algorithm. The structure was solved and refined using the Bruker SHELXTL software package version 2018/2, and the cell data and refinement parameters are summarized in Table 1.

Table 1. Crystal and intensity collection data for $\{fac\text{-Pt}(\text{CH}_3)_3\}_2(\mu\text{-I})_2(\mu\text{-adenine}) \cdot \text{CHCl}_3 \cdot \text{CH}_3\text{CH}_2\text{OH}$.

formula	$\text{C}_{14}\text{H}_{30}\text{Cl}_3\text{I}_2\text{N}_5\text{OPt}_2$
molecular weight, g mol^{-1}	1034.76
temperature, K	100.00
wavelength, Å	0.71073
lattice	triclinic
space group	P_{-1}
cell constants	
a , Å	10.1669(9)
b , Å	10.5929(9)
c , Å	13.0266(11)
α , deg.	92.574(3)
β , deg.	109.885(3)
γ , deg.	103.182(4)
volume, Å ³	1272.97(19)
Z	2
ρ (calc.) g cm^{-3}	2.700
absorption coefficient, mm^{-1}	13.732
$F(000)$	940
crystal size, mm^3	$0.337 \times 0.189 \times 0.066$
θ range	1.992 to 33.778°

Table 1. Cont.

formula	$C_{14}H_{30}Cl_3I_2N_5OPt_2$	
index ranges	$-15 \leq h \leq 15$	
	$-16 \leq k \leq 16$	
	$-20 \leq l \leq 20$	
reflections collected	83,153	
independent reflections	10,210 [$R_{int} = 0.0328$]	
coverage, independent reflections	100%	
absorption correction	Multi scan	
max. and min. transmission	0.7467 and 0.3892	
refinement method	Full-matrix least-squares on F^2	
data/restraints/parameters	10,210/1/263	
goodness-of-fit on F^2	1.085	
final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0223$	$wR_2 = 0.0460$
R indices (all data)	$R_1 = 0.0282$	$wR_2 = 0.0475$
largest difference peak and hole	1.413 & −1.998 e. $\text{\AA}^{-3}$	

In vitro Sulforhodamine B Assays. In vitro assays involving sixty human tumor cell lines were carried out by staff members in the National Cancer Institute's Developmental Therapeutics Program following a standardized procedure [38–40].

3. Results

3.1. Synthesis and Spectroscopic Data

The new compound $\{fac\text{-Pt}(\text{CH}_3)_3\}_2(\mu\text{-I})_2(\mu\text{-adenine})$ (abbreviated Pt_2ad) was prepared by treating $[\text{Pt}(\text{CH}_3)_3(\mu_3\text{-I})]_4$ with two equivalents of adenine, as shown in Figure 1, and was isolated as an air-stable crystalline solid. The two platinum atoms in Pt_2ad were chemically inequivalent, so the $^{195}\text{Pt}\{^1\text{H}\}$ NMR spectrum featured two peaks at δ −2863 and −2929 ppm. Four sets of methyl peaks appeared with their ^{195}Pt satellites in the ^1H NMR spectrum at δ 1.396 ppm (6H, $^2J = 75$ Hz), 1.399 ppm (6H, $^2J = 75$ Hz), 1.68 ppm (3H, $^2J = 74$ Hz), and 1.76 ppm (3H, $^2J = 74$ Hz); similarly, the $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum revealed four sets of methyl groups with their ^{195}Pt satellites at δ −8.04 ppm ($^1J = 659$ Hz), −7.16 ppm ($^1J = 661$ Hz), 10.19 ppm ($^1J = 708$ Hz), and 11.68 ppm ($^1J = 718$ Hz). NMR and infrared spectra are included in the Supplementary Materials.

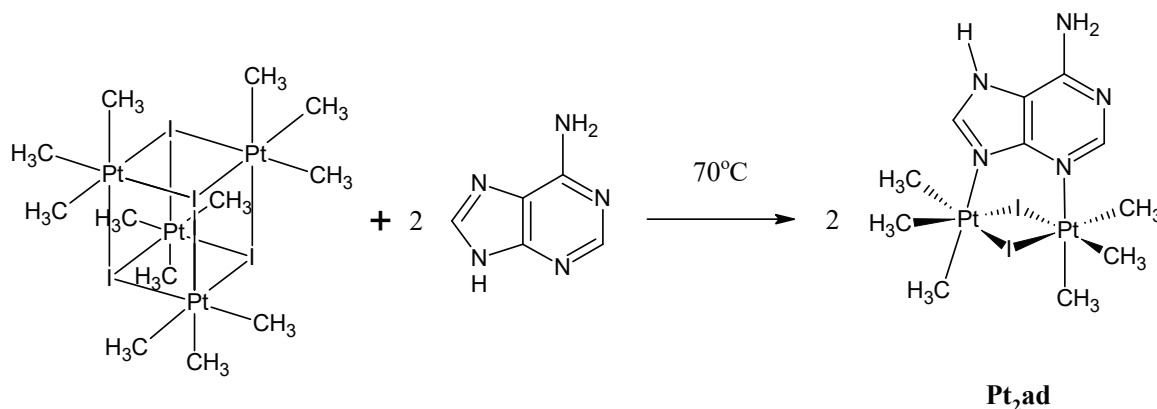


Figure 1. Synthesis of $\{fac\text{-Pt}(\text{CH}_3)_3\}_2(\mu\text{-I})_2(\mu\text{-adenine})$ (Pt_2ad).

3.2. Single Crystal X-Ray Structure

Pt₂ad was structurally characterized by single-crystal X-ray diffraction. Figure 2 shows a thermal ellipsoid plot (50% probability) with the nonhydrogen atoms labeled. Table 1 shows the crystal and intensity collection data, while Table 2 shows select metrical data.

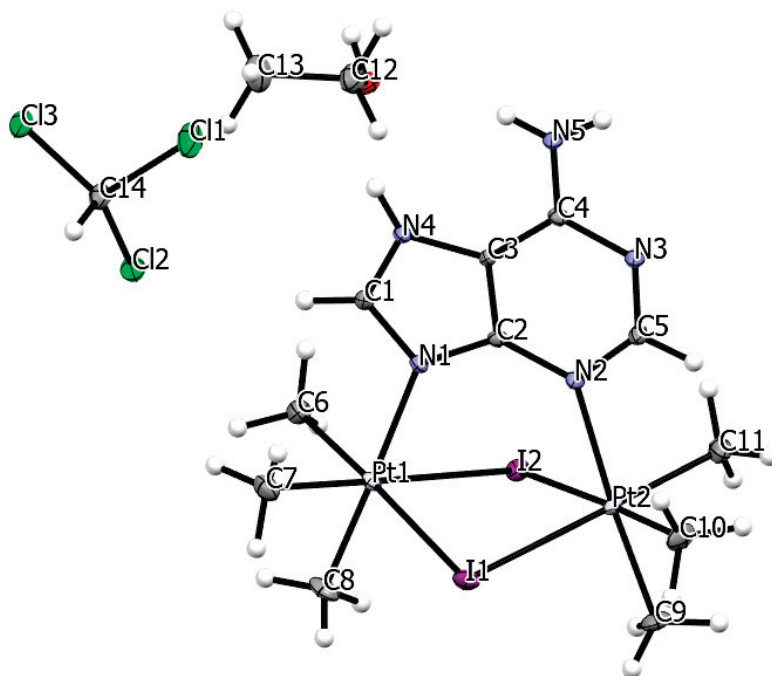


Figure 2. Thermal ellipsoid plot (50%) from the X-ray structure of $[fac\text{-Pt}(\text{CH}_3)_3]_2(\mu\text{-I})_2(\mu\text{-adenine}) \cdot \text{CHCl}_3 \cdot \text{CH}_3\text{CH}_2\text{OH}$.

Table 2. Selected metrical data.

Bond	Bond Length (Å)	Bond Angle	Degrees
Pt(1)–N(1)	2.203(2)	Pt(1)–I(1)–Pt(2)	87.111(9)
Pt(1)–I(1)	2.7548(3)	N(1)–Pt(1)–I(1)	89.22(6)
Pt(1)–I(2)	2.8004(3)	N(1)–Pt(1)–I(2)	87.14(6)
Pt(1)–C(6)	2.064(3)	N(1)–Pt(1)–C(6)	93.04(10)
Pt(1)–C(7)	2.055(3)	N(1)–Pt(1)–C(7)	91.37(10)
Pt(1)–C(8)	2.051(3)	N(1)–Pt(1)–C(8)	178.54(11)
Pt(1) ----- Pt(2)	3.805	C(6)–Pt(1)–I(1)	177.73(9)
Pt(2)–N(2)	2.207(2)	C(6)–Pt(1)–I(2)	91.30(8)
Pt(2)–I(1)	2.7672(3)	C(6)–Pt(1)–C(7)	89.70(12)
Pt(2)–I(2)	2.7887(3)	I(1)–Pt(1)–I(2)	88.976(8)
Pt(2)–C(10)	2.054(3)	Pt(2)–I(2)–Pt(1)	88.966(9)
Pt(2)–C(11)	2.053(3)	N(2)–Pt(2)–I(1)	89.78(6)
Pt(2)–C(9)	2.050(3)	N(2)–Pt(2)–I(2)	90.31(6)
		N(2)–Pt(2)–C(10)	91.23(10)
		N(2)–Pt(2)–C(11)	90.09(10)
		N(2)–Pt(2)–C(9)	176.21(10)
		C(10)–Pt(2)–I(1)	89.51(9)
		C(10)–Pt(2)–I(2)	177.83(9)
		C(10)–Pt(2)–C(11)	91.58(13)
		I(1)–Pt(2)–I(2)	88.966(9)

Adenine is known to coordinate with both platinum(II) and platinum(IV) through a variety of diverse coordination modes [31]. The particular coordination mode in Pt₂ad involves bridging two Pt(IV) centers by a neutral tautomer of adenine, as shown in Figure 2. A similar coordination mode was proposed for [Pt^{IV}Cl₃(H₂O)]₂(μ-Cl){μ-adenine(−1)}, where adenine(−1) is a deprotonated adenine anion, but this compound was not structurally characterized by X-ray crystallography [41].

The coordination geometry for each Pt(IV) center in Pt₂ad is octahedral. The plane defined by I(1)–Pt(1)–I(2) intersects the plane defined by I(1)–Pt(2)–I(2) at an angle of 32.49°. The three-center four-electron Pt–I bond lengths in Pt₂ad are intermediate between the terminal two-center two-electron Pt–I bonds in Pt(CH₃)₂I₂{2,2′-bipyridine} (2.6355(5) and 2.6569(5) Å) [32], and the four-center six-electron bonds in cubic [Pt(CH₃)₃(μ₃-I)]₄ · ½ CH₃I (2.8105(8) to 2.8366(7) Å) [42]. The Pt–N bond distances in Pt₂ad closely resemble those in trinuclear [(Pt^{IV}(CH₃)₃(μ-9-methyladenine(−1)))₃] · O=C(CH₃)₂ (2.18(1)–2.25(1) Å) and in trinuclear [(Pt^{IV}(CH₃)₃(μ-9-methyladenine(−1)))₃] · 1.5 Et₂O · 2 H₂O (2.180(7)–2.226(7) Å) [43]. The Pt(1)–Pt(2) distance in Pt₂ad (3.805 Å) is within the sum of the van der Waals radii of two platinum atoms [44], suggesting an intramolecular electronic interaction between the two platinum atoms.

This study selected Adenine as an ancillary ligand because it was thought that such a ligand would naturally intercalate into tumor DNA, facilitating the interaction of the platinum centers with natural nucleobases. Intermolecular π–π contacts between the adenine ligand and the DNA nucleobases were expected to drive this intercalation. However, the X-ray structural study revealed that there were two independent molecules of Pt₂ad in the unit cell, and that there were no intermolecular π–π interactions between these two molecules.

3.3. In Vitro Anticancer Activity

The National Cancer Institute’s Developmental Therapeutics Program (NCI/DTP) tested the anticancer activity of Pt₂ad against 60 cancer cell lines by an in vitro sulforhodamine B assay. Specifically, there were six leukemia cell lines, nine non-small cell lung cancer cell lines, seven colon cancer cell lines, six central nervous system cancer cell lines, nine melanoma cell lines, seven ovarian cancer cell lines, eight renal cancer cell lines, two prostate cancer cell lines, and six breast cancer cell lines. Pt₂ad exhibited cytotoxicity toward some but not all cancer cell lines. The results involving only those cell lines affected by Pt₂ad are summarized in Table 3. For comparison, the results involving cisplatin against the same cell lines are also included in Table 3, as values in parentheses.

Table 3. In vitro sulforhodamine B assay results for Pt₂ad. The values in parentheses are the results for cisplatin.

Type of Cancer	Cell Line, Reference	GI ₅₀ , μM	TGI, μM	LC ₅₀ , μM
Non-Small Cell Lung	NCI-H522, [45]	1.51	3.70	18.6
Colon Cancer	HCC-2998, [46]	13.1 (10.7)	31.7 (25.5)	76.8 (60.8)
	HCT-116, [47]	3.43 (9.24)	15.5 (>100)	52.5 (>100)
	HT29, [48]	5.99 (7.81)	21.2 (>100)	63.6 (>100)
	SW-620, [49]	3.37 (2.72)	11.4 (>100)	39.2 (>100)
Central Nervous System	SF-539, [50]	1.47 (0.600)	2.76 (7.67)	5.20 (>100)
Melanoma	LOX IMVI, [51]	3.85 (0.961)	18.4 (7.93)	58.2 (>100)
	Malme-3M, [52]	1.95 (1.85)	8.19 (4.58)	39.2 (>100)
	M14, [53]	2.08 (1.71)	14.8 (7.68)	63.4 (35.1)

Table 3. Cont.

Type of Cancer	Cell Line, Reference	GI ₅₀ , μ M	TGI, μ M	LC ₅₀ , μ M
	MDA-MB-435, [54]	6.19 (2.77)	18.4 (14.4)	46.2 (42.7)
	SK-MEL-28, [55]	5.10 (2.99)	17.4 (12.8)	42.4 (99.5)
	UACC-62, [56]	3.18 (1.34)	15.7 (9.39)	42.0 (37.2)
Ovarian Cancer	OVCAR-5, [57,58]	2.35 (4.38)	11.9 (46.4)	35.2 (>100)
Renal Cancer	A498, [59]	19.7 (12.7)	37.8 (26.4)	72.6 (54.9)
	RXF-393, [40]	-----	2.63 (4.58)	5.99 (24.2)
Breast Cancer	MDA-MB-231, [60]	1.62 (28.1)	8.79 (>100)	60.2 (>100)
	BT-549, [61]	3.56 (3.36)	16.5 (44.9)	61.2 (>100)
	MDA-MB-468, [62]	1.49 (0.233)	3.85 (0.744)	19.2 (4.50)

These in vitro cell viability assays clearly underscore the importance of using multiple cell lines of the same type of cancer. For instance, Pt₂ad was particularly lethal toward the central nervous system (CNS) cancer cell line SF-539, with an LC₅₀ = 5.20 μ M, but Pt₂ad was completely inactive toward five other CNS cancer cell lines (SF-268, SF-295, SNB-19, SNB-75, and U251). In comparison, cisplatin was inactive toward all six CNS cancer cell lines. The SF-539 cell line was derived from glioblastoma multiforme cells located in the right temporoparietal region of the brain of a 34-year-old white female in 1986 [50]. Glioblastoma multiforme is the deadliest of all brain cancers. Combined with radiotherapy, temozolomide [63] is a first-line standard of care chemotherapy drug for treating glioblastoma multiforme, but, interestingly, the NCI/DTP assays showed that temozolomide was completely inactive (LC₅₀ > 100 μ M) against the SF-539 cell line. Indeed, the NCI/DTP tests showed that temozolomide was inactive against all six CNS cell lines. Thus, Pt₂ad is significantly more cytotoxic toward the SF-539 cell line than either cisplatin or temozolomide.

Pt₂ad is also more cytostatic than either cisplatin or temozolomide against CNS SF-539 cancer cells, as seen by comparing the growth inhibition values in Table 3. For total growth inhibition (TGI), the concentration of Pt₂ad needed to be only 2.76 μ M compared with the required concentration of 7.67 μ M for cisplatin. However, for 50% growth inhibition (GI₅₀) of a population of SF-539 cells, a cisplatin solution with a concentration of only 0.600 μ M was needed, compared to a 1.47 μ M solution of Pt₂ad. Interestingly, for temozolomide, GI₅₀ > 100 μ M and TGI > 100 μ M.

In addition to demonstrating cytotoxicity toward cancer cells in vitro, a good chemotherapeutic drug for treating glioblastoma multiforme must be able to penetrate the blood–brain barrier. The free web tool Swiss ADME [64] predicts that Pt₂ad should be able to penetrate the blood–brain barrier, and that the gastrointestinal tract would highly absorb Pt₂ad. The Swiss ADME calculation also revealed the low water-solubility and high molar mass of Pt₂ad as limitations on the usefulness of this compound as a chemotherapy drug.

Pt₂ad was also particularly cytotoxic and cytostatic toward the renal cancer cell line RXF-393 [40], with an LC₅₀ = 5.99 μ M and TGI = 2.63 μ M. For this same cell line, the LC₅₀ = 24.2 μ M and TGI = 4.58 μ M for cisplatin. Conversely, cisplatin was more cytotoxic and cytostatic than Pt₂ad against the renal cancer cell line A498. Although notably less cytotoxic toward ovarian and breast cancer cell lines, Pt₂ad was nonetheless more cytotoxic than cisplatin toward both ovarian OVCAR-5 cells and the triple-negative breast cancer cell lines MDA-MB-231 and BT-549.

4. Discussion

Although Pt₂ad is the first adenine-stabilized Pt(IV) complex to be tested as an anticancer drug, several Pt(II) complexes stabilized by chemically modified derivatives of adenine have been tested against various cell lines of breast cancer [22–29], leukemia [24,27–29], non-small cell lung cancer [22,25], melanoma [22,23,25,27–29], osteosarcoma [22,23,25–29] ovarian cancer [21–23,25], cervical cancer [22,23,25], and urinary bladder cancer [30]. In most cases, the platinum(II) complex displayed cytotoxicity comparable to or slightly better than cisplatin. Pt₂ad also displayed cytotoxicity against cell lines of breast cancer, ovarian cancer, and melanoma that were comparable to or slightly better than cisplatin. However, Pt₂ad displayed the greatest cytotoxicity against the central nervous system glioblastoma multiforme cell line SF-539 and the renal adenocarcinoma cell line RXF-393. The cytotoxicity of the platinum(II) complexes against central nervous system and renal carcinoma cell lines was not reported.

Pt₂ad is particularly cytotoxic toward the CNS cell line SF-539 but notably less cytotoxic toward the other five CNS cell lines (SF-268, SF-295, SNB-19, and U251) used in this study. Similarly, Pt₂ad was cytotoxic toward the renal adenocarcinoma RXF-393 cell line but much less cytotoxic toward the other seven renal cancer cell lines (786-0, A498, ACHN, CAKI-1, SN12C, TK-10, and UO-31) used in this study. These differences in anticancer activity toward different cell lines of the same cancer are not understood.

5. Conclusions

Pt₂ad joins a growing list of organometallic platinum(IV) derivatives that exhibit anticancer properties [31,65–70].

Pt₂ad was more cytotoxic than cisplatin against three colon cancer cell lines (HCT-116, HT29, and SW-620), the glioblastoma multiforme SF-539 cell line, three melanoma cell lines (LOX IMVI, Malme-3M, and SK-MEL-28), the ovarian cancer cell line OVCAR-5, the renal cancer cell line RXF-393, and the triple-negative breast cancer cell lines MDA-MB-231 and BT-549. Nevertheless, there were also some cell lines for which cisplatin was more cytotoxic than Pt₂ad, and a number of cell lines against which Pt₂ad displayed little to no cytotoxicity. When assessing the anticancer properties of a new compound in vitro, the use of as many different cell lines as possible is necessary to accurately gauge its chemotherapeutic potential.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/compounds5020016/s1>. Figure S1: Portion of the ¹H NMR spectrum of Pt₂ad, showing only those peaks representing the [Pt–CH₃] moieties. Solvent = CD₃OD; Figure S2: Portion of the ¹H NMR spectrum of Pt₂ad, showing only those peaks representing the [C–H] groups in the bridging adenine ligand. Solvent = CD₃OD; Figure S3: Portion of the ¹³C{¹H} NMR spectrum of Pt₂ad, showing only those peaks representing the [Pt–CH₃] moieties. Solvent = CD₃OD; Figure S4: Portion of the ¹³C{¹H} NMR spectrum of Pt₂ad, showing only those peaks representing the carbon atoms in the bridging adenine ligand. Solvent = CD₃OD; Figure S5: ¹⁹⁵Pt{¹H} NMR spectrum of Pt₂ad, from –4500 to 500 ppm. Solvent = CD₃OD; Figure S6: Infrared spectrum of Pt₂ad, in a KBr pellet.

Author Contributions: Conceptualization, writing, project administration, W.A.H.; synthesis, spectroscopic characterization, A.M.O.; X-ray crystallography, K.A.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, under deposition number CCDC 2298069. These data can be obtained free of charge at <https://www.ccdc.cam.ac.uk/> or from the Cambridge Crystallographic Data Centre, 12,

Union Road, Cambridge CB2 1EZ, UK; email: deposit@ccdc.cam.ac.uk; fax: +44 (0)1223-336408. Any other data are available on request from the corresponding.

Acknowledgments: The authors thank the Department of Chemistry and Biochemistry at the University of Alaska Fairbanks (UAF) for financial support of this work. At UAF, the 600 MHz NMR spectrometer was purchased with funding from the US Army Medical Research and Material Command (05178001), and the 300 MHz NMR spectrometer was purchased with funding from the National Science Foundation (DUE-9850731). Undergraduate research support for A.M.O. and support for maintaining the 600 MHz NMR spectrometer at UAF were supplied by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under grant number P20GM103395. The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH. A.M.O. also acknowledges undergraduate research support from the UAF Office of Undergraduate Research and Scholarly Activity (URSA). UAF is an affirmative action/equal employment opportunity employer and education institution: www.alaska.edu/nondiscrimination. The National Science Foundation's Major Research Instrumentation is acknowledged for their support (1827313) in the purchase of the Bruker D8 Venture X-ray diffractometer at Whitworth University. The authors thank the National Cancer Institute Developmental Therapeutics Program (NCI/DTP) and acknowledge NCI/DTP (<https://dtp.cancer.gov>) for providing the in vitro sulforhodamine B assay data for Pt₂ad (NSC 842139), cisplatin (NSC 119875), and temozolomide (NSC 362856/32).

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

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Communication

Quinaldehyde *o*-Nitrobenzoylhydrazone: Structure and Sensitization of HepG2 Cells to Anti-Cancer Drugs

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Abstract: A quinoline unit is present in many natural products and is an attractive pharmacophore for the development of clinical drugs, including antineoplastics. The title compound (QN) was synthesized via the condensation reaction between quinoline-2-carboxaldehyde and 2-nitrobenzhydrazide. QN's structure was examined by X-ray diffraction and features extensive stacking interactions in the crystal. The compound is weakly toxic to HepG2 cells, with an IC₅₀ exceeding 400 μ M for 48 h exposure. QN at 50 μ M, with the dose reduction index in the range of 1.9–4.4, potentiated the cytotoxicity of several clinical chemotherapeutic drugs, including doxorubicin and other topoisomerase inhibitors, vincristine, and carboplatin, but not cisplatin or 5-fluorouracil. The calculated ADME parameters predict satisfactory drug-like properties for QN.

Keywords: hepatoblastoma; hydrazide–hydrazone; Schiff base; crystal structure; cytotoxicity; potentiation; synergism

1. Introduction

Liver cancer is one of the most common cancers by incidence and the third leading cause of cancer-related mortality globally [1]. Moreover, these already unfortunate statistics have increased by 25% in the last decade [2]. To counter this alarming trend, major efforts are being dedicated to the development of new therapeutic agents targeting hepatoma in adults [3], but the clinical treatment of liver cancer in children has not progressed beyond standard chemotherapy so far [4]. Therefore, pursuing the enhancement of the therapeutic efficacy of chemotherapeutic drugs in children and the mitigation of their severe side effects remains a high priority in the field of pediatric oncology research.

The sensitization of cancer cells to clinical anti-cancer drugs is an important task in medicinal chemistry. It aims at overcoming primary or acquired cancer drug resistance and lowering the burden of systemic toxicity, which are typical for chemotherapy [5,6]. Although a large number of natural products have been proposed as chemosensitizers [7], consistently favorable clinical outcomes have been achieved when molecular mediators of drug resistance have been identified and specifically targeted. Consequently, the application of drug combinations in clinics has proven beneficial over many years and has become a cornerstone of modern cancer therapy.

A significant number of successful clinical and experimental drugs for cancer treatment contain quinoline units. For instance, a family of topoisomerase inhibitors derived from the plant metabolite camptothecin, such as topotecan, irinotecan, and exatecan, and a line of tyrosine kinase inhibitors for targeted cancer therapy, including lenvatinib, cabozantinib, bosutinib, foretinib, and neratinib, use quinoline as a scaffold (Figure 1).

In addition, the quinoline pharmacophore constitutes a privileged structure for many antimalarial drugs (quinine, chloroquine, mefloquine, amodiaquine) or antibacterials (bedaquiline, ciprofloxacin), some of which are being considered for repurposing in cancer therapy [8,9]. Another interesting class of pharmaceuticals marked for repurposing are nitroaromatics [10], which include a variety of antimicrobials (nitrofurans), antiparasitics (niclosamide), and others (e.g., dantrolene). Several *ortho*-nitrophenyl derivatives have displayed promising activity in experimental cancer models [11,12].

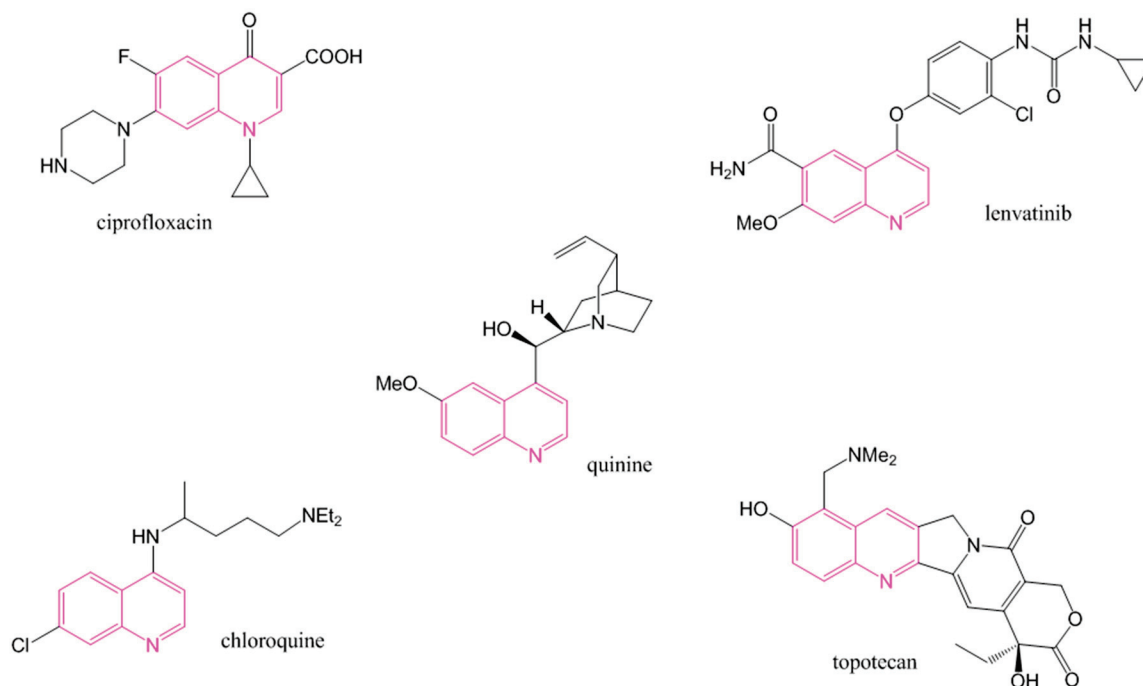


Figure 1. Natural metabolite quinine and examples of synthetic quinoline derivatives—clinical drugs.

As a part of our search for sensitizers of tumor cells [13,14], we prepared quinaldehyde *o*-nitrobenzoylhydrazone (QN) and report here the details of its molecular and crystal structure, as well as evidence of QN sensitization of human hepatoblastoma line HepG2 to chemotherapeutic drugs that are currently used to treat this pediatric cancer in clinics [4,15].

2. Materials and Methods

All commercial reagents and cell culture media were purchased from the companies Thermo Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

The HepG2 (passage 74) human hepatoblastoma cell line was purchased from the American Type Culture Collection. The cells were stably transfected with a viability reporter continuously producing destabilized green fluorescent protein from a copepod (cGFP), as reported earlier [16]. The original HepG2 and its reporter transfect were maintained in Gibco OptiMEM-I (Thermo Fisher Scientific) media supplemented with 5% Newborn Calf Serum (NCS) and a penicillin/streptomycin antibiotic cocktail. All cells were kept at 100% humidity, 5% CO₂, and 37 °C. The cells were subcultured at a 1:4 ratio upon reaching near confluency.

2.1. Synthesis and Crystallization of 2-Nitro-*N'*-(Quinolin-2-ylmethylene)benzohydrazide

The synthesis of QN was carried out by following a general protocol for the preparation of hydrazones [16]. Briefly, 2-nitrobenzhydrazide was prepared by the hydrazinolysis of methyl 2-nitrobenzoate [17]. In the next step, 362 mg (2 mmoles) of 2-nitrobenzhydrazide and 314 mg (2 mmoles) of 2-formylquinoline were completely dissolved in 10 mL of 95% EtOH and left at room temperature overnight. Crystallization started within one

hour, depositing off-white plates of chromatographically (TLC on silica gel F₂₅₄, R_f = 0.7 with 1:20 MeOH/CHCl₃ irrigant) pure crystalline QN, yield 487 mg (76%). The material was recrystallized once from 95% ethanol and used for spectroscopic characterization, diffraction studies, and cytotoxicity experiments. M.p. 193–193.5 °C.

2.2. Mass-Spectrometric and NMR Characterization

QN was dissolved in methanol at room temperature to 2 mg/mL and diluted 1:100 in water/MeOH (1:1 *v/v*) containing 0.1% formic acid. An accurate mass measurement was acquired with an LTQ Orbitrap XL high-resolution mass spectrometer equipped with an electrospray ionization (ESI) source and Xcalibur software v.2.1.0 (Thermo NA, Thermo Fisher Scientific, Waltham, MA, USA). Calc for C₁₇H₁₃N₄O₃ ([M + H]⁺ ion) *m/z* = 321.0982; found *m/z* = 321.0984. See Supplementary Figure S1.

H-1 and C-13 NMR spectra of a 0.2 M QN solution in *d*₆-DMSO were recorded at, respectively, 800.1 and 201.2 MHz using a Bruker AMX800 instrument (Bruker Corporation, Billerica, MA, USA). Data are given for the major component. H-1 NMR: 7.335 (d, 1H, *J* = 8.6 Hz, arom.), 7.741 (dd, 1H, *J* = 7.6; 1.4 Hz, arom.), 7.748 (m, 1H, *J* = 8.2; 1.1 Hz, arom.), 7.586 (m, 1H, *J* = 7.1; 0.8 Hz, arom.), 7.798 (m, 1H, *J* = 7.5; 1.3 Hz, arom.), 7.898 (d, 1H, *J* = 7.8 Hz, arom.), 7.908 (t, 1H, *J* = 7.5 Hz, arom.), 8.039 (d, 1H, *J* = 8.4 Hz, arom.), 8.184 (s, 1H, azomethine), 8.226 (d, 1H, *J* = 8.2 Hz, arom.), 8.253 (d, 1H, *J* = 8.6 Hz, arom.), 12.463 (s, 1H, NH). C-13 NMR: 116.38, 123.61, 127.41, 127.71, 127.93, 128.84, 129.72, 130.17, 130.73, 131.02, 134.48, 136.74, 145.12, 146.92, 147.21, 152.80, 168.10. See Supplementary Tables S1 and S2 and Supplementary Figures S2–S4.

2.3. X-Ray Diffraction Studies

Crystal data and experimental details of the crystallographic studies are given in Supplementary Table S3. The crystal structure was solved with the direct methods program SHELXS [18] and refined by full-matrix least squares techniques using the SHELXL suite of programs [19], with the help of Olex2 [20]. Data were corrected for Lorentz, polarization, and absorption effects. Non-hydrogen atoms were refined with anisotropic thermal parameters. The hydroxyl and ammonium hydrogen atoms were located in difference Fourier maps and were allowed to refine freely. The remaining H-atoms were placed at calculated positions and included in the refinement using a riding model. All hydrogen atom thermal parameters were constrained to ride on the carrier atoms (*U*_{iso}(methine, methylene H) = 1.2*U*_{eq} and *U*_{iso}(amide H) = 1.5*U*_{eq}). Structure visualization was performed with the Mercury program v.3.8 [21].

2.4. Cellular Proliferation Assay

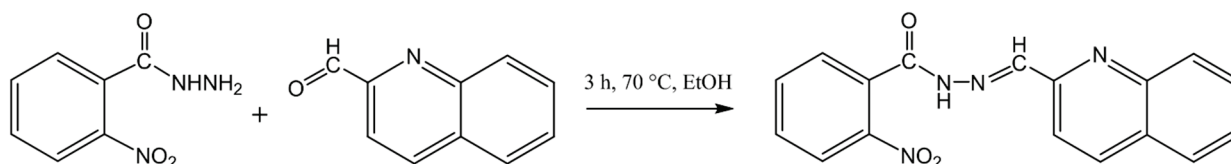
The cellular proliferation assay is based on measurements of destabilized GFP, which is continuously produced and degraded in the reporter cells. In the typical manner, reporter HepG2 cells were seeded in wells of a cell culture-treated 96-well plate at 1 × 10⁴ cells per well in the maintenance medium. The cells were allowed to adhere and establish clusters for 42 h. Next, the maintenance medium was replaced with the Phenol Red-free 5:5:1 DMEM/F12 Ham/RPMI-1640 mixture, supplemented with 1 g/L BSA, 2 mg/L insulin, 2 mg/L transferrin, 2 µg/L selenite, and the pen/strep (the test medium). The cells were allowed to adapt for the next 6 h; then, they were exposed to fresh test medium, now containing test agents, their combinations, or the drug carrier (0.5% DMSO) alone, for 48 h. Immediately after treatments with cytotoxic agents, the adherent reporter cells in wells were washed and lysed in 70 µL of the reporter lysis buffer [22] overnight at 8 °C. The lysates' fluorescence was measured at the 482(9)/512(17) nm wavelength (slit width) setup. All measurements were performed using a Synergy MX (BioTek Instruments, Winooski, VA, USA) plate reader.

2.5. Molecular Modeling, Dose-Effect Analysis, ADME Predictions, and Statistical Analysis

The Hirshfeld surfaces analyses and DFT calculations of the crystal lattices, at the B3LYP/6-31G(d,p) theory level, were performed using CrystalExplorer v.17.5 software [23,24]. Dose–effect analysis of the cell proliferation data was performed by CalcuSyn v.2 (Biosoft, Ferguson, MO, USA). ADME parameters and drug target predictions were performed using the online tools SwissADME and SwissTargetPrediction [25,26]. Statistical tests and plots were performed by using SigmaPlot v.11.0.

3. Results

Quinaldehyde *o*-nitrobenzoylhydrazone was readily synthesized via the condensation reaction between quinaldehyde and *o*-nitrobenzhydrazide in ethanol (Scheme 1).



Scheme 1. Molecular and crystal structure of QN.

The title compound crystallizes in the monoclinic space group $P2_1/c$, with four molecules per unit cell (Supplementary Tables S3–S5). The asymmetric unit contains one molecule of the hydrazone QN, as shown in Figure 2. All bond lengths and angles are within their expected ranges (Supplementary Table S6), with an exception for the C1–C2 bond, whose length, at 1.465(3) Å, is shorter than typical σ -bonds and is longer than typical aromatic bonds. This deviation is likely due to the C1 participation in an extended delocalized π -bonding, which involves the quinoline ring system, the C1–N2 azomethine π -electrons, a pair of p -electrons at the sp^2 -hybridized amide N3, and the C11–O1 acyl π -bond. Indeed, a portion of the QN molecule that includes the aforementioned components is flat (Supplementary Table S7), with the greatest deviation from the mean plane, among the non-hydrogen atoms, found for atom C11 at 0.223 Å. The nitrophenyl ring is at 67.31° to the quinoline plane. The configuration around the azomethine bond is *trans*; thus, the systematic name of the structure is (*E*)-2-nitro-*N'*-(quinolin-2-ylmethylene)benzohydrazide. The configuration around the amide bond is *cis*, which is uncommon and which may be stabilized by a short intramolecular contact, the C3—H3A...O2, between the electronegative nitro group and the quinoline ring (Figure 2, Supplementary Table S8).

The conventional hydrogen bonding in the crystal structure of QN is limited to just one intermolecular heteroatom contact (Supplementary Table S8) involving the hydrazide groups only and is shown in Figure 3. The hydrogen-bonding pattern consists of isolated $R^2_2(8)$ rings. This homodromic motif is formed by centrosymmetric dimers of QN molecules linked by the N3—H3A...O1 hydrogen bonds. In addition, one short intermolecular contact, the C15—H15...O3, which satisfies the distance and directionality conditions for a potential hydrogen bond (Supplementary Table S8), may also contribute to the stability of molecular packing in the crystal. The intermolecular non-polar interactions are dominated by the aromatic ring π - π contacts between quinoline residues; the shortest of these contacts, occurring between two neighboring rings #2, is about 0.14 Å less than the sum of the Van der Waals radii (Supplementary Table S9). These interactions form a pattern of infinite stacks, propagating in the [010] direction, perpendicular to the H-bonded rings (Figure 3).

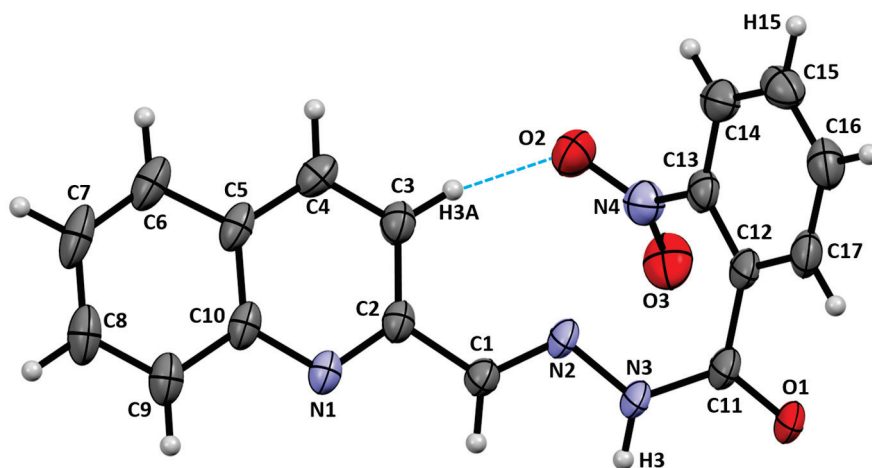


Figure 2. Atom numbering and displacement ellipsoids at the 50% probability level for quinaldehyde *o*-nitrobenzoylhydrazone. An intramolecular C—H...O interaction is shown as a dashed line.

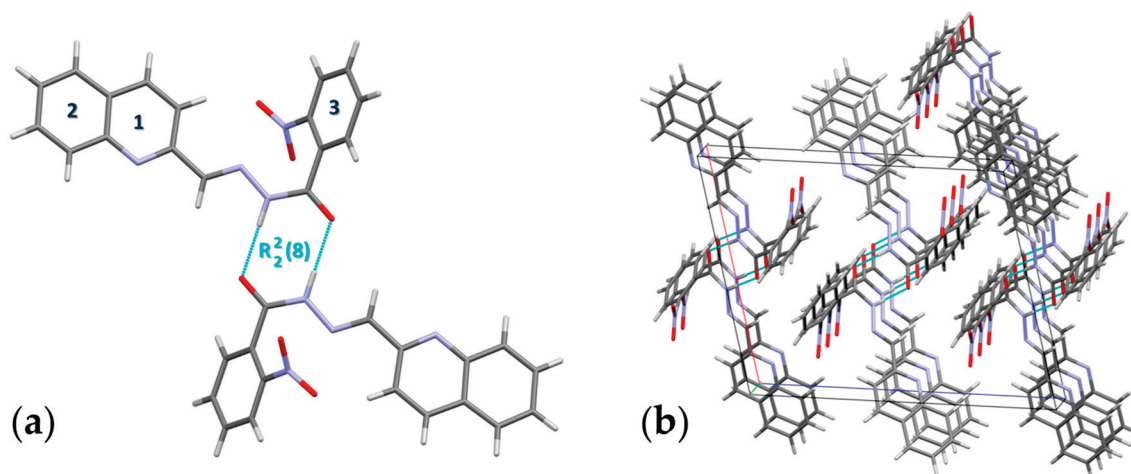


Figure 3. (a) Ring numbering and formation of H-bonded homodimers for quinaldehyde *o*-nitrobenzoylhydrazone. (b) Molecular packing of QN. Hydrogen bonds are shown as cyan dotted lines. Crystallographic axes' color codes: a—red; b—green; c—blue.

To evaluate the contributions of these and other intermolecular contacts to the energetics of the crystal lattice in QN, we calculated pairwise interaction energies for all unique contacts found in the crystal structure. The results are shown in Figure 4. It follows from these data that electrostatic interactions within the dimers and the π - π stacking are the major contributors to the packing forces in the crystal of QN. The pairwise interaction energies calculated for QN can be visualized as energy framework diagrams for the contributions of electrostatic and dispersion forces, and for the total energy. The diagrams and crystal packing are shown in Figure 4 and demonstrate that the main crystal packing forces are those that form sheets of the dimers parallel to the crystallographic plane (101) and those that keep stacks of the sheets together.

In DMSO solution, QN undergoes isomerization, as evidenced by the presence of two sets of signals, with a 2:1 signal intensity ratio, both in the proton- and carbon-13 NMR spectra (Supplementary Tables S1 and S2, Supplementary Figures S2–S4). Based on previous studies of *N'*-arylhydrazone stereochemistry [27,28], it is conceivable that QN dissolved in DMSO retains the *E*-configuration around the azomethine C1=N2 bond, but establishes an equilibrium between two conformers, namely *syn*- and *antiperiplanar* in respect to the mutual disposition of the N2 and O1 atoms, due to a slow rotation around the amide C11–N3 bond. Furthermore, relative values of chemical shifts of the amide and the

azomethine protons are indicative [27] of the specific rotamers: the upfield resonances of the amide were ascribed as corresponding to the *synperiplanar* conformer, while the downfield signals were attributed to its *antiperiplanar* counterpart. For azomethine protons, the order is reversed. In the H-1 NMR spectrum of QN, the amide proton singlets are located at 12.463 ppm (major) and 12.459 ppm (minor), while the azomethine proton resonates at 8.184 ppm (major) and 8.414 ppm (minor). This observation suggests that the *antiperiplanar* conformation of QN existing in a crystalline state (Figure 2) dominates the equilibrium in DMSO solution as well.

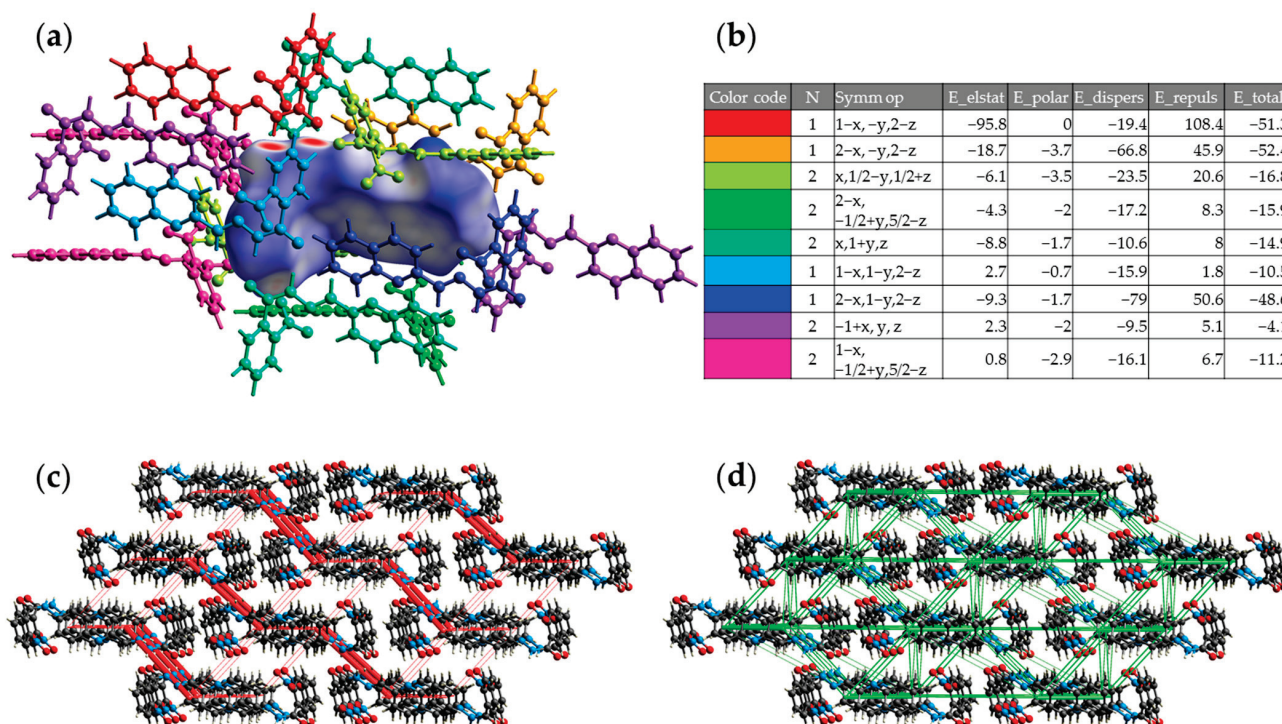


Figure 4. Interaction energies in the crystal structure of QN. (a) A view of the interactions between a central molecule, shown as its Hirshfeld surface, and 14 molecules that contact the central molecule. (b) Calculated energies (electrostatic, polarization, dispersion, repulsion, and total) of pairwise interactions between the central molecule and those encoded by respective colors. Energy frameworks for (c) electrostatic and (d) dispersion contributions to the total pairwise interaction energies in QN. The cylinders link molecular centroids, and the cylinder thickness is proportional to the magnitude of the energies. For clarity, the cylinders corresponding to energies < 6 kJ/mol are not shown.

Interactions of QN and Cytotoxic Chemotherapeutic Agents in HepG2 Cells

The initial evaluation of QN cytotoxicity in the hepatoblastoma cell line HepG2 revealed that this compound can inhibit cell proliferation significantly, over 10%, in the range of concentrations exceeding 100 μ M, as shown in Figure 5. We next questioned whether this hydrazone could sensitize HepG2 to cytotoxic drugs approved for hepatoblastoma, namely, doxorubicin, vinblastine, cisplatin, carboplatin, and 5-fluorouracil. Three out of five agents from this list showed a clear interaction with QN, so we have expanded the number of tested drugs to seventeen, including an extended set of topoisomerase inhibitors and sorafenib, which is the first-line chemotherapeutic drug for hepatocellular carcinoma. Experimental dose–response curves recorded for treatments of the cells with individual drugs and the drug combinations with 50 μ M of QN are shown in Figure 5 and Supplementary Figures S5–S8. Based on these data, we determined IC₅₀ values for the individual agents and calculated the dose reduction indexes for the combinations, which are summarized in Table 1. It follows from these experiments that QN sensitized HepG2

cells to most of the tested agents, with moderate DRI (arbitrarily set in the 1.9–4.4 range) obtained for nine agents, low DRI (1.4–1.7) observed for three agents, and no sensitization by QN (DRI < 1.3) found for five drugs, including cisplatin and 5-fluorouracil.

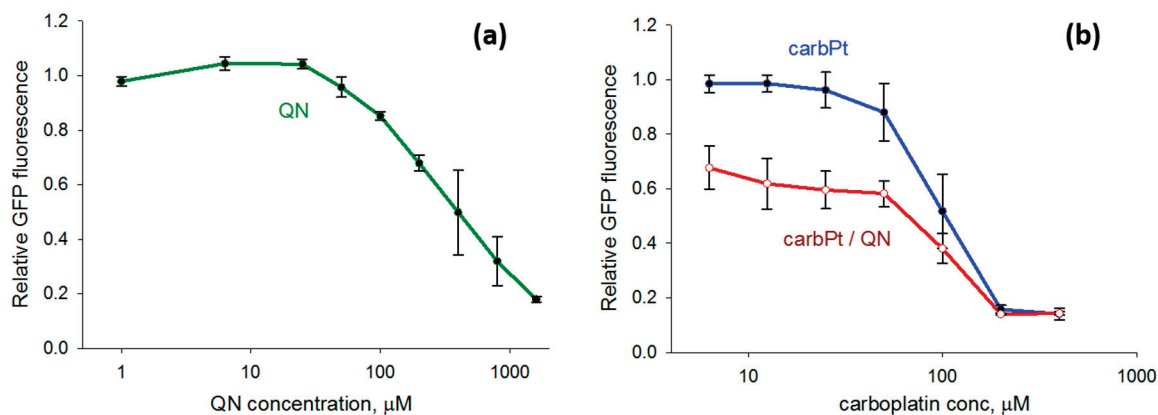


Figure 5. Proliferation, expressed as GFP transcriptional activity, of HepG2 cells treated for 48 h with (a) quinaldehyde *o*-nitrobenzoylhydrazone (QN) alone, (b) carboplatin alone (blue dose–response curve), or in combination with 50 μM of QN (red dose–response curve). Error bars are SDs for $n = 3$.

Table 1. Inhibition of HepG2 proliferation¹ by anti-cancer agents alone and in combination with 50 μM of QN.

Agent	Mechanism	Single Agent		Combination with QN		DRI
		IC ₅₀ , μM	r	IC ₅₀ , μM	r	
Doxorubicin	topoisomerase inh	0.792	0.960	0.410	0.961	1.9
Daunorubicin	topoisomerase inh	0.650	0.954	0.319	0.966	2.0
Epirubicin	topoisomerase inh	3.00	0.980	1.16	0.941	2.6
Idarubicin	topoisomerase inh	0.213	0.935	0.155	0.953	1.4
Mitoxanthrone	topoisomerase inh	0.884	0.934	0.718	0.936	1.2
Topotecan	topoisomerase inh	4.47	0.947	2.27	0.967	2.0
Etoposide	topoisomerase inh	132	0.980	79.7	0.959	1.7
Actinomycin D	topoisomerase inh	0.015	0.990	0.0046	0.844	3.3
Docetaxel	microtubule stabil	0.145	0.953	0.070	0.909	2.1
Vincristine	microtubule inh	1.80	0.900	0.406	0.905	4.4
Cisplatin	DNA crosslink	1.59	0.954	2.41	0.941	0.7
Carboplatin	DNA crosslink	132	0.962	65.6	0.942	2.0
Oxaliplatin	DNA crosslink	17.4	0.982	24.3	0.975	0.7
Mitomycin C	DNA crosslink	4.26	0.993	2.23	0.987	1.9
5-Fluorouracil	antimetabolite	137	0.929	140	0.983	1.0
Sorafenib	multi-kinase inh	13.2	0.876	7.55	0.948	1.7
Vorinostat	HDAC inhibitor	4.77	0.996	4.42	0.901	1.1
QN		418	0.998			

¹ Measured for 48 h exposure.

In addition to the drug combination studies, where the dose of QN was fixed at a non-toxic 50 μM, we performed a checkerboard experiment, with the doses of both doxorubicin and QN being varied. This experiment allowed us to probe whether QN, at cytotoxic concentrations, could interact with doxorubicin synergistically. Specifically, we built isobolographic curves, shown in Figure 6, which are concave towards the origin point and thus suggest a synergistic interaction between doxorubicin and QN. To quantify the synergism, we calculated the Combination Index (CI) based on the dose–effect analysis empowered by the CalcuSyn v.2 software [29]. It follows from the analysis that the synergistic interaction between doxorubicin and QN is dose-dependent and significantly increases

when the dose–effect (inhibition of the cell proliferation) of the drug combination exceeds 50%, as illustrated in Figure 6.

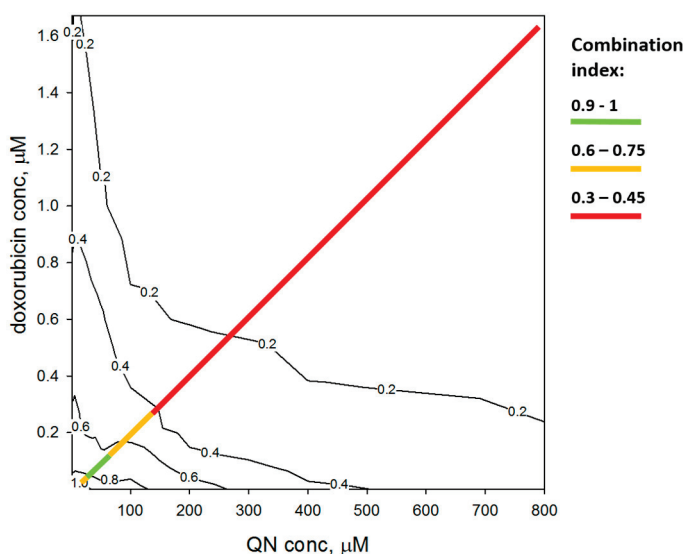


Figure 6. Analysis of the cytotoxic interactions between doxorubicin and QN in HepG2 cells exposed to doxorubicin/QN combinations for 48 h. The numbers on the isobolographic curves correspond to the fractions of surviving cells. The color pattern on the diagonal bar encodes approximate regions of weak-to-no interaction (green), moderate synergism (yellow), or strong synergism (red) based on the calculated Combination Index values.

4. Discussion

Hydrazone is a useful structure that is widely employed in medicinal chemistry. Dozens of clinical and experimental drugs contain a hydrazone unit, and hundreds more have demonstrated interesting biological activities in the lab [30]. Although falling short of matching the potential of “click” chemistry, Schiff base formation between carbonyl and hydrazine derivatives is quantitative and provides readily attainable coupling between desirable pharmacophores, thus enabling the assembly of combinatorial and dynamic libraries [16,31]. Indeed, our interest in the structure and bioactivity of QN was inspired by preliminary data obtained as a result of running a small combinatorial library against doxorubicin-treated HepG2.

Although this small study did not aim to unveil the molecular mechanism(s) of QN’s potentiation of doxorubicin and other chemotherapeutics, the literature provides a few insights on the matter. For instance, several quinoline hydrazones have shown low-to-submicromolar cytotoxicity against HepG2, which was attributed to the inhibitory potential of these structures against epidermal growth factor receptor (EGFR) activity [32]. EGFR is a tyrosine kinase implicated in tumorigenesis and, along with a number of other kinases, in the intrinsic drug resistance of hepatic cancer cells [33]. As a matter of fact, several tyrosine kinase inhibitors built around the quinoline core, such as lenvatinib (multi-kinase inhibitor), cabozantinib (multi-kinase inhibitor), bosutinib, and neratinib (HER1/2/4 inhibitor), have been recently approved for targeting cancer therapies. Using the online server SwissTargetPrediction, we evaluated probabilities for QN to inhibit pharmacologically significant proteins. As illustrated in Figure 7, kinases were predicted to be more likely targets for the QN structure.

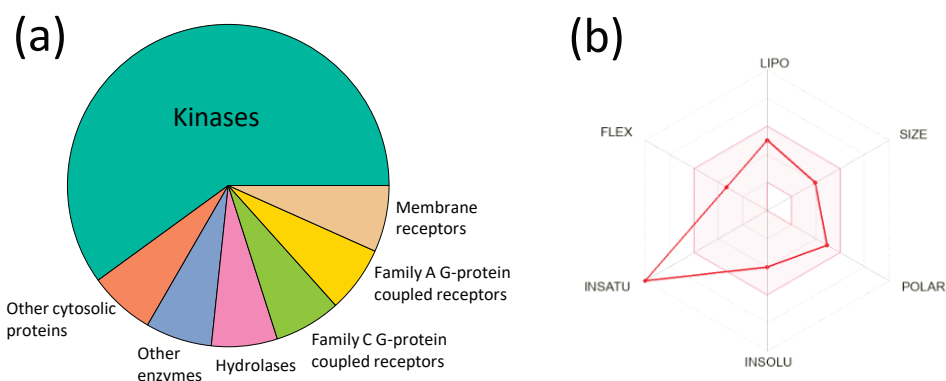


Figure 7. (a) The classes of the top 15 potential targets for QN, as predicted by SwissTargetPrediction. (b) Bioavailability radar predicted by SwissADME for QN, with the pink zone signifying the optimal range of molecular properties. The red line connects individual molecular property scores.

Additionally, we evaluated the bioavailability and other ADME parameters for QN and found that this molecule is expected to possess pharmacokinetic properties favorable for drug development (Figure 7, Table 2).

Table 2. Some ADME parameters and drug-likeness predicted for QN.

Parameter	Value
GI absorption	High
BBB permeability	Low
P-glycoprotein substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	Yes
CYP2C9 inhibitor	Yes
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeability)	−5.94 cm/s
Lipinski rule violations	0
PAINS alerts	0
Bioavailability score	0.55

5. Conclusions

In conclusion, quinaldehyde *o*-nitrobenzoylhydrazone is a novel structure whose crystal structure is dominated by π - π stacking, with the total lattice energy estimated to be −225.7 kJ/mol. The structure is conformationally unstable and undergoes the *cis/trans* isomerization around the amide bond in solution. In vitro, QN demonstrated the ability to sensitize a hepatoblastoma cancer cell line to 13 out of 17 tested common chemotherapeutic agents, with the maximum effect, at DRI = 4.4, observed for its combination with vincristine and the synergistic interaction, at CI = 0.3, demonstrated for its combination with doxorubicin. The compound is likely to target kinases and to have favorable ADME parameters. It may serve as a hit structure for further drug development.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/compounds5030024/s1>, Table S1: Tentative assignments of resonances in a H-1 NMR spectrum of QN; Table S2: Tentative assignments of resonances in a C-13 NMR spectrum of QN; Table S3: Details of X-ray diffraction experiment for QN; Table S4: Fractional atomic coordinates and isotropic or equivalent isotropic displacement parameters for QN; Table S5: Atomic displacement parameters for QN; Table S6: Bond distances and angles for QN; Table S7: Torsion angles for QN; Table S8: Hydrogen bond geometry for QN; Table S9: π - π stacking geometry for

QN; Figure S1: ESI-MS of QN; Figures S2 and S3: ¹H-NMR spectrum of QN; Figure S4: ¹³C-NMR spectrum of QN; Figures S5–S8: Proliferation of HepG2 cells treated with anti-cancer drugs alone or in combination with 50 µM of QN.

Author Contributions: Conceptualization, V.V.M. and T.P.M.; methodology, chemical synthesis, V.V.M.; structural data collection and curation, S.P.K.; biological data collection and analysis, V.V.M.; writing—original draft preparation, V.V.M.; review and editing, S.P.K. and T.P.M.; project administration and funding acquisition, T.P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded, in part, by the University of Missouri Experiment Station Chemical Laboratories and by the USDA National Institute of Food and Agriculture, Hatch project 1023929.

Data Availability Statement: Supporting data are included in the Supplementary Information. The complete crystallographic data for the structural analysis have been deposited in the Cambridge Crystallographic Data Centre, CCDC #2359763. Copies of this information may be obtained free of charge from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033, e-mail: deposit@ccdc.cam.ac.uk, or via: www.ccdc.cam.ac.uk). All other data are available from the corresponding author upon reasonable request.

Acknowledgments: The authors thank Deborah L. Chance and Shaokai Jiang for their help with obtaining the MS and NMR spectra.

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

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Article

Reduction Potential Predictions for Thirty-Seven 1,4-di-N-Oxide Quinoxaline-2-Carboxamide Derivatives with Anti-Tuberculosis Activity

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Abstract: The ability for density functional theory with the B3LYP functional with the lanl2dz basis set to predict the 1st (Wave 1) and 2nd (Wave 2) reductions of the diazine ring in a series of thirty-seven (37) 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives in dimethylformamide was examined. The B3LYP/lanl2dz method had a strong correlation and low correlation to the experimental potentials for Wave 1 and Wave 2, respectively. There are nine identifiable analogs based on similarities of structure. The predicted reduction potentials for the derivatives of each analog generally fit the modified Hammett equation. The B3LYP/lanl2dz method is shown to be useful in accurately predicting the Wave 1 potentials for quinoxaline-di-N-oxide derivatives. For derivatives with assessable anti-tuberculosis activity, the predicted Wave 1 potentials have a similar correlation with the bioactivity when compared to the experimental wave 1 potentials.

Keywords: quinoxaline-di-N-oxide derivatives; voltammetry; anti-tuberculosis; reduction potential; computational prediction; DFT: B3LYP/lanl2dz; lanl2dz; modified Hammett equation

1. Introduction

Over the last several decades, scientists have been investigating quinoxaline derivatives for a variety of medical applications. Quinoxaline derivatives have been found to have medicinal effects including anti-cancer, anti-trypanosomal, anti-microbial and anti-viral activities [1–16]. The biomechanisms for the medicinal applications is not known but is often linked to the ease of reduction of the quinoxaline derivatives [6,17,18]. In 2011, Crawford et. al. studied the electrochemical properties of a series of thirty-seven (37) 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives shown in Table 1 [18]. The ease of reduction was compared to the molecular structure, and it was observed that the addition of an electron-withdrawing group resulted in a positive shift for the reduction potential. Likewise, the addition of an electron-donating group resulted in a negative shift in the reduction potential. The thirty-seven derivatives can be assigned to nine analogs based on similarities in structure. The anti-tuberculosis activities of the derivatives were measured and compared to the ease of reduction. The two analogs containing benzyl (compounds 1–5) and 2-phenylethyl (compounds 6–12) were previously found to have significant anti-tuberculosis activity. For these derivatives, Crawford et al. demonstrated an exponential correlation between the anti-tuberculosis activity and the ease of reduction for this subset of derivatives [18].

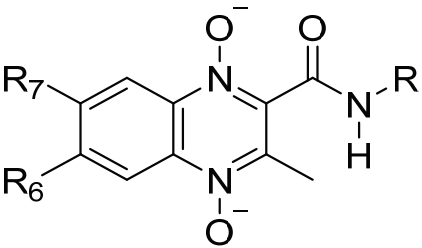
The common correlation between bioactivity and ease of reduction has made cyclic voltammetry a common analytical technique to study quinoxaline derivatives [6,17–21]. We have proposed that simple computational methods provide a powerful tool allowing for the prediction of the electrochemical properties of quinoxaline derivatives. Furthermore, the ability to accurately predict the electrochemical properties could become a powerful tool to help synthetic chemists design potential derivatives. As a proposed

tool for synthetic chemists, we are looking for the fastest reliable computational methods. Several computational studies have investigated the HOMO/LUMO gap in small organic molecules [20,22–26]. Additionally, studies have investigated electronic properties, thermodynamic properties, effects of substitutions, and polymerization of quinoxalines [20,24,27,28].

In our previous studies, we investigated twenty 3-aryl-quinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives that demonstrated varying degrees of cytotoxic activity [29,30]. Voltametric methods were used to measure the electrochemical properties of the 20 quinoxaline derivatives in dimethylformamide (DMF) [30]. All but one of the first reductions of the n-oxide derivatives (Wave 1) were found to be reversible or quasireversible, while the second reductions (Wave 2) were found to be irreversible. There was good correlation between molecular structure and the reduction potential of these derivatives. Surprisingly, no correlation was found between the ease of reduction and the cytotoxic activity for the twenty 3-aryl-quinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives studied [30]. We also tested the ability of DFT: B3LYP/6-31G [29], DFT: B3LYP/lanl2dz [29] and DFT: B3LYP/cc-pVTZ (unpublished) [31] to predict the electrochemical properties for the same twenty 3-aryl-quinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives. For all basis sets, a strong correlation with similar R^2 values and standard error was found between the predicted and experimental potentials for wave 1 when considering the sixteen derivatives without a nitro functional group. The main advantage of DFT: B3LYP/cc-pVTZ was the ability to predict wave 1 for four (4) derivatives with a nitro functional group. None of the methods were able to accurately predict the wave 2 or nitro wave reductions.

In this study, we investigate the ability for Density Functional Theory (DFT) [32] calculation at the B3LYP/lanl2dz level of theory to accurately predict the 1st (Wave 1) and 2nd (Wave 2) reduction potentials for the diazine ring for the thirty-seven (37) 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives from the 2011 Crawford study [18]. The DFT: B3LYP/lanl2dz level of theory was used as none of the current derivatives contain a nitro functional group. The correlation between the DFT: B3LYP/lanl2dz predicted potentials and the anti-tuberculosis activity and compared the results to the correlation of the experimental potentials.

Table 1. The parent structure of the 1,4-di-N-oxide quinoxaline-2-carboxamide derivative and the list of the R, R7, and R6 substituents [18,33].



Compounds Code	R	R7/R6
1	Benzyl	CH ₃ /H
2		Cl/H
3		Cl/Cl
4		F/H
5		CF ₃ /H
6	2-Phenylethyl	H/H
7		CH ₃ /H
8		Cl/H
9		Cl/Cl
10		CF ₃ /H
11		F/H
12		F/F

Table 1. Cont.

Compounds Code	R	R7/R6
13	p-Methoxybenzyl	H/H
14		CH ₃ /H
15		Cl/H
16	p-Trifluoromethylbenzyl	H/H
17		CH ₃ /H
18		Cl/H
19	p-Chlorobenzyl	H/H
20		CH ₃ /H
21		Cl/H
22		Cl/Cl
23	p-Bromobenzyl	H/H
24		CH ₃ /H
24		Cl/H
26		Cl/Cl
27	p-Methylbenzyl	H/H
28		CH ₃ /H
29		Cl/H
30	2,2-Diphenylethel	H/H
31		CH ₃ /H
32		Cl/H
33		Cl/Cl
34	Benzo[d] [1,3] dioxol-5-ylmethyl	H/H
35		CH ₃ /H
36		Cl/H
37		Cl/Cl

2. Materials and Methods

2.1. Drawing the Derivatives

The molecular structures for the 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives were drawn using GaussView 5 [34]. For each derivative, three structures were drawn including the original neutral molecule, the anion product from the first N-oxide reduction (Wave 1), and the dianion product from the second N-oxide reduction (Wave 2). Figure 1 shows 2D structures created with ChemDraw for the three individual structures for Compound 20 (Table 1). The original neutral molecule is shown in Figure 1a. The anion product structures for the product of wave 1 had the radical attached to the carbon adjacent to the amide group (Figure 1b). The dianion product from wave 2 is shown in Figure 1c.

2.2. DFT Reduction Potential Predictions

All density functional theory (DFT) with the B3LYP functional and the lanl2dz basis set [35,36] calculations were carried out in Gaussian 09 [37] following the procedure outlined in our previous papers [29,30]. All the Gaussian 09 settings remained the default settings unless explicitly indicated. Each structure was optimized in the gas phase using the lanl2dz basis set to find the most stable configuration with the lowest energy (E_{opt}). Next, a frequency calculation was run from the optimized structures to determine the thermal correction factor ($E_{Thermal}$) needed to correct for thermal artifacts. The energy of the structure in the gas phase (E_g) was determined by combining the E_{opt} and $E_{Thermal}$ according to Equation (1).

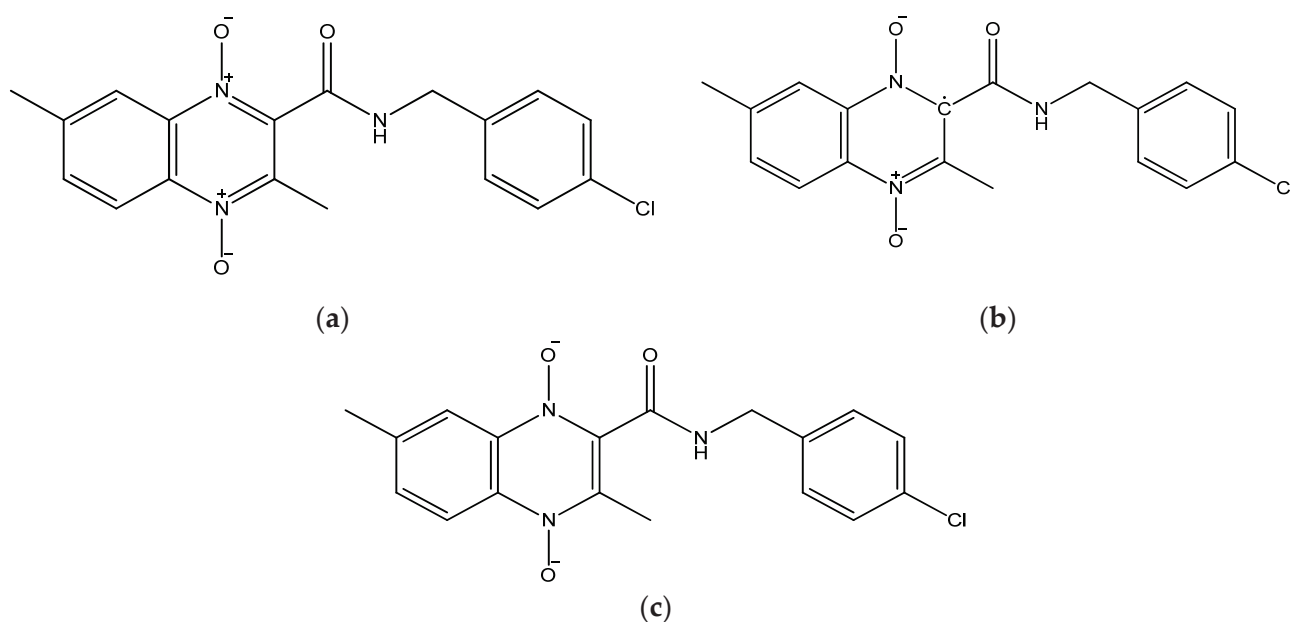


Figure 1. The molecular structures of Compound 20 [18] in (a) neutral, (b) the anion product from the 1st N-oxide reduction (Wave 1), and (c) the dianion product from the 2nd N-oxide reduction (Wave 2) drawn with ChemDraw [33].

$$E_g = E_{opt} + E_{Thermal} \quad (1)$$

The change in Gibbs Free Energy from the reduction of the derivative is found by comparing the energy of the structures in gas phase before and after the reduction of the derivative. For wave 1, the change in Gibbs Free Energy ($\Delta G_{red, wave1(g)}$) is the difference between the energy of the anion ($E_{g,a}$) and neutral forms ($E_{g,n}$) of the derivative (Equation (2)).

$$\Delta G_{red, wave1(g)} = E_{g,a} - E_{g,n} \quad (2)$$

Energy calculations were run from the optimized structures in the gas phase in the solvent N, N-dimethylformamide (DMF) to find the solvated energy (E_{solv}). The default solvent in Gaussian 09 is the Integral equation formalism variant of the Polarizable Continuum Model (IEFPCM) [38–40]. The Gibbs Free Energy of Solvation was found by subtracting E_g from E_{solv} (Equation (3)).

$$\Delta G_{solv} = E_{solv} - E_g \quad (3)$$

Figure 2 shows the thermodynamic cycle used to calculate the change in Gibbs Free energy associated with the reduction wave 1 and wave 2, $\Delta G_{red, wave1(solv)}$ and $\Delta G_{red, wave2(solv)}$, respectively. For wave 1, $\Delta G_{red, wave1(solv)}$ is calculated from $\Delta G_{solv,n}$, $\Delta G_{solv,a}$ and $\Delta G_{red, wave1(g)}$ according to equation 4 where n is the neutral and a is the anion form of the derivative.

$$\Delta G_{red, wave1(solv)} = -\Delta G_{solv,n} + \Delta G_{red, wave1(g)} + \Delta G_{solv,a} \quad (4)$$

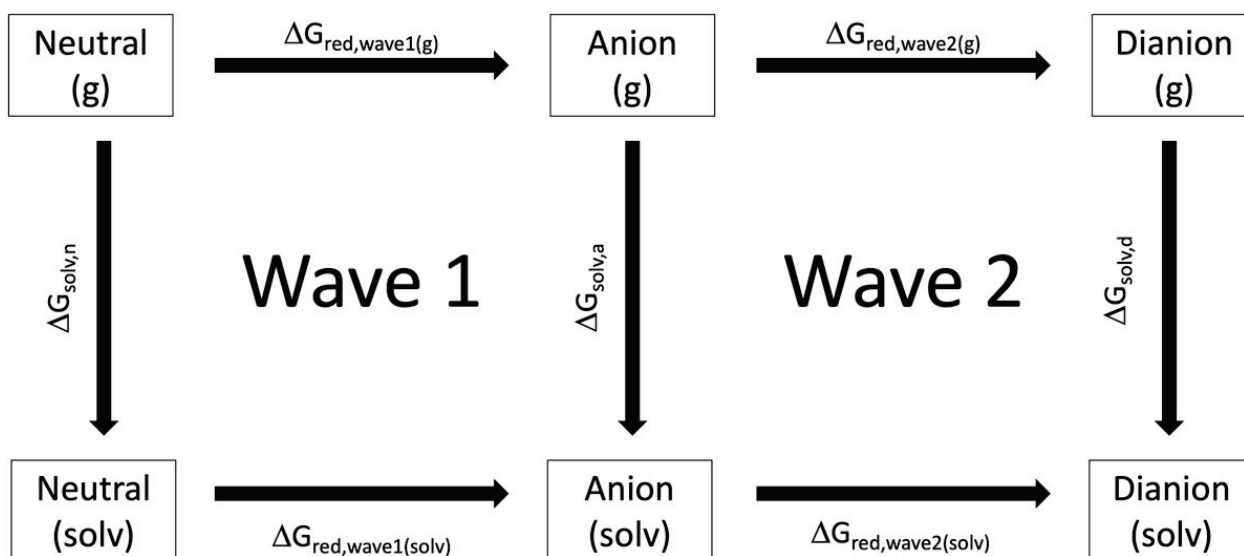


Figure 2. The thermodynamic cycle used to calculate the electrochemical cell potentials for wave 1 and wave 2.

The change in Gibbs Free energy $\Delta G_{red,wave1(solv)}$ is converted to a half-cell reduction potential ($\epsilon_{red,wave1}$) using Equation (5),

$$\epsilon_{red,wave1} = \frac{-\Delta G_{red,wave1(solv)}}{nF} \quad (5)$$

where n is the number of electrons transferred and F is Faraday's constant (96,485 C/mole). Computational values for the ferrocene (Fc) and ferrocenium (Fc⁺) ion reference system have been previously calculated [22] and were used in this study. The cell reduction potentials (ϵ_{cell}) were calculated by comparing the half-cell reduction potentials of the derivative and the Fc/Fc⁺ reference system (Equation (6)).

$$\epsilon_{cell} = \epsilon_{red,wave1} - \epsilon_{Fc/Fc+} \quad (6)$$

lastly, the reduction potential was modified to set the ferrocene/ferrocenium reference system to zero (ϵ'_{cell}) by subtracting 0.7 V, the difference between the ferrocene/ferrocenium reference system and the standard hydrogen electron in DMF (Equation (7)).

$$\epsilon'_{cell} = \epsilon_{cell} + 0.72 \text{ V} \quad (7)$$

3. Results and Discussion

3.1. Computational Raw Chemical Potentials

The B3LYP/lanl2dz predicted half-cell reduction potentials for the 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives are shown in Table 2. The calculated reduction potentials were within the expected range. The appearance of the neutral and anion structures of the compounds appeared as expected. The dianion structures of some chlorine or Br containing derives is observed to have C–Cl or C–Br bond lengthening. For the derivatives with C–Cl bond lengthening, the C–Cl of the dianion was found to be between 1.87 Å and 1.89 Å. The derivatives with C–Br bond lengthening the C–Br in the dianion structure was between 2.05 Å and 2.3 Å.

Table 2. The predicted half-cell reduction potentials for the 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives calculated using Gaussian 09 at the DFT: B3LYP/lanl2dz level of theory [32,37].

Compounds	Wave 1 Half-Cell Potentials (V)	Wave 2 Half-Cell Potentials (V)
1	3.34	2.17
2	3.54	1.75 *
3	3.65	2.49
4	3.52	2.35
5	3.66	2.47
6	3.37	2.19
7	3.27	1.47
8	3.51	1.67 *
9	3.63	2.48
10	3.64	2.46
11	3.50	1.65
12	3.60	2.43
13	3.37	2.15
14	3.32	1.49
15	3.52	2.33 *
16	3.42	2.13
17	3.37	2.11
18	3.57	2.35
19	3.40	1.48 *
20	3.34	1.45 *
21	3.55	1.73 *
22	3.68	1.89
23	3.40	1.70 *
24	3.34	1.70 *
25	3.55	2.24 *
26	3.67	2.46
27	3.38	2.19
28	3.32	1.50
29	3.52	2.36 *
30	3.38	1.43
31	3.34	1.48
32	3.53	1.65 *
33	3.65	2.40
34	3.40	2.18
35	3.33	2.16
36	3.54	1.73 *
37	3.66	2.48

* C–Cl bond length > 1.87 Å or C–Br bond length > 2.0 Å.

The optimized dianion structure of Compound 20 is shown in Figure 3. The C–Cl bond was missing, indicating that the chlorine atom had moved far enough away from the derivative that GaussView 5 [34] no longer draws the Cl as bonded. The C–Cl bond distance increases as the compound is reduced. After optimization the C–Cl bond length was found to be 1.76 Å, 1.837 Å, and 1.87 Å for the Compound 20 derivative, after wave 1, and after wave 2, respectively. This result was not totally unexpected, since we observed a the C–Cl bonding lengthening and even Cl atom dissociating from pyrazine in molecule E4 in our previous study [29]. In the 2019 study, the DFT: B3LYP/lanl2dz the C–Cl distance was found to be 1.81 Å, 1.83 Å, and 1.91 Å for the E4 before reduction, after wave 1, and after wave 2, respectively. The effect was more pronounced with the DFT: B3LYP/6-31g. The C–Cl distance was 1.81 Å, 1.84 Å, and 10.9 Å for the original E4 before reduction, after wave 1, and after wave 2, respectively [29]. Since the lengthening of the C–Cl bond is seen in more than one study across different quinoxaline analogs, this could indicate a real structural change. Given the electron withdrawing nature of chlorine, it is not unreasonable to think some C–Cl bond lengthening may occur as the molecule is reduced and has an increased electron density on the chlorine atom.

3.2. Computationally Predicted Electrochemical Cell Reaction Potentials

The calculated and experimental electrochemical cell potentials (ϵ_{cell}) versus Fc/Fc+ with the Fc/Fc+ potential set to zero are shown in Table 3. The comparison of the calculated electrochemical cell potentials (ϵ'_{cell}) with the ferrocene/ferrocenium electrode (Fc/Fc+) reduction potential adjusted to zero for wave 1 is between -1.1261 and -1.5159 V but for wave 2 is in a lower range, from -2.3236 to -3.3491 V (Table 3).

Table 3. The DFT: B3LYP/lanl2dz-predicted electrochemical potential, experimental [18] electrochemical cell potentials, and anti-tuberculosis activities [18] for the 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives.

Compounds	DFT Predicted		Experimental [18]		Anti-Tuberculosis Activity [18]	
	Wave 1 ϵ'_{cell} (V)	Wave 2 ϵ'_{cell} (V)	Wave 1 ϵ'_{cell} (V)	Wave 2 ϵ'_{cell} (V)	IC ₉₀ (µg/mL)	SI
1	−1.46	−2.63	−1.658	−1.91	4.21	>23.77
2	−1.26	−3.05 *	−1.482	−1.786	0.43	>230.94
3	−1.14	−2.30	−1.211	−1.391	<0.2	>500
4	−1.27	−2.44	−1.517	−1.642	1.52	>65.7
5	−1.13	−2.32	−1.414	−1.516	0.41	>246.3
6	−1.43	−2.60	−1.625	−1.848	3.05	>32.81
7	−1.52	−3.32	−1.649	−1.907	8.62	>11.6
8	−1.28	−3.12 *	−1.525	−1.72	<0.2	>500
9	−1.16	−2.31	−1.387	−1.557	<0.19	>153.84
10	−1.16	−2.33	−1.439	−1.543	1.153	>26.02
11	−1.29	−3.14	−1.551	−1.696	0.504	>198.41
12	−1.19	−2.37	−1.485	−1.664	0.517	>58.03
13	−1.42	−2.65	−1.598	−1.866	2.85	>10.54
14	−1.47	−3.30	−1.618	−1.876	8.9	>3.37
15	−1.27	−2.46 *	−1.489	−1.79	—	—
16	−1.37	−2.66	−1.594	−1.9	16.81	—
17	−1.42	−2.68	−1.599	−1.9	>100	—
18	−1.22	−2.44	−1.46	−1.778	6.13	—
19	−1.39	−3.32 *	−1.53	−1.738	11.04	>6.52
20	−1.45	−3.34 *	−1.57	−1.798	14.56	—
21	−1.24	−3.07 *	−1.47	−1.737	29.68	—
22	−1.12	−2.90	−1.374	−1.582	51.86	—
23	−1.39	−3.09 *	−1.54	−1.795	15.61	—
24	−1.45	−3.09 *	−1.58	−1.806	78.22	—
25	−1.24	−2.56 *	−1.445	−1.685	5.33	>7.5
26	−1.12	−2.34	−1.363	−1.564	6.92	>5.78
27	−1.42	−2.61	−1.58	−1.798	6.76	>5.92
28	−1.48	−3.30	−1.59	−1.809	99.91	—
29	−1.27	−2.43	−1.489	−1.787	32.04	—
30	−1.42	−3.36	−1.591	−1.855	15.99	—
31	−1.45	−3.32	−1.64	−1.87	16.79	—
32	−1.27	−3.14 *	−1.491	−1.802	60.43	—
33	−1.15	−2.39	−1.41	−1.531	66.54	—
34	−1.40	−2.61	−1.611	−1.854	22.75	—
35	−1.46	−2.63	−1.624	−1.887	13.22	—
36	−1.25	−3.06 *	−1.488	−1.76	6.99	>5.72
37	−1.13	−2.31	−1.403	−1.525	34.92	—

* C–Cl bond length > 1.87 Å or C–Br bond length > 2.0 Å.

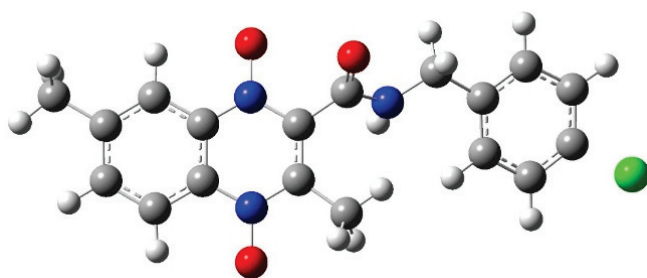


Figure 3. The carbon—chlorine bond length increases to 1.87 Å for Compound 20 during the optimization of the dianion species. Chemical structure created in GaussView 5 [34] and optimized in Gaussian 09 [37]. Carbon is shown in grey, hydrogen in white, nitrogen in blue, oxygen in red, and chlorine in green.

3.3. Comparison to Experimental Reduction Potentials

To investigate any correlation between calculated and experimental data, two diagrams based on the electrochemical cell potentials (E'_{cell}) were plotted for both wave 1 and wave 2. Figure 4a is a comparison between the experimental [18] and calculated electrochemical potentials for wave 1. The wave 1 slope has a value of 1.16 ± 0.09 (slope $\pm$ standard error of slope) and has an R^2 value of 0.831, indicating a significant correlation between the calculated and experimental data. However, the slope value is greater than 1 meaning that the computational values are changing in response to the chemical differences more than the experimental values. Additionally, we observed that calculated values are more positive than the experimental values. In Table 3, the calculated electrochemical cell potentials (E'_{cell}) for wave 1 lie in the range of -1.12 and -1.52 V. In contrast, the experimental values ranged from -1.211 to -1.658 V [18].

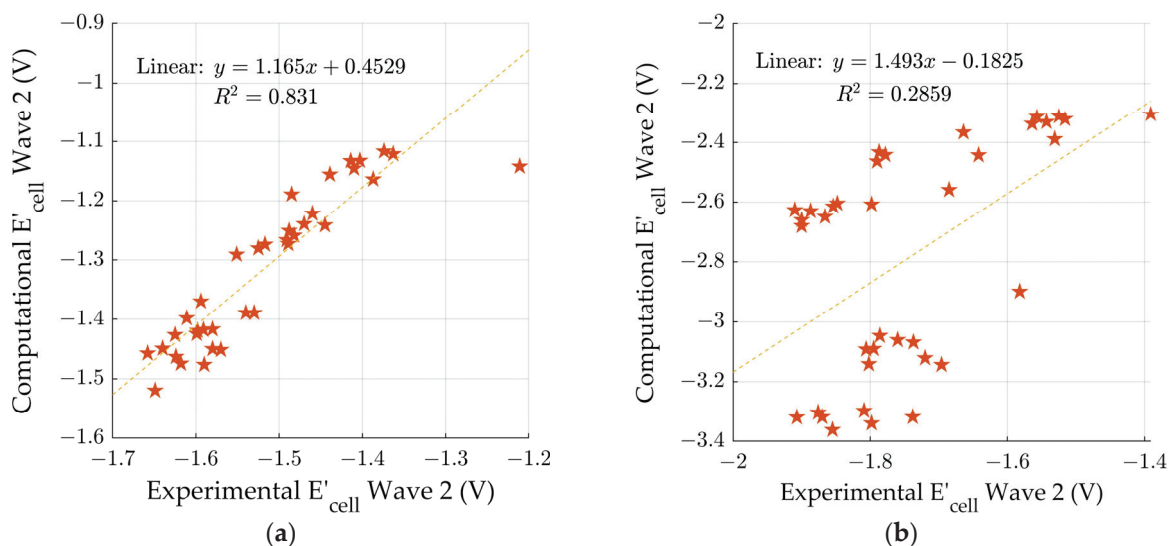


Figure 4. Comparison of computational electrochemical cell potentials vs. experimental electrochemical cell potentials [18] for (a) wave 1, and (b) wave 2.

Figure 4b is a comparison between the experimental [18] and calculated electrochemical potentials for wave 2. The wave 2 comparison has a slope of 1.5 ± 0.5 (slope $\pm$ standard error of slope) and an R^2 value of 0.285, indicating little to no correlation between the experimental and computational waves to electrochemical potentials. For wave 2, we observed that the calculated electrochemical cell potentials (E'_{cell}) were significantly more negative than the experimental values (Table 3). The calculated electrochemical cell potentials (E'_{cell}) for wave 2 lie in the range -2.30 V to -3.36 V. In contrast, the experimental values ranged from -1.391 V to -1.91 V [18].

The inability for DFT: B3LYP/lanl2dz to predict the electrochemical potentials for wave 2 was also observed in our previous study [29]. We have also tested the ability for DFT: B3LYP/cc-pVTZ to predict the electrochemical potentials for the twenty (20) 3-aryl-quinoline-2-carbonitrile 1,4-di-N-oxide derivatives from our 2019 study (unpublished) [31]. The DFT calculations with the higher B3LYP/cc-pVTZ level of theory is also unable to accurately predict the wave 2 electrochemical potentials. Given the combination of our studies on both sets of derivatives, we hypothesize that the inability to predict the wave 2 potentials is an indication that there are likely other structural changes during reduction. It would be expected that the addition of the second electron leads a highly reactive species that could react with solvent, an electrolyte, or another quinoline molecule. Unfortunately, structural changes during reduction were not investigated by the experimentalists.

3.4. Comparison to the Modified Hammett Equation

The Hammett Equation is used to model the impact of the substitution of hydrogens with electron withdrawing or electron donating groups on the reaction constant for the reduction of the aromatic ring system [41]. The predicted Wave 1 potentials generally fits the modified Hammett equation for the different homologous series. The modified Hammett equation (Equation (8)) shows the relationship between change in electrochemical potentials $\Delta\epsilon_{cell}$ with $\rho_{\pi R}$, the reaction constant, and σ_x , the polar inductive electronic substituent constant. It is recommended to use the average of the para position polar inductive substituent constant, σ_{p-x} , and the meta position polar inductive substituent constant, σ_{m-x} , for quinolines, $\frac{1}{2}(\sigma_{p-x} + \sigma_{m-x})$ [42]. The Hammett constants are listed in Table 4.

$$\Delta\epsilon_{cell} = \rho_{\pi R} * \sigma_x \quad (8)$$

Table 4. Hammett substituent constants [41].

Compound	R7/R6	σ_{p-x}	σ_{m-x}	$\frac{1}{2}(\sigma_{p-x} + \sigma_{m-x})$
6	H/H	0	0	0
7	CH ₃ /H	−0.17	−0.07	−0.12
8	Cl/H	0.23	0.37	0.30
9	Cl/Cl	0.46	0.74	0.60
10	CF ₃ /H	0.55	0.41	0.48
11	F/H	0.06	0.34	0.20
12	F/F	0.12	0.68	0.40

The derivatives within each analog generally fit the modified Hammett equation. Figure 5 shows the comparison between the Hammett constant $\frac{1}{2}(\sigma_{p-x} + \sigma_{m-x})$ [41] and the electrochemical potentials. This analysis was repeated for all the analogs. For the DFT: B3LYP/lanl2dz predicted electrochemical potentials, the R^2 values range between 0.8873 and 0.9972 for series with at least four (4) derivatives. Although the qualitative trend is observed for the series with three derivatives, the R^2 was not calculated due to a lack of data points. The correlation between DFT: B3LYP/lanl2dz predicted wave 2 electrochemical potentials and the Hammett constants [41] weakens drastically when compared to wave 1. Compounds 6–12 have a R^2 of 0.950 (Figure 5) for wave 1 but only an $R^2 = 0.502$ for wave 2 (not shown). This correlation decrease is not observed with the experimental data [18] as the wave 2 R^2 remains >0.9.

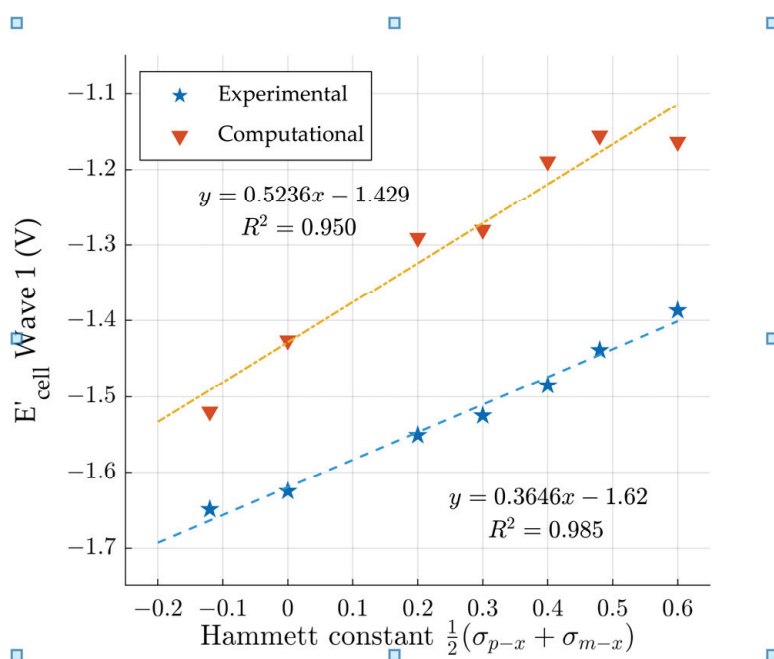


Figure 5. Comparison of the Hammett constant $\frac{1}{2}(\sigma_{p-x} + \sigma_{m-x})$ [41] vs computationally predicted and experimental [18] electrochemical wave 1 cell potentials.

3.5. Comparison to Experimental Anti-Tuberculosis Data

In 2011, Crawford et al. used IC_{90} and CC_{50} assays to quantify the anti-tuberculosis activity of these compounds [18]. The IC_{90} was defined as a reduction in fluorescence of 90% compared to a control against *M. tuberculosis* H₃₇Rv (ATCC 27294) using the Microplate Alamar Blue Assay. Compounds with $IC_{90} \leq 10$ $\mu\text{g/mL}$ were considered “active” for anti-tuberculosis activity and evaluated with the CC_{50} assay. The CC_{50} was determined using the VERO cell cytotoxicity assay. A selectivity index (SI) value was found by dividing CC_{50} by IC_{90} and SI values ≥ 10 were recommended for further anti-tuberculosis activity testing. The thirty-seven (37) derivatives were assigned to nine (9) analogs based on their structures and attached R-group. For two of the analogs (Compounds 1–5 and Compounds 6–12), all the derivatives were considered “active.” For these 12 compounds, a correlation was observed between the ease of reduction and the anti-tuberculosis activity [18].

Figure 6 shows the comparison of predicted and experimental potentials [18] with the measured anti-tuberculosis activity [18]. In Figure 6a, the predicted and experimental potentials are plotted versus $\log(IC_{90})$. The R^2 values are determined to be 0.6 and 0.7 respectively, demonstrating that there is a moderate exponential correlation between the ease of reduction and the IC_{90} . The correlation between predicted ease of reduction and IC_{90} suggests that DFT: B3LYP/lanl2dz can moderately predict anti-tuberculous activity for some derivatives with new R6/R7 variations. Figure 6b shows comparison of the predicted and experimental potentials and the selectivity index (SI). The R^2 values are determined to be 0.2 and 0.4 respectively, demonstrating that there is low correlation between the ease of reduction and the selectivity index. The SI values are reported as a range. The range is necessary as SI is a ratio of CC_{50} and IC_{90} . The CC_{50} values are reported versus a concentration maximum [18]. The CC_{50} values are listed as >100 for Compounds 1–8 and 11 [18]. Given the lower certainty of the SI values, it is not surprising that there is less correlation with the ease of reduction.

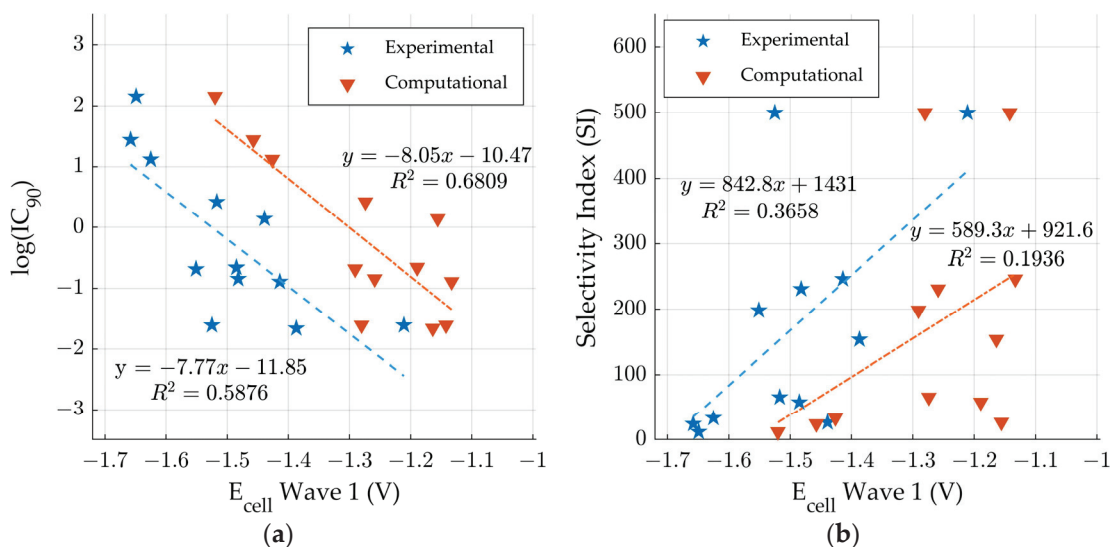


Figure 6. Comparison of computationally predicted and experimental electrochemical cell potentials [18] vs. anti-tuberculosis activity for compounds 1–12 [18]. (a) $\log\text{IC}_{90}$ vs wave 1 and (b) SI vs wave 1.

While it is likely that the ease of reduction is important for the mechanism for reaction for the quinoxaline derivatives through the formation of the free radical in wave 1 [18], an expectation for a “perfect” correlation between ease of reduction and activity would be unrealistic. For in vivo systems, additional factors including solubility, membrane permeability, absorption, stereochemistry, and active site binding will play a role in observed anti-tuberculosis activity.

4. Conclusions

For the 1st n-oxide reduction (wave 1), the DFT: B3LYP/lanl2dz calculations were able to predict the electrochemical properties for thirty-seven 1,4-di-N-oxide quinoxaline-2-carboxamide derivatives with good agreement to the experimental electrochemical potentials. The correlation was improved over our original study of twenty 3-aryl-quinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives that includes some derivatives with a nitro group [29]. The lanl2dz basis set is a useful computational tool for making a first-level prediction for electrochemical properties for quinoxaline 1,4-di-N-oxide derivatives without attached nitro-groups. The predicted wave 1 electrochemical potentials had a moderate exponential correlation to the experimental IC_{90} values similar to the experimental potentials. This correlation suggests that DFT: B3LYP/lanl2dz predictions of the wave 1 electrochemical potentials could moderately predict new R6/R7 derivatives with anti-tuberculosis activity.

Author Contributions: Conceptualization, F.P., P.W.C. and C.M.R.; methodology, F.P. and C.M.R.; software, F.P., J.M.K. and C.M.R.; validation, F.P.; formal analysis, F.P., P.W.C., J.M.K. and C.M.R.; investigation, F.P., J.M.K. and C.M.R.; resources P.W.C., J.M.K. and C.M.R.; data curation, F.P., J.M.K. and C.M.R.; writing—original draft preparation F.P., P.W.C., J.M.K., G.R.C. and C.M.R.; writing—review and editing F.P., P.W.C., J.M.K., G.R.C. and C.M.R.; visualization, F.P., J.M.K. and C.M.R.; supervision, P.W.C. and C.M.R.; project administration, C.M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to data size.

Acknowledgments: The authors acknowledge Victor Batista from Yale University for his guidance. Some of the computation for this work was performed on the high-performance computing infrastructure provided by Research Support Solutions and in part by the National Science

Foundation under grant number CNS-1429294 at the University of Missouri, Columbia MO. DOI: <https://doi.org/10.32469/10355/69802>.

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

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Article

Identification of Constituents and Evaluation of Biological Activity of *Piptadenia stipulacea* (Benth.) Ducke Ethanol Extract

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Abstract: Secondary metabolites such as flavonoids bring a range of biological properties to natural products, making them potential candidates for the pharmaceutical industry. *Piptadenia stipulacea* (Benth.) Ducke is well known in Brazil as Jurema Branca, and yet few studies have investigated its biological and phytochemical properties. This study aimed to characterize and evaluate the biological properties of ethanolic extract obtained from the bark of Jurema Branca. Characterization was performed by qualitative phytochemistry, HPLC, and mass spectroscopy. The antibacterial properties were investigated by microdilution method, cytotoxicity by MTT method, biocompatibility testing with human erythrocytes was performed, and antioxidant properties were investigated using DPPH and ABTS radical scavenging. The phytochemical tests demonstrated that rhamnetin and luteolin were the main constituents of the extract. This is the first report of these compounds in this species. The extract presented activity against *Staphylococcus aureus* (MIC = 500 µg/mL) and demonstrated activity against human colorectal adenocarcinoma (HCT-116), prostate adenocarcinoma (PC-3), and acute myeloid leukemia (HL-60) cell lines with IC₅₀ of 37.96, 37.6, and 27.82 µg/mL, respectively, for this *Piptadenia* genus. Additionally, the extract presented excellent biocompatibility and antioxidant activity (IC₅₀ = 956.7 and 147.2 µg/mL in DPPH and ABTS methods, respectively). These results are novel for the *Piptadenia* genus and pave the way for further evaluations regarding the biological importance of this species.

Keywords: Fabaceae; phytochemistry; flavonoids; antibacterial; cytotoxicity; antioxidant

1. Introduction

Throughout human history, natural plant products have remained attractive alternatives for the prevention and treatment of disease, and they play an important role in the development of modern medicine. This is mainly because of the many chemical structures present in their compositions [1]. Plants belonging to the Fabaceae and Mimosoidae subfamilies are richly constituted with secondary metabolites such as tannins,

flavonoids, alkaloids, and saponins [2]. The antimicrobial, antioxidant, antitumor, antiparasitic, and anti-inflammatory properties of these constituents are well-documented in the literature [3–5].

Piptadenia stipulacea (Benth.) Ducke, popularly known as Jurema Branca, belongs to the Fabaceae family and Mimosoideae subfamily. The plants or shrubs range from 2 to 4 m high, with light brown bark, alternating leaves, and white flowers [6]. *P. stipulacea* is endemic to the Caatinga and is widely found in northern and northeastern Brazil. The species has been used historically as a hallucinogen in indigenous rituals in mixtures such as Jurema wine and Ayuhasca. It is also used in agriculture to produce biomass [7], charcoal [8], for feeding goats, and as pasture [9].

The Brazilian northeast presents great biodiversity, harboring a wide variety of plant species with medicinal properties that are traditionally used by the local population. By analyzing chemical composition and pharmacological activity, it is possible to identify active substances and understand their therapeutic effects. This not only provides a scientific basis for the use of these plants in traditional medicine but also paves the way for the discovery of new bioactive compounds with pharmacological potential.

The literature reports that all parts of *P. stipulacea* present biological properties that can be attributed to the presence of secondary metabolites, principally tannins and flavonoids [10]. In popular medicine, the species is applied as a cicatrizing agent [6], as a sedative, and as an anti-inflammatory [11]. Due to its wide range of biological properties, and its antioxidant [12], antimicrobial [13], and antitumor potential [14], secondary metabolites obtained from *P. stipulacea* may be an alternative source for many biologically active agents.

Considering its regional prevalence and the lack of studies regarding the pharmacology of this plant, this study aimed to characterize and identify the major constituents of the ethanolic extract of *P. stipulacea* and investigate its antibacterial, cytotoxic, hemolytic, and antioxidant properties.

2. Materials and Methods

2.1. Collection and Identification of the Plant Material

The species *P. stipulacea* is represented in Figure 1. The plant was collected in April 2017 in the municipality of Poranga, in the state of Ceará, Brazil (coordinates: 4°45'25" S / 40°57'26" W). The plant material was identified, and the exsiccate was archived at the Parnaíba Delta Herbarium (HDELTA) of the Parnaíba Delta Federal University—UFDPA, under voucher specimen 4814. The genetic access was registered on the Sisgen platform, with registration number A11C0A7.

2.2. Preparation of the *P. stipulacea* Ethanolic Extract (EEPs)

The *P. stipulacea* bark was washed, dried in an oven at 50 °C for 4 days, and ground in a manual mill (IKA A11, BIOVERA®, Ramos, Brazil). The ethanolic extract was obtained by cold extraction [15]. The grounded material was mixed in ethanol P.A. (1:10, *w/v*) and left under stirring for 3 days (5 min/day). Subsequently, the content was filtered using a qualitative QUALY® filter paper (80 g/m²) and dried in a water bath at 50 °C. The resulting solution was then placed in a vacuum centrifuge (Eppendorf Vacufuge Plus 5305 Concentrator Lab Centrifuge) to remove the excess solvent. The EEPs presented a 30% yield and was used for phytochemical characterization tests and evaluation of biological properties. The material was protected from light during all processes.



Figure 1. Images of *Piptadenia stipulacea* (Benth.) Ducke (A), barks (B) and leaves (C).

2.3. Characterization

2.3.1. Qualitative Phytochemistry

The presence of secondary metabolites in the EEPs was evaluated qualitatively [15]. Tests evaluated the presence of tannins and phenols by adding 1% FeCl_3 . The formation of a dark precipitate with a blue shade indicated the presence of hydrolysable (pyrogallol) tannins, while green tones suggested the presence of phlobaphenic (condensed) tannins. Additionally, anthocyanins, anthocyanidins, flavonoids, chalcones, and aurones were identified based on pH changes. Acidification was performed by adding 5% HCl to reach pH 3, while alkalization was achieved by adding 10% NaOH to reach pH 8.5 and pH 11, respectively. The presence or absence of flavonoids (by class) was expressed according to colorimetric change reactions [15]. Saponins constitution was observed by vigorous shaking of the extract solubilized in distilled water for 5 min in a hermetically sealed tube. The presence of saponins could then be observed by formation of a stable layer for more than half an hour. Alkaloids were detected by addition of a 5% HCl solution to the extract and adding Bouchardat and Dragendorff reagents, with the presence of alkaloids confirmed through respective formations of a reddish-orange precipitate and brick red precipitates. Polysaccharides and anthraquinones were detected using lugol and solutions of toluene and 10% NH_4OH , respectively. The appearance of a blue color indicates the presence of polysaccharides, and a pink, red, or violet color indicates the presence of anthocyanidins.

2.3.2. High Performance Liquid Chromatography (HPLC)

The chromatographic profile of the EEPs was obtained with a Shimadzu liquid chromatograph (Shimadzu®, Tokyo, Japan), equipped with a UV-vis detector (SPD-20A), a binary pump system (LC-6AD), and an automatic sampler (SIL-10AF). Sample separation was performed with a Phenomenex-Luna C18 RP column (250×4.6 mm, $5 \mu\text{m}$) at room temperature (25 ± 2 °C). The mobile phase consisted of acetonitrile + 0.05% trifluoroacetic acid (pump A) and ultrapure water + 0.1% formic acid (pump B). Samples were analyzed using a 0–100% gradient condition, with a 1 mL/min flow and 50 min runtime. UV-vis detection was performed at 218 nm and 269 nm. For the analysis, samples were dissolved

in ethanol (1 mg/mL), filtered with 0.45 µm PTFE syringe filters, and injected (20 µL). All solvents used were of HPLC grade.

2.3.3. Mass Spectrometry

The mass spectra were obtained by the direct infusion of the sample (1 mg of EEPs to 10 mL of MeOH) into a mass spectrometer with an ion trap analyzer (Amazon X, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (DI-ESI-IT-MSn) in the negative ion mode, an m/z range of 100–2000 Da, syringe flow of 3.0 µL min^{−1}, capillary voltage of 4.5 kV, an 8.0 L min^{−1} drying gas flow (N₂), nebulization pressure of 10.0 psi, and a source temperature of 220 °C.

2.4. Biological Activity

2.4.1. Determination of Antibacterial Potential by Microdilution Broth

Bacterial strains were maintained on Mueller–Hinton agar and incubated at 37 °C for 24 h. Antibacterial activity was determined against *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) [16] using similar [5] concentrations and conditions. Oxacillin and Meropenem were used respectively as positive controls for the Gram-positive and Gram-negative strains. The plates were incubated under aerobic conditions, at 37 °C, for 24 h and the minimum inhibitory concentration capable of inhibiting visible bacterial growth (MIC) was determined.

2.4.2. Evaluation of Cytotoxicity by MTT Method

The cytotoxic potential of the EEPs was tested against four human tumor cell lines, obtained from the National Cancer Institute. HL-60 (Acute Myeloid Leukemia), HCT-116 (Colorectal Adenocarcinoma), PC-3 (Prostate Adenocarcinoma), and SF-295 (Glioblastoma) were cultivated in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) and supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin) at 37 °C and 5% CO₂.

The MTT method [17] was performed under similar [5] concentrations and conditions. Briefly, a serial dilution (ratio 2 to 50 µg/mL) EEPs was added to the human cell lines for 69 h. An MTT solution (0.5 mg/mL) was then added for 3 h, totaling 72 h of treatment. Absorbance was read at 595 nm after solubilization of formazan crystals using DMSO, in a Beckman Coulter Inc., spectrophotometer—model DTX-880, and the OSoftware program 1.0 (Beckman Coulter Inc., Brea, CA, USA). Results were expressed in IC₅₀ (half maximal inhibitory concentration). Doxorubicin was used as the positive control.

2.4.3. Hemolysis Assay for Human Biocompatibility

To determine the hemolytic potential of the EEPs, a hemolysis assay was performed according to [18] under the same concentrations and conditions as [5]. Triton X (0.1%) and saline (0.85%) were used as positive and negative controls, respectively. The EC₅₀ (half-maximum effect) was calculated using GraphPad Prism 8. The investigations were performed following the rules of the Declaration of Helsinki of 1975, which was revised in 2013, and the procedure was approved by the research ethics committee of the Parnaíba Delta Federal University, under protocol number 5.114.527.

2.4.4. In Vitro Antioxidant Activity

Antioxidant Potential—DPPH Radical Scavenging

DPPH (1,1-diphenyl-β-picrylhydrazine, Sigma Aldrich) method was performed [19] under the same concentrations and conditions as [5]. Absorbance was measured at 517 nm using an ELISA reader (SPECTRAMAX 290). Trolox (6-hydroxy 2,5,7,8tetramethylchroman-

2-carboxylic acid) was used as negative control and the IC₅₀ values were calculated using GraphPad Prism 8, with 95% confidence.

Antioxidant Potential—ABTS Radical Scavenging

The ABTS radical (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was prepared according to [19] and under the same concentrations and conditions as [5]. Absorbance was measured at 734 nm (SPECTRAMAX 290 ELISA plate). Trolox was used as the reference antioxidant. The IC_{50s} values were calculated by GraphPad Prism 8, with 95% confidence.

2.5. Statistical Analysis

The experiments were analyzed according to the mean $\pm$ standard deviation of the mean (SD) of percentage growth inhibition using the GraphPad Prism 8 program from two independent experiments, in triplicate.

2.6. Reagents

The following reagents were utilized: Ethyl alcohol P.A (C₂H₆O, Dinamica, Indaiatuba, São Paulo, Brazil), Iron(III) chloride solution (FeCl₃, Dinamica), Hydrochloric acid (HCl, Dinamica), Sodium Hydroxide (NaOH, Dinamica), Bouchardat reagent, Dragendorff reagent, Lugol (Dinamica), Toluene (Dinamica), Ammonium Hydroxide (NH₄OH, Êxodo Científica, Sumaré, São Paulo, Brazil), Acetonitrile HPLC grade (CH₃CN, Êxodo Científica), Trifluoroacetic Acid HPLC grade (CF₃CO₂H, Dinamica), Formic Acid (CH₂O₂, Sigma Aldrich), Methanol HPLC grade (MeOH, Sigma-Aldrich), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Invitrogen, Waltham, MA, USA), DMSO (Dimethyl sulfoxide, Êxodo Científica), Triton-X-100 (C₁₄H₂₂O(C₂H₄O)_n(n = 9–10), Êxodo Científica), DPPH (1,1-diphenyl- β -picrylhydrazine, Sigma Aldrich), Trolox (6-hydroxy 2,5,7,8tetramethylchroman-2-carboxylic acid, Sigma Aldrich), ABTS radical (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, Sigma Aldrich)).

3. Results

3.1. Phytochemical Characterization

A qualitative phytochemical assay to determine the presence of secondary metabolites was performed and the results are represented in Table 1. It is possible to observe that flavonic compounds, such as flavones, flavonoids, and xanthenes, are constituents present in the *P. stipulacea* ethanolic extract (EEPs). We did not observe the presence of tannins, phenols, antocyanins and antocyanidins, chalcones, aurones, saponins, alkaloids, polysaccharides, or anthraquinones, possibly because of the extraction method or some environmental factor.

Table 1. Phytochemical characterization of *Piptadenia stipulacea* (Benth.) Ducke ethanol extract. (-) Not detected; (+) Detected.

Secondary Metabolites	EEPS
Flobabenic Tannins	-
Pyrogalic Tannins	-
Phenols	-
Antocyanins and Antocyanidins	-
Flavones, Flavonols, Xanthenes	+
Chalcones and Aurones	-
Saponins	-
Alkaloids	-
Polysaccharides	-
Anthraquinones	-

The chromatographic profile of the EEPS was obtained by high-performance liquid chromatography (HPLC) and is represented in Figure 2. It is possible to observe the presence of different peaks with retention times from 38 to 48 min and different intensities, with a majority peak at 42 min. The difference in the intensity of the peaks probably indicates a difference in the concentration of the compounds.

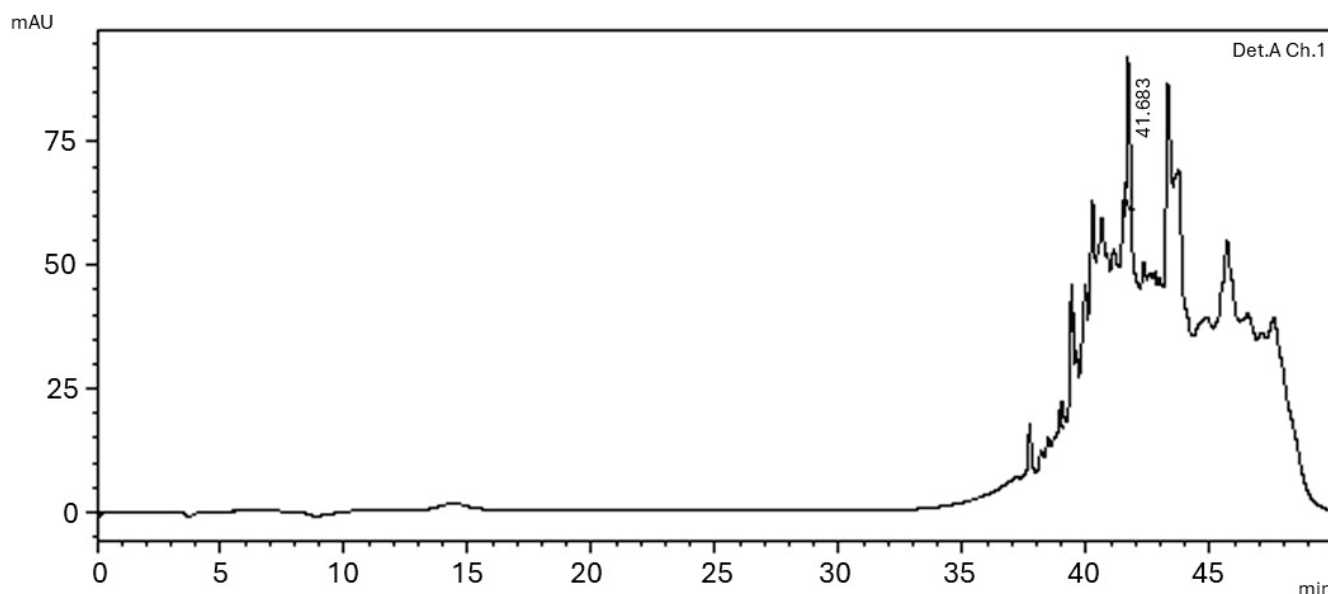


Figure 2. Chromatographic profile of the *Piptadenia stipulacea* (Benth.) Ducke ethanolic extract. Mobile Phase: ACN- $\text{CF}_3\text{CO}_2\text{H}$ (0.05%)/ H_2O - HCO_2H (0.1%), gradient: 0–50 min, 0–100% ACN, injection volume 20 μL , flow rate 1 mL min^{-1} and $\lambda = 218 \text{ nm}$.

We also performed a full Mass Spectroscopy scan with Ion Trap Analyzer and Electrospray Ionization Source (DI-ESI-IT-MS) to identify the compounds in the EEPs. The results are represented in Figure 3 and Table 2. The mass spectrum presented 20 peaks and intensities corresponding to 20 metabolites.

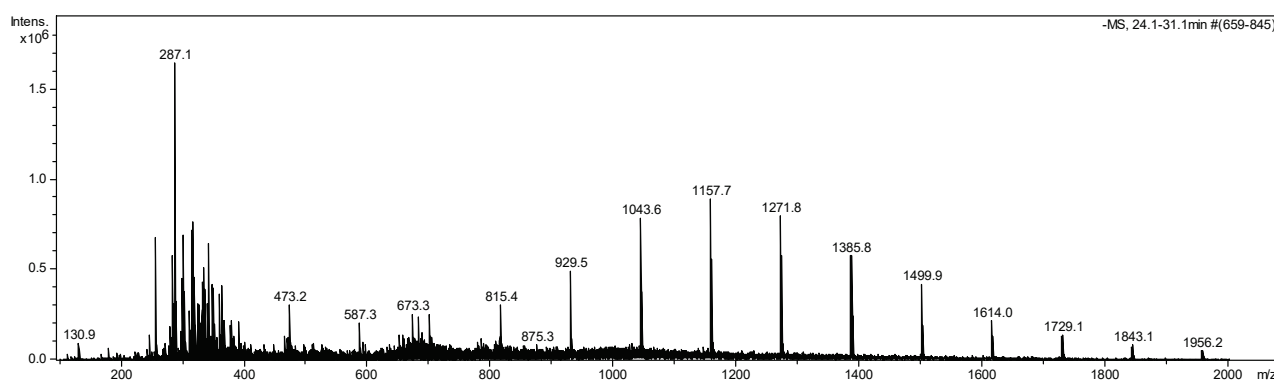
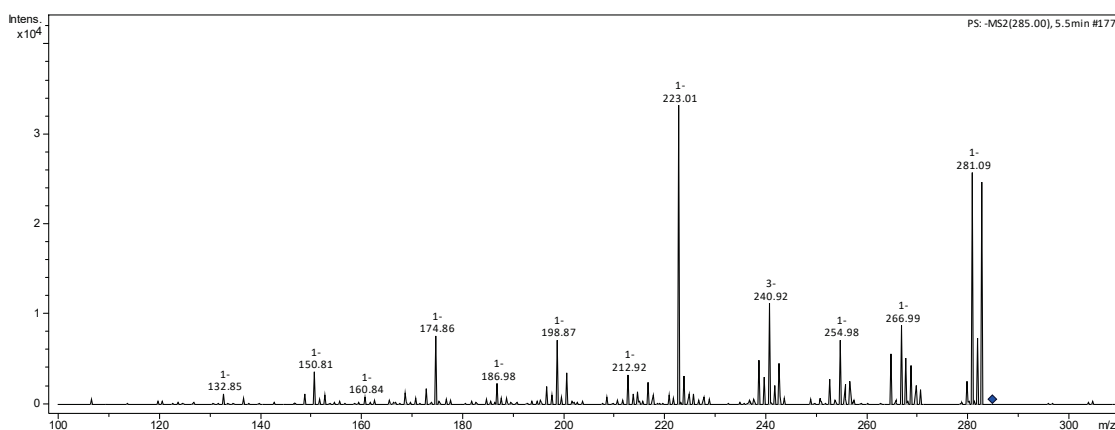


Figure 3. Full scan DI-ESI-MS/MS $[\text{M}-\text{H}]^-$ from the *Piptadenia stipulacea* (Benth.) ethanolic extract, m/z 100–2000. Fingerprint obtained by DI-ESI-IT-MS/MS in negative mode.

Considering the fragmentation patterns, deprotonated molecule ions, and fragments, it was possible to observe five compounds, three being flavonoids (compounds 1–3) and two being caffeic acid derivatives (4–5). Based on the literature consulted, these compounds correspond to luteolin (1) [20]; rhamnetin (2) [20,21]; a luteolin derivative (3) [20]; caffeic acid hexoside-O-pentoside (4) [22]; and caffeic acid hexoside dimer (5) [23]. The spectra of the five noted compounds are shown in Figures 4–8.

Table 2. Annotated chemical constituents of the ethanolic extract of the stem bark of *P. stipulacea* by DI-ESI-IT-MSⁿ; N.I.: not identified.

[M–H] [–]	MS ⁿ (m/z)	Identification	Reference
285	267 (30), 255 (24), 241 (33), 223 (100), 217 (19) 199 (13), 175 (19), 151 (16), 133 (8)	luteolin	[20]
287	269 (24), 255 (16), 241 (35) 223 (100), 189 (9), 175 (37), 151 (11)	N.I.	
301	284 (100), 271 (24), 257 (60) MS ³ [257] 239 (77), 215 (34), 189 (83), 201 (35), 175 (100)	N.I.	
315	300 (59), 271 (100), 267 (19), 223 (15), 203 (3), 125 (4)	rhamnetin	[21]
359	311 (48), 285 (79), 267 (15), 245 (100), 223 (18), 131 (91) MS ³ [245] 131 (100), 113 (7)	luteolin derivative	
447	415 (40), 401 (44), 347 (48), 293 (100), 285 (30), MS ³ [415] 371 (21), 347 (100)	N.I.	
473	397 (21), 359 (63), 341 (100), 245 (51) MS ³ [341] 179 (100), 161 (23), 143 (20), 113 (24) MS ⁴ [359] 245 (50), 131 (80)	caffeic acid hexoside-O-pentoside	[22]
587	473 (42), 391 (23), 359 (42), 341 (100), 245 (50) MS ³ [473] 359 (100), 245 (71) 131 (33)	N.I.	
609	571 (29), 517 (16) 477 (100), 341 (51) 255 (82) MS ³ [477] 293 (100)	N.I.	
673	341 (22), 331 (25), 287 (100) MS ³ [331] 301 (13), 287 (100)	N.I.	
683	347 (18), 341 (100), 331 (28), 287 (51) MS ³ [341] 179 (100), 161 (23), 143 (18), 113 (14)	caffeic acid hexoside dimer	[23]
701	655 (16), 587 (52), 473 (45), 359 (100), 341 (53), 287 (59) MS ³ [587] 473 (100), 359 (65), 245 (39)	N.I.	
815	701 (47), 683 (26), 587 (39), 473 (100), 359 (46) MS ³ [701] 587 (100), 473 (42), 359 (49), 245 (71)	N.I.	
929	815 (63), 701 (53), 587 (100), 473 (64), 359 (59) MS ³ [815] 701 (100), 587 (34), 473 (48), 359 (98), 245 (26)	N.I.	
1043	929 (69), 815 (68), 701 (100), 587 (77), 473 (82), 359 (74) MS ³ [929] 815 (63), 701 (27), 587 (100), 473 (99), 359 (40)	N.I.	
1157	1043 (75), 929 (65), 815 (93), 701 (92), 587 (87), 473 (100), 359 (70) MS ³ [1043] 929 (57), 815 (100), 701 (47), 587 (49), 473 (88), 359 (32)	N.I.	
1271	1157 (55), 1043 (63), 929 (89), 815 (64), 587 (77), 473 (100), 359 (35) MS ³ [1157] 929 (37), 815 (28), 473 (100)	N.I.	
1385	1271 (73), 1157 (43), 1043 (100), 929 (65), 815 (77), 701 (84), 587 (75), 473 (63) MS ³ [1043] 929 (38), 587 (100)	N.I.	
1501	1385 (39), 1271 (34), 1157 (100), 1043 (39), 929 (40), 815 (57), 701 (45), 587 (44), 473 (31)	N.I.	
1615	1501 (33), 1385 (22), 1271 (100), 1157 (30), 1043 (21), 929 (39), 815 (40), 701 (53), 587 (35), 473 (23) MS ³ [1271] 815 (100), 701 (26)	N.I.	

**Figure 4.** MS2 mass spectrum of ion m/z 285 referring to luteolin (1).

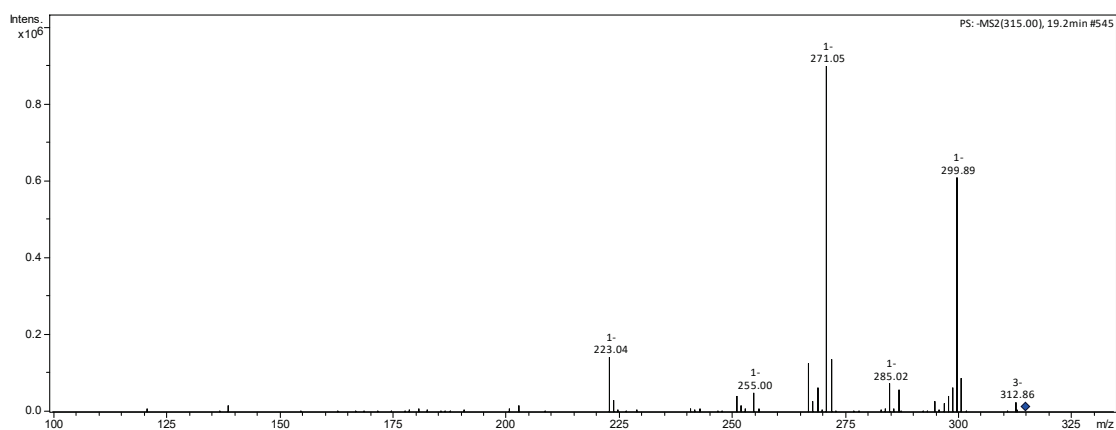


Figure 5. MS2 mass spectrum of ion m/z 315 referring to rhamnetin (2).

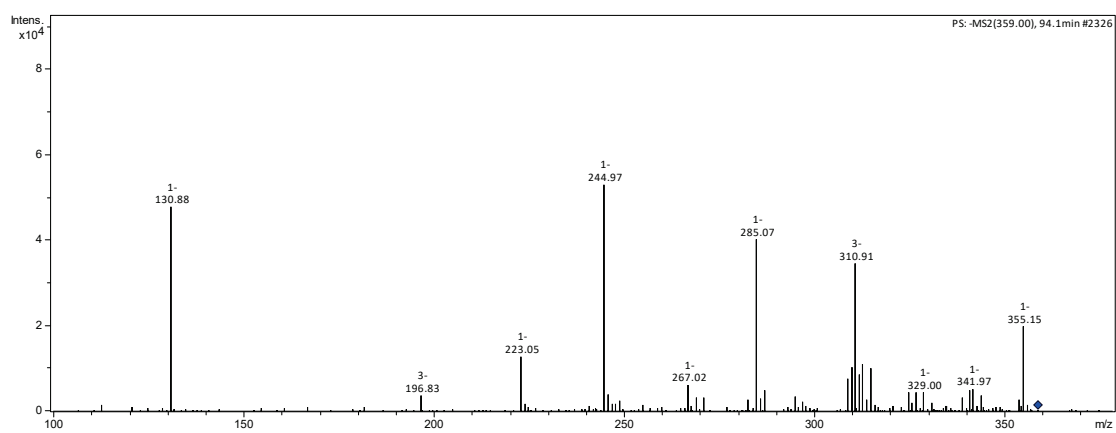


Figure 6. MS2 mass spectrum of ion m/z 359 referring to luteolin derivative (3).

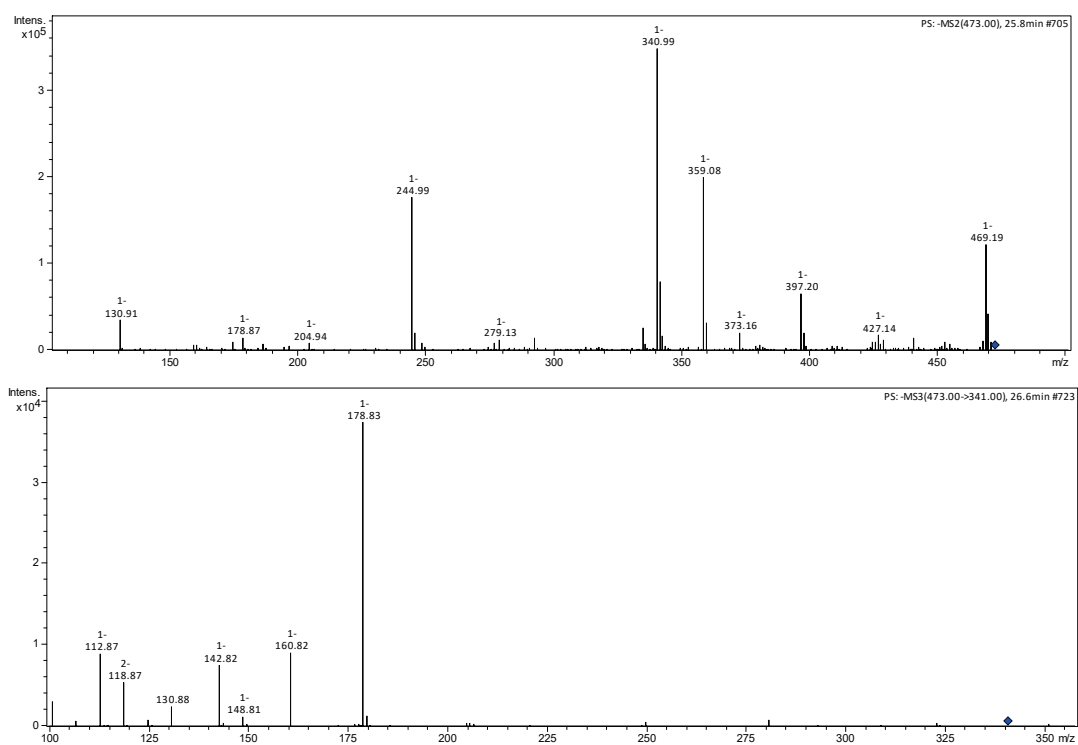


Figure 7. MS2 and MS3 mass spectra of ions m/z 473 and 341 referring to hexoside-O-pentoside caffeic acid (4).

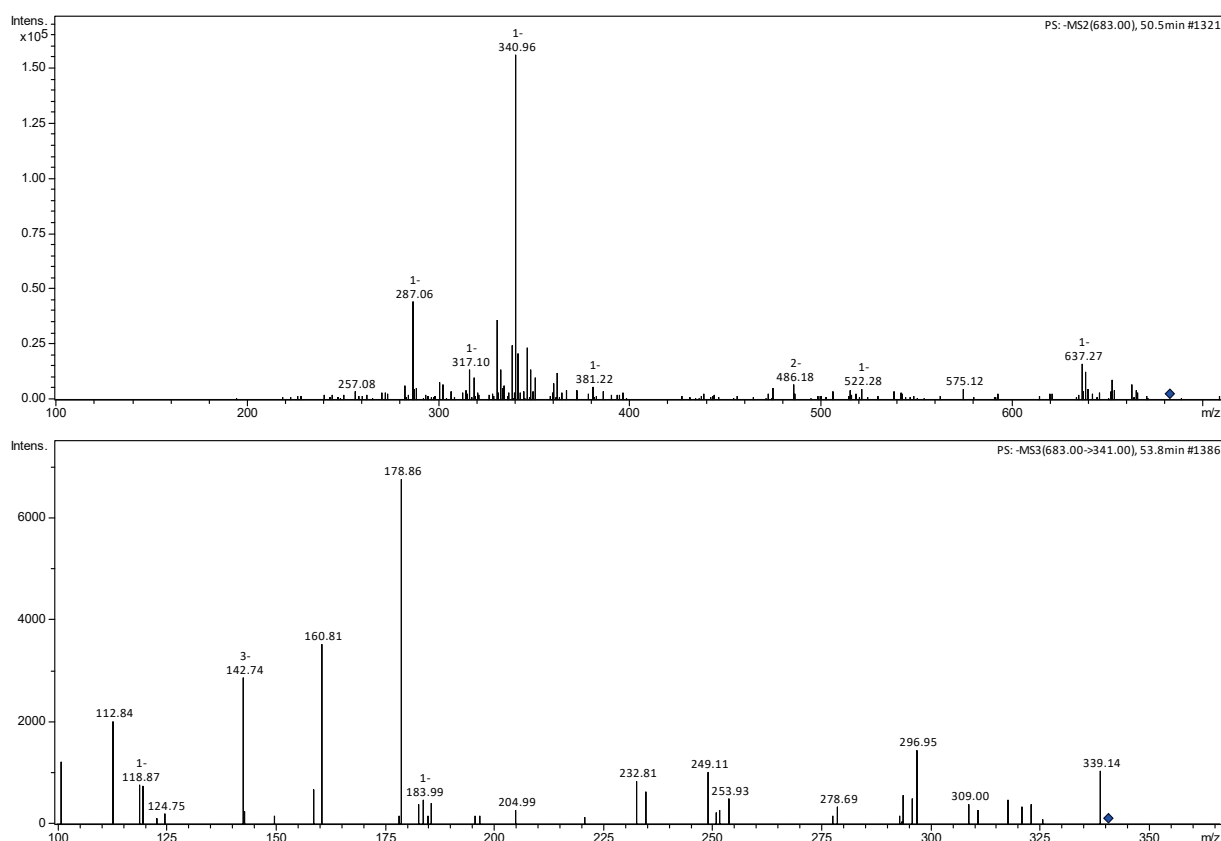


Figure 8. MS2 and MS3 mass spectra of ions m/z 683 and 341 referring to hexoside caffeic acid dimer (5).

Compounds **1** and **2** present a fragmentation pattern characteristic of flavonoids belonging to the flavone class. Compound **1** presented as a deprotonated molecule with m/z 285 $[M-H]^-$ and fragmentation ions at m/z 151 and m/z 125, produced by RDA cleavage of the C ring. The neutral loss of H_2O generating the ion m/z 267 was identified as luteolin (Figure 2). Compound **2** presented the ion m/z 359 $[M-H]^-$ and fragment ions m/z 285, 267, 241 and 223, similar to those of the previous compound, suggesting that this compound was derived from luteolin [20].

Compound **3** was identified as rhamnetin. Compound **3** produced m/z 315 $[M-H]^-$ with fragmentation ions m/z 300 $[M-H-CH_3]^-$, 271 $[M-H-CO_2]^-$ and 125 from RDA cleavage of the C ring [20,21].

Compound **4** presented an ion of the deprotonated molecule at m/z 473 $[M-H]^-$, with characteristic fragmentation ions at m/z 455 $[M-H-H_2O]^-$, 427 $[M-H-CO_2]^-$ and 341 $[M-H-pentose]^-$. The MS3 spectrum of the m/z 341 ion presented fragmentation ions m/z 179 $[M-H-Hexose]^-$, corresponding to aglycone, and 161 $[M-H-Hexose-H_2O]^-$, corresponding to the loss of a water molecule, and being identified as caffeic acid hexoside-O-pentoside [22]. Compound **5** was identified as a dimer of caffeic acid hexoside by the m/z 683 $[M-H]^-$ ion and fragmentation ions m/z 341, corresponding to a unit of caffeic acid hexoside and 179 corresponding to the aglycone [22,23].

It was also possible to characterize the structure of compounds **1** (luteolin), **2** (rhamnetin), and **4** (hexoside-O-pentoside caffeic acid) (Figure 9), identified in the ethanolic extract, based on comparisons of DI-ESI-IT-MSn data with the corresponding fragmentation patterns and the pathways reported by [20,21,23].

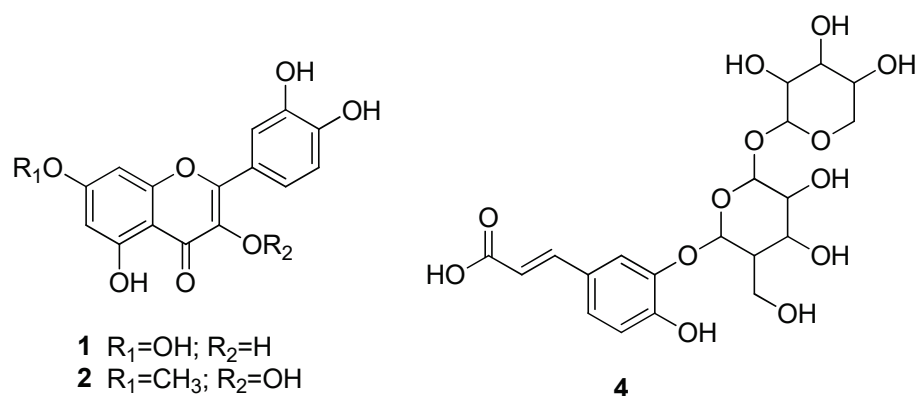


Figure 9. Structure of compounds **1** (Luteolin), **2** (Rhamnetin), and **4** (hexoside-O-pentoside caffeic acid), identified in the ethanol extract of *P. stipulacea* stem bark.

We then conducted a comprehensive investigation into the antibacterial, cytotoxic, hemolytic, and antioxidant effects of EEPS. The objective was to gain insights into the broad and traditional utilization of *P. stipulacea* and facilitate in-depth studies on its properties, considering the dearth of reports in the existing literature.

3.2. Biological Activities

3.2.1. Evaluation of Antibacterial Properties

Plants belonging to the Fabaceae family are known for antimicrobial effects, usually attributed to its phenolic compounds, which were also detected in our study. We evaluated the antibacterial potential of the EEPS tested against two bacterial strains of clinical relevance (*S. aureus*—ATCC29213 and *E. coli*—ATCC25922). The minimum inhibitory concentration (MIC) is shown in Table 3.

Table 3. Minimum Inhibitory Concentration (MIC) of *P. stipulacea* (Benth.) Ducke ethanol extract against Gram-positive and Gram-negative bacterial strains. Positive control: Oxacillin^a and Meropenem^b.

Bacterial Strains	MIC (µg/mL)	
	EEPS	Positive Control
<i>S. aureus</i> (ATCC 29213)	500	<0.25 ^a
<i>E. coli</i> (ATCC 25922)	>2000	<0.5 ^b

These results demonstrated that EEPS presents antibacterial effects against Gram-positive strain *S. aureus* at 500 µg/mL. The tested concentrations had no effect against the Gram-negative strain *E. coli*.

3.2.2. Cytotoxic Activity of EEPS

To determine the cytotoxicity of EEPS, the MTT test was performed against four human cancer cell lines for 72 h: HCT-116 (Colorectal Adenocarcinoma), PC-3 (Prostate Adenocarcinoma), HL-60 (Acute Myeloid Leukemia), and SF-295 (Glioblastoma). The results are presented in Table 4.

The MTT assay showed that the EEPS in concentrations ranging 0.39–50 µg/mL caused cytotoxicity in three out of the four human cancer cell lines evaluated. All the cell lines presented similar IC₅₀ values, but HL-60 was slightly more sensitive to the extract, with an IC₅₀ of 27.82 µg/mL. The extract did not present activity against SF-295 at the tested concentrations.

The hemolytic potential of the EEPS against human erythrocytes was analyzed to determine the concentration that induces half the maximum effect (EC₅₀). Hemolysis was

not observed in any of the tested concentrations of the EEPS, presenting an EC_{50} greater than 1000 $\mu\text{g/mL}$.

Table 4. Cytotoxic activity of *P. stipulacea* (Benth.) Ducke ethanol extract on human cancer cell lines and human erythrocytes. Doxorubicin was used as a positive control.

Cell Lines	IC ₅₀ ($\mu\text{g/mL}$) 95% Confidence Interval	
	EEPS	Doxorubicin
HCT-116	37.96 (35.10–41.04)	0.12 (0.09–0.16)
PC-3	37.6 (33.72–41.92)	0.44 (0.34–0.54)
SF-295	>50	0.23 (0.12–0.27)
HL-60	27.82 (24.16–32.04)	0.02 (0.005–0.01)

3.2.3. EEPS Antioxidant Activity

The antioxidant potential of EEPS was evaluated using ABTS and DPPH radicals scavenging assays. The results are represented in Table 5.

Table 5. Antioxidant potential of *P. stipulacea* (Benth.) Ducke ethanol extract in both DPPH and ABTS methods. Trolox was used as a positive control.

Samples	IC ₅₀ ($\mu\text{g/mL}$) 95% Confidence Interval	
	DPPH	ABTS
EEPS	956.7 (815.9–1122)	147.2 (127.0–170.6)
TROLOX	63.66 (57.80–69.93)	73.91 (66.19–82.32)

The results demonstrated that although EEPS did not show significant antioxidant activity in the DPPH assay (IC_{50} of 956.7 $\mu\text{g/mL}$), in the ABTS assay, the EEPS obtained an IC_{50} of 147.2 $\mu\text{g/mL}$, with a 95% confidence interval of 127–170.6 $\mu\text{g/mL}$, an antioxidant effect close to the positive control Trolox, which presented IC_{50} of 73.91 $\mu\text{g/mL}$.

4. Discussion

4.1. Phytochemical Characterization

Secondary metabolites play an important role in plant defense systems and have been used in popular medicine for therapeutic purposes due to their extensive biological activities. For this reason, secondary metabolites are one of the main sources of new drugs [24]. Phytochemical studies assess the constitution of these metabolites and certify their benefits as potential herbal medicines.

The presence of tannins, phenols, anthocyanidins, and saponins has been reported as being the main compounds obtained from *P. stipulacea* bark ethanolic extracts [6]. The presence of tannins in this type of extract [10,25] has also been reported. However, collection sites, seasonality, temperatures, soil fertility, and other factors directly influence the biosynthesis and thus the relative composition of secondary metabolites in plants [26].

Phytochemical components such as flavonoids, due to their numerous biological potentials [27], have value in medicine. Flavonoids from different plant parts (fruits, veg-

etables, stems, grains, and bark) are phenolic compounds recognized for having anticancer properties [28]; they are also antimicrobial, antiproliferative, and antioxidant agents [29], in addition to exerting neuroprotective effects [30]. Depending on their chemical structure, flavonoids are classified as flavones, flavonols, isoflavonoids, and chalcones [31].

Qualitative phytochemistry and HPLC indicated the presence of these flavonoid classes in the EEPS. Mass Spectroscopy determined that the extract was composed of the flavonoid luteolin (1), and derivatives (3) of rhamnetin (2), as well as hexoside caffeic acid-O-pentoside (4) and hexoside caffeic acid dimer (5). To our knowledge, this is the first report identifying these metabolites in *Piptadenia stipulacea* (Benth.) Ducke.

Luteolin is a natural flavone with neuroprotective [32] and antiviral properties [33]. It is important to anticancer therapies against various cell lines. In breast cancer, for example, luteolin inhibits proliferation, and protects against carcinogenic stimuli, by activating cell cycle arrest [34].

Rhamnetin is a distinguished category of flavonoids with recognized cardioprotective [35] and anti-inflammatory benefits. In cancer therapy, rhamnetin presents inhibitory effects on cell proliferation and epithelial-mesenchymal transition in lung tumor cell lines. It also induces apoptosis in breast tumor cell lines [36].

The HPLC chromatogram, shown in Figure 2, demonstrates the presence of peaks with differing intensities, and could indicate the presence of other compounds in varied concentrations. With the mass spectroscopy fingerprint, we determined the presence of caffeic acid (CA) derivatives and the flavonoids Luteolin and Rhamnetin, as previously mentioned.

Caffeic acid (CA) and its derivatives are phenolic compounds widely synthesized by plant species with known antimicrobial, antioxidant, anti-inflammatory, and anticancer properties. The anticancer effects of CA are related to the anti- and pro-oxidant capacities of its chemical structure [37].

Briefly, the methodologies employed in the current investigation confirmed the presence of phenolic compounds, particularly flavonoids, thus laying the groundwork for further biological evaluation.

4.2. Biological Activities

4.2.1. Evaluation of Antibacterial Properties

Plants belonging to the Fabaceae family are known for antimicrobial effects, usually attributed to phenolic compounds, which were also detected in our study. Refs. [6,10] demonstrated that the ethanolic extract of *P. stipulacea* bark had low antibacterial activity against Gram-negative and Gram-positive strains, such as *E. coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *S. aureus*, and *Aeromonas caviae*.

Tannins isolated from the bark of *P. stipulacea* showed a better antibacterial effect against the Gram-positive strain *S. aureus* [38]. In this aspect, it is possible to infer that the presence of tannins improves the antimicrobial effect of *P. stipulacea*. These secondary metabolites were not detected in the EEPS, which could explain the lower antibacterial activity detected in gram-negative bacteria. It is also important to emphasize that Gram-negative bacteria have a more complex outer membrane structure than Gram-positive bacteria; this characteristic provides more resistance against antimicrobial agents [39] and could also explain our results.

4.2.2. Cytotoxic Activity of EEPS

To the best of our knowledge, there are no reports of the antitumor potential of *P. stipulacea* on human cancer cell lines in literature. However, the cytotoxic potential of other species belonging to the *Piptadenia* genus has already been reported.

Hydroalcoholic and hexane extracts and dichloromethane and ethyl acetate fractions of *Piptadenia moniliformes* leaves, fruits, fruit peel, and seeds were evaluated against three human tumor cell lines, HCT-116 (colon), OVCAR-3 (ovary), and SF295 (glioblastoma), for cytotoxic activity. However, they did not present satisfactory toxicity [40]. *Piptadenia adiantoides* crude extract has also been shown to be ineffective against UACC-62, MCF-7, and TK-10 human cell lines to a concentration of 20 µg/mL [41].

The cytotoxic potential attributed to Fabaceae family is usually associated with flavonoids. These compounds have great cytotoxic potential against cancer cells, mainly by cell cycle arrest and inhibition of cell permeability [42]. In this study, the cytotoxic potential of EEPS is likely attributable to luteolin and rhamnetin, since these flavonoid compounds present cytotoxicity, as already described [43,44]. Our results indicate that among the *Piptadenia* genus, *P. stipulacea* presents promising cytotoxic activities and the application of the extract in cancer therapy is plausible.

Hemolysis assay is a preliminary trial that evaluates the cytotoxicity of many compounds. It is widely used to evaluate blood biocompatibility and the potential of samples to cause premature lysis or disruption of red blood cells of human or animal origin [45]. In the present study, hemolysis was not observed in any of the evaluated EEPS concentrations, with EC₅₀ greater than 1000 µg/mL. This effect is probably related to the diverse pool of molecules presented in the secondary metabolites of EEPS [6]. The widely varied phytochemical composition highlighted in this study could influence the biological properties of the extract.

The hemolytic activity of *P. stipulacea* ethanolic extract leaves against sheep erythrocytes has been evaluated [46,47]. It was noted that the extract did not present lysis at any of the concentrations tested. These results, together with the present study, reveal that *P. stipulacea* is safe and biocompatible. It is also important to note that this is the first study to evaluate the hemolytic properties of *P. stipulacea* against human erythrocytes.

4.2.3. EEPS Antioxidant Activity

Flavonoids play an important role in oxidative stress in plants, besides exerting potential antioxidant activity and free radical scavenging capacity [48]. Previous studies by our group have indicated that plants of the Fabaceae family exert great antioxidant potential [5].

It has been observed that alcoholic and ethyl acetate fractions of *Piptadenia moniliformis* fruit peels have antioxidant potential [40], and the aqueous fraction of *P. moniliformis* also has antioxidant potential [49]. It is important to note that the present study is the first work demonstrating the antioxidant potential of *P. stipulacea*, and that these effects may be related to the presence of the flavonoids luteolin and rhamnetin.

The antioxidant potential of *P. stipulacea* can be linked to its cytotoxic activity. Flavonoids are natural compounds capable of promoting redox and ROS level alterations in cancer cells, and subsequently suppress signaling pathways responsible for tumor survival and tumorigenesis [50].

Some studies have reported that luteolin and rhamnetin exhibit antioxidant mechanisms in cancer cells, showing correlations with mitochondrial dysfunction, alterations in epithelial-to-mesenchymal transition (EMT), and changes in key biomarkers such as malondialdehyde (MDA), superoxide dismutase (SOD), and the antioxidant enzyme catalase (CAT) [51,52]. Based on these studies, it can be suggested that the flavonoids luteolin and rhamnetin, present in the ethanol extract of *Piptadenia stipulacea* (EEPS), may target redox imbalances in tumor cells. However, further studies are needed to confirm these potential mechanisms.

5. Conclusions

In the present study, we obtained an ethanolic extract of *P. stipulacea* primarily composed of phenolic compounds. Mass spectrometry identified three flavonoids (rhamnetin, luteolin, and a derivative) as well two caffeic acid derivatives, the latter identified for the first time in this species.

The biological assays demonstrated firsthand a desirable cytotoxic effect against three human cancer cell lines (HCT-116, PC-3, and HL-60) with good antioxidant activity, antibacterial activity against gram-positive bacteria *S. aureus*, and with no observed hemolytic effects. These findings may be related to the presence of the identified flavonoids. This study opens the way for further evaluations to deepen our knowledge concerning the use of this plant in biomedicine.

Author Contributions: Conceptualization: S.A.d.N.M.S. and J.D.B.M.F.; Methodology: S.A.d.N.M.S., A.B.B., J.M.T.S., A.F.M., R.F.S. and E.d.S.M.F.; Validation: A.R.d.A., M.H.C., D.A.d.S., A.J.A. and J.D.B.M.F.; Formal analysis: J.D.B.M.F. and A.J.A.; Investigation: S.A.d.N.M.S.; Resources: A.R.d.A., M.H.C., D.A.d.S., A.J.A. and J.D.B.M.F.; Data curation: S.A.d.N.M.S., A.B.B., J.M.T.S., A.F.M., R.F.S. and E.d.S.M.F.; Writing-original draft: S.A.d.N.M.S.; Writing-review and editing: S.A.d.N.M.S., J.D.B.M.F., A.J.A. and J.M.T.S.; Visualization: S.A.d.N.M.S.; Supervision: J.D.B.M.F.; Project administration: J.D.B.M.F. and A.J.A.; Funding acquisition: J.D.B.M.F. and A.J.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ); Instituto Nacional de Ciência e Tecnologia em Biodiversidade e Produtos Naturais (INCT-Bionat) [CNPQ Grant Number 465637/2014-0, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grant Number 2014/50926-0].

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Instituto Nacional de Ciência e Tecnologia em Biodiversidade e Produtos Naturais (INCT-Bionat), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Piauí (FAPEPI) for the support of this work.

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

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Article

Protective Effect of Biflavones from *Platonia insignis* Mart. against Ethidium Bromide-Induced Lethality in *Staphylococcus aureus*

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Abstract: Brazilian flora are highly diverse and have great potential for research into new medicines of plant origin. *Platonia insignis* Mart. is a fruit tree from the Amazon region with several potential pharmacological applications already demonstrated. The present study aimed to investigate the protective effect of a mixture of the biflavones volkensiflavone and moreloflavone obtained from *P. insignis* flowers against the lethality induced by ethidium bromide in efflux pump overproducer strains of *Staphylococcus aureus*. Microdilution tests were performed to determine the minimum inhibitory concentration of ethidium bromide in the absence or presence of biflavones. The addition of biflavones to the growth medium increased the minimum inhibitory concentration of ethidium bromide against all strains tested, indicating that biflavones protected the bacterial cells against cell damage induced by this biocide agent. Comparative analysis of the infrared absorption spectra of each isolated compound, as well as of the associated compounds, demonstrated the complexation reaction, indicating that there was a preferential interaction between the H₂N- groups of primary amines of ethidium bromide with the carbonyls of biflavones that formed imines -C=NH. Such complexation could reduce the amount of free ethidium bromide molecules available for DNA intercalation, resulting in the observed protective effect. These results suggest a possible technological application of the biflavones from *P. insignis* as protective agents against cell damage induced by ethidium bromide.

Keywords: natural products; flavones; cytoprotection; genotoxicity

1. Introduction

Ethidium bromide (EtBr) is a tricyclic aromatic compound of the phenanthridine class showing aniline groups on either side of a pyridine, represented by the formula C₂₁H₂₀BrN₃. It is also a strong DNA intercalating agent [1]. Intercalation of EtBr in the DNA double helix results in increased fluorescence emission, and for this reason, this compound is widely used as an organic fluorescent dye for visualizing DNA on agarose gel [2,3]. These changes in the spectrum contradict what was expected, which was that its fluorescence would be partially reduced by interactions with the solvent's molecules when in solution [4]. Studies indicate that binding between EtBr and DNA occurs mainly through intercalation between base pairs in DNA, but electrostatic binding can also occur [5].

Despite its wide use, this molecule has high toxicity, mutagenic activity and ability to inhibit RNA synthesis [6]. Other studies report that EtBr is capable of inhibiting DNA synthesis as well as causing selective inhibition of mitochondrial DNA and kinetoplasmic

DNA. EtBr is able to inhibit the formation of mitochondrial cytochrome oxidase and to selectively inhibit specific phases of RNA synthesis or processing [7]. It is also an inhibitor of DNA polymerase enzyme and DNA-dependent RNA polymerase enzyme by binding to template DNA [2]. Furthermore, it inhibits cell growth in tissue cultures, induces mutation by altering the gene reading frame and causes the development of chromosomal abnormalities [6].

Species of Brazilian flora are widely evaluated in relation to the pharmacological effects of their plant extracts and essential oils as well as their isolated phytoconstituents, aiming at the development of new drugs. *Platonia insignis* Mart. (Clusiaceae) is a fruit and lumber plant from the Amazon rainforest, popularly known as bacurizeiro [8]. The distribution of this species was recorded in the states of Amazonas, Amapá, Pará, Maranhão, Piauí, Tocantins and Mato Grosso [9,10]. In Piauí, its occurrence was reported in a wide area of cerrado in the north-central region of the state [11].

Different biological activities were reported for the species *P. insignis*, such as leishmanicidal activity against *Leishmania amazonensis* [12], a hypotensive effect on the cardiovascular system of rats [13] and antioxidant activity [14]. It also has anticonvulsant, antidepressant, stimulatory and protective effects on the central nervous system [15,16] as well as cytotoxic activity [17].

In a previous study performed by our research group, we isolated and identified a mixture of biflavonoids (volkensiflavone and moreloflavone) from the hydroethanolic extract of the *P. insignis* flowers, which inhibited the efflux pumps NorA, QacA/B, TetK and MsrA and thereby enhanced the activity of norfloxacin, tetracycline and erythromycin against *Staphylococcus aureus* [18]. This mixture of biflavonoids also demonstrated antileishmanial activity and increased the lysosomal activity of macrophages against internalized amastigote forms of *Leishmania amazonensis*, suggesting a possible modulating effect [19]. Several biological activities are attributed to volkensiflavone, such as analgesic, antibacterial and antitumor activities [20]. Moreloflavone showed anti-viral [21], antioxidant [22], anti-inflammatory [23] and antiparasitic activities [24].

Another study verified that a higher concentration of EtBr was needed to inhibit *S. aureus* strains when quercetin was added in the growth medium, suggesting a protector effect caused by this flavonoid against cell damage induced by EtBr [25]. Hesperetin and naringenin were able to displace EtBr complexed to calf-thymus DNA [26], indicating that the molecular interactions of these flavonoids with EtBr could result in DNA protection. However, there are no data available in the literature demonstrating that biflavonoids volkensiflavone and moreloflavone could protect prokaryotic cells against cell damage induced by EtBr. In the present study, we investigated the protective effect of the mixture of biflavones (BFs) volkensiflavone and moreloflavone (Figure 1) obtained from the *P. insignis* flowers against the lethality induced by ethidium bromide in efflux pump-producing *Staphylococcus aureus* strains.

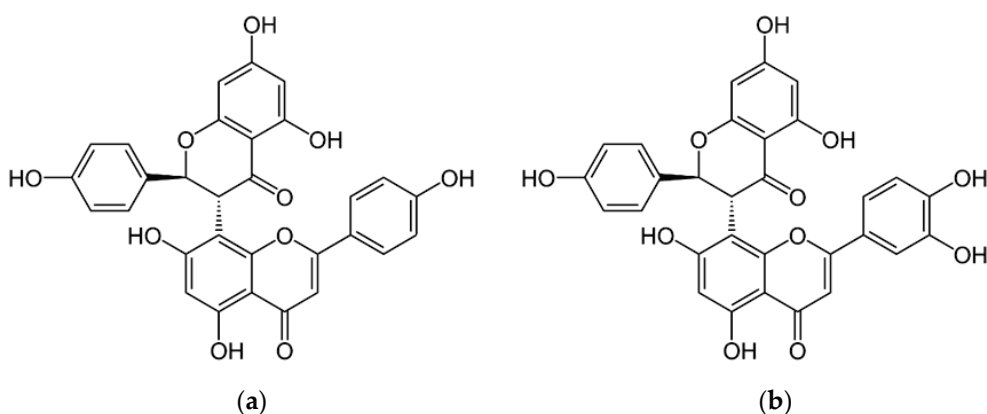


Figure 1. Chemical structure of the biflavones mixture obtained from the hydroethanolic extract of *Platonia insignis* flowers: (a) volkensiflavone and (b) moreloflavone.

2. Materials and Methods

2.1. Bacterial Strains and Chemical Compounds

Assays to evaluate the protective effect against the antibacterial activity of EtBr were carried out with the following strains of *Staphylococcus aureus*: SA1199B (overproducer of the NorA efflux pump), strain SA1199 (parental wild-type producer of NorA at basal levels), IS-58 (producer of the TetK efflux pump), K2068 (producer of the MepA efflux pump), K4100 (producer of the QacC efflux pump) and RN4220 (producer of the MsrA efflux pump). These strains were kindly provided by Dr. Glenn W. Kaatz, John Dingell VA Medical Center, Detroit, MI, USA. Bacterial strains were maintained in Brain Heart Infusion Agar (BHIA, Himedia, Mumbai, India) slanted at 4 °C. Prior to assays, cells were subcultured into tubes containing Brain Heart Infusion medium (BHI, Himedia, Mumbai, India) and incubated at 37 °C for 24 h. EtBr and chlorpromazine (CPZ) were obtained from Sigma Chemical Corp., St. Louis, MO, USA. EtBr and CPZ were dissolved in sterile distilled water.

2.2. Extraction of the Biflavones Mixture

The biflavones mixture (BFs) used to perform the biological assays was obtained from the hydroethanolic extract of the *P. insignis* flowers by developing the work of Silva et al. [18]. The flowers of *Platonia insignis* Mart. were collected in the municipality of Parnarama, Maranhão state, in August 2014. The exsiccateae were deposited in the Graziella Barroso Herbarium of the Federal University of Piauí (UFPI) under the ICN number TEPB 27174. The petals of fresh flowers of *P. insignis* were ground and macerated in a solvent mixture EtOH/H₂O in the ratio of 7:3 (*m/v*) and in the ratio of 1:5 plant material/solvent (*m/v*) at room temperature for 9 days. Subsequently, the hydroalcoholic extract (EHPi) was rotaevaporated and lyophilized. The EHPi was resuspended in MeOH/H₂O 9:1 (*v/v*) and subjected to liquid–liquid partitioning with solvents in increasing order of polarity. The ethyl acetate fraction, FAcOEt, was obtained with a mass of 3.82 g and $\eta = 26.56\%$. From mass spectrometry with electrospray ionization in negative mode coupled with a mass spectrometer (ion trap hybrid) (ESI-IT-MS) in scan mode, a mixture of biflavonoids, moreloflavone and volkensiflavone, stood out as majorities in the hydroalcoholic extract. To identify the constituents present in the FAcOEt fraction, silica dry column chromatography was performed, thereby obtaining a subfraction eluted with MeOH/H₂O 1:1 (*v/v*) and presenting as a yellow amorphous solid. After ESI-MS analysis in the negative ionization mode and NMR, the moreloflavone compounds were identified, with the majority molecular form being C₃₀H₁₉O₁₁; the volkensiflavone was also identified, and its molecular form was C₃₀H₁₉O₁₀.

2.3. Determination of the Minimum Inhibitory Concentration

Test solutions of BFs and CPZ were prepared in DMSO, which was followed by dilution in sterile distilled water to a final concentration of 1024 µg/mL. Minimal inhibitory concentrations (MIC) were determined using a microdilution assay in BHI broth with bacterial suspensions of approximately 10⁵ CFU/mL and test product ranging from 8 to 512 µg/mL. Microtiter plates were incubated at 37 °C for 24 h, and then 20 µL of resazurin (0.01 % *w/v* in sterile distilled water) was added to each well to detect bacterial growth as indicated by a color change from blue to pink. MIC was defined as the lowest concentration at which no bacterial growth was observed.

2.4. Evaluation of the Protective Effect of BFs against Lethality Induced by EtBr in *Staphylococcus aureus*

To assess whether BFs were able to protect *S. aureus* strains against the lethal effect induced by EtBr, bacterial cells were treated with EtBr in the presence or absence of BFs at subinhibitory concentrations (MIC 1/8, 128 µg/mL). EtBr concentrations ranged from 0.125 to 128 µg/mL. Microtiter plates were incubated at 37 °C for 24 h, and readings were performed with resazurin as described above. As a control, assays were performed replacing BFs with CPZ (MIC 1/8, 8 µg/mL), a known inhibitor of the NorA efflux pump [27].

2.5. Spectroscopic Analysis in the Infrared Region

Absorption spectra of EtBr, BFs and EtBr/BFs (1:1, m/m) in the infrared region were obtained with a Cary 630 FTIR spectrometer and ATR accessory (both from Agilent Technologies, Santa Clara, CA, USA).

2.6. Statistical Analysis

The experiments were performed in triplicate, and the results were normalized by calculating the geometric mean of the MICs. The standard error and standard deviation of the geometric mean were determined. Statistical analyses were performed using the GraphPad Prism program, version 5.02 (San Diego, CA, USA). The occurrence of differences between EtBr treatment in the absence or presence of BF was examined using one-step analysis of variance (ANOVA). The differences mentioned above were analyzed using the Bonferroni post-test and were considered statistically significant when $p < 0.05$.

3. Results and Discussion

Figure 2 shows the results of the assays for comparing the antibacterial activity of EtBr either alone or associated with BFs against the SA1199B strain that overproduces the NorA efflux pump. It can be verified that the MIC value obtained for EtBr isolated against this strain was 64 $\mu\text{g/mL}$. On the other hand, when the SA1199B strain was treated with EtBr associated with the BFs in subinhibitory concentration, the EtBr MIC increased to 128 $\mu\text{g/mL}$ (a $2\times$ increase), indicating that the BFs protected the SA1199B strain against the lethal effect of EtBr. Such protection was not exercised by CPZ in subinhibitory concentrations, which, unlike BFs, potentiated the action of EtBr, possibly by inhibiting the NorA efflux pump.

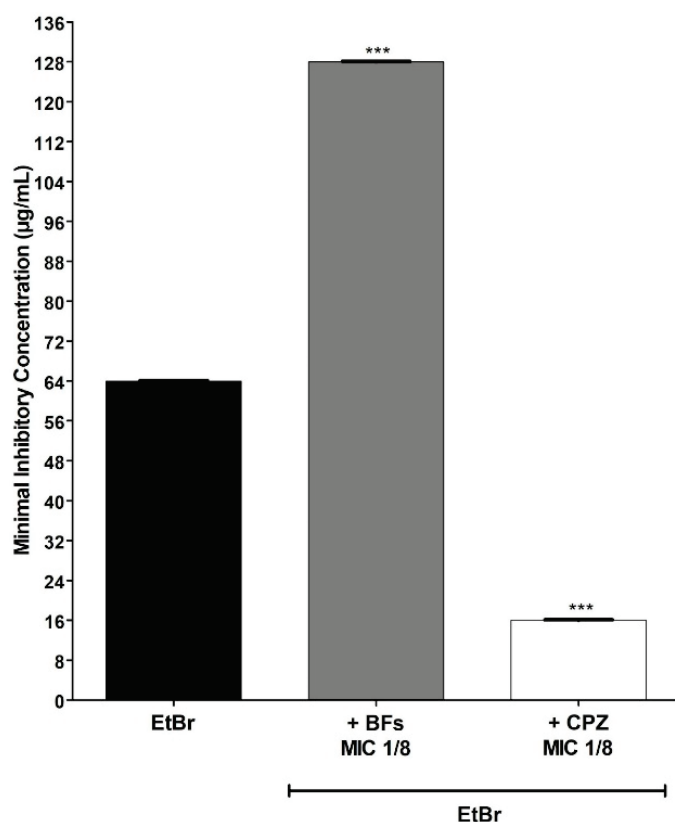


Figure 2. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* SA1199-B (NorA+++) in the absence or presence of biflavones (BF, 128 $\mu\text{g/mL}$) or chlorpromazine (CPZ, 8 $\mu\text{g/mL}$). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$).

Similar results were obtained with the wild-type parent SA1199 strain that produced the NorA efflux pump at baseline levels (Figure 3). For this strain, the MIC value obtained for EtBr isolated against the strain was 16 $\mu\text{g/mL}$, whereas for EtBr combined with the BFs, the MIC value was raised to 32 $\mu\text{g/mL}$ (a $2\times$ increase). On the other hand, these results suggest that the protective effect exerted by the BFs against EtBr-induced lethality in *S. aureus* occurs independently of the NorA efflux pump's expression level. Such protection was not exercised by CPZ, which, unlike BFs, potentiated the action of EtBr against the SA1199B strain, possibly by inhibiting the NorA efflux pump.

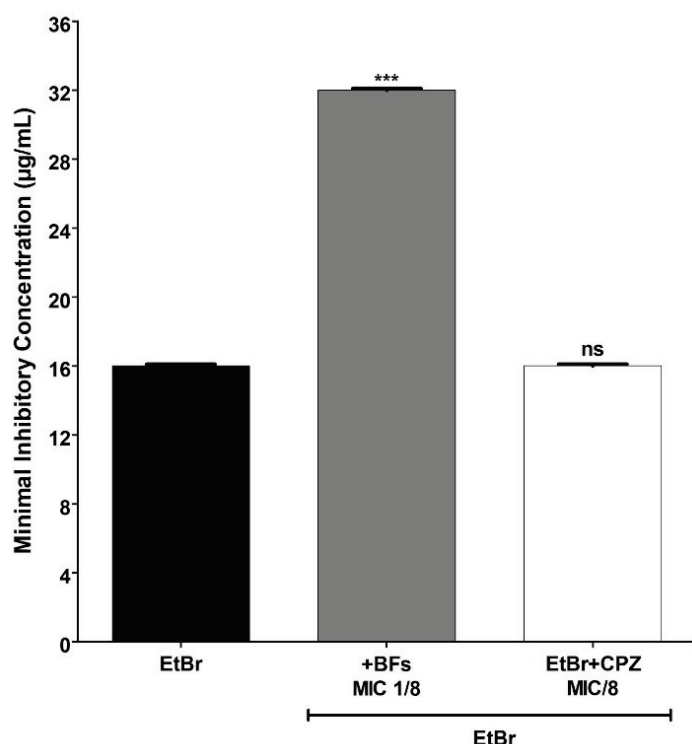


Figure 3. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* SA1199 (NorA+) in the absence or presence of biflavones (BFs, 128 $\mu\text{g/mL}$) or chlorpromazine (CPZ, 8 $\mu\text{g/mL}$). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$). ns: not significant.

The BFs also increased by $2\times$ the concentration of EtBr necessary to inhibit the growth of the IS-58 strain overproducer of the efflux pump TetK (Figure 4), the strain K2068 overproducer of the efflux pump MepA (Figure 5) and the strain K4100 overproducer of the efflux pump QacC (Figure 6) as well as the strain RN4220 overproducer of MsrA efflux pump (Figure 7). The NorA and TetK proteins are proton-motive force-dependent efflux pumps and members of the MFS (Major Facilitator superfamily) [28]. The MepA efflux pump is a proton-motive force-dependent transporter of the MATE superfamily (Multidrug and toxin Extrusion), whereas the QacC protein is a carrier protein belonging to the SMR (Small multidrug Resistance) [29]. On the other hand, the MsrA efflux pump is ATP-dependent and belongs to the ABC superfamily [30]. These results indicate that the protective effect exerted by the BFs against EtBr-induced lethality in *S. aureus* occurs regardless of the type of efflux pump expressed by this pathogen.

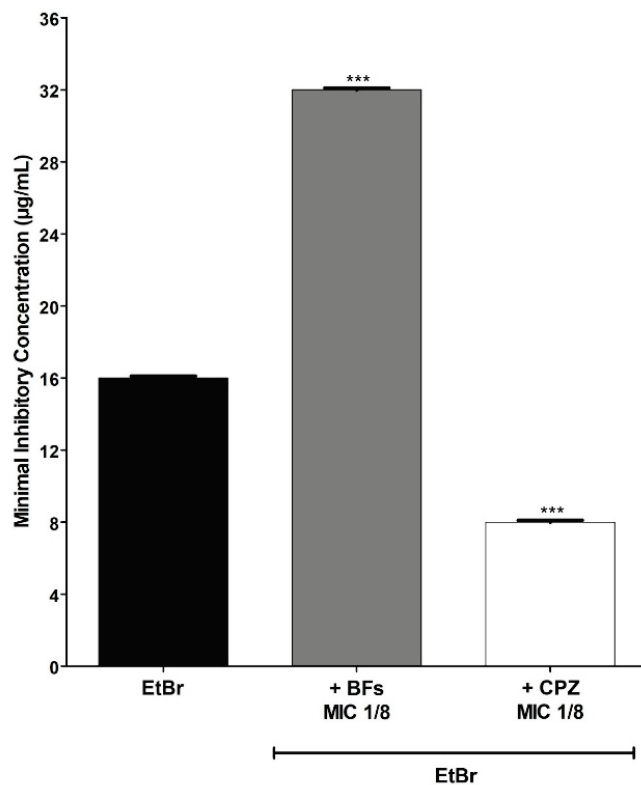


Figure 4. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* IS-58 (tetK) in the absence or presence of biflavones (BFs, 128 µg/mL) or chlorpromazine (CPZ, 8 µg/mL). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$).

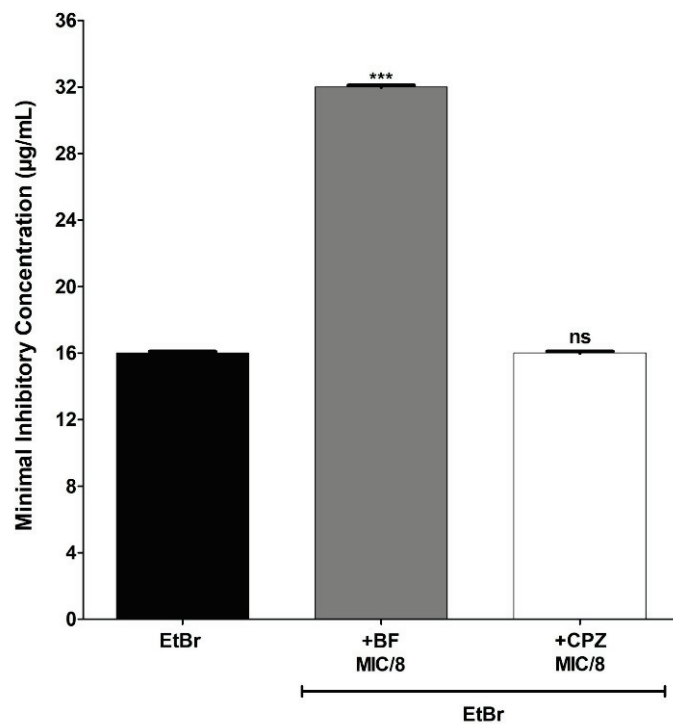


Figure 5. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* K2068 (MepA) in the absence or presence of biflavones (BFs, 128 µg/mL) or chlorpromazine (CPZ, 8 µg/mL). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$). ns: not significant.

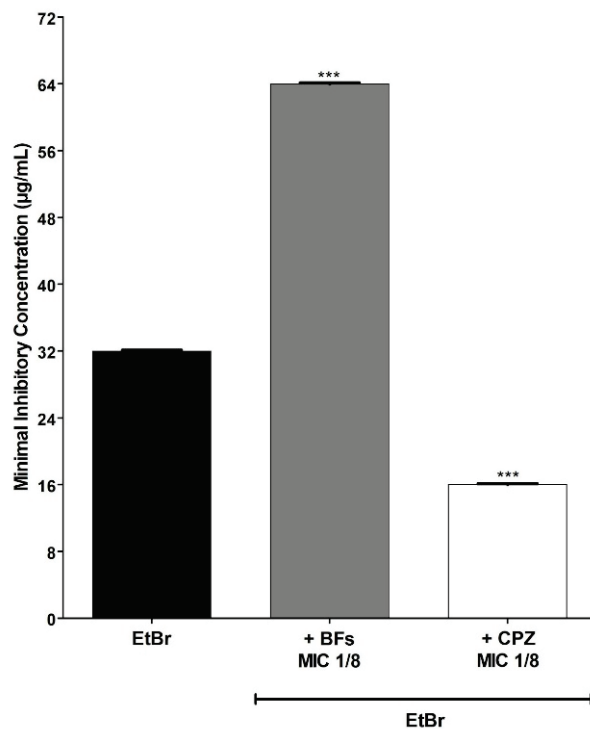


Figure 6. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* K4100 (QacC) in the absence or presence of biflavones (BFs, 128 µg/mL) or chlorpromazine (CPZ, 8 µg/mL). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$).

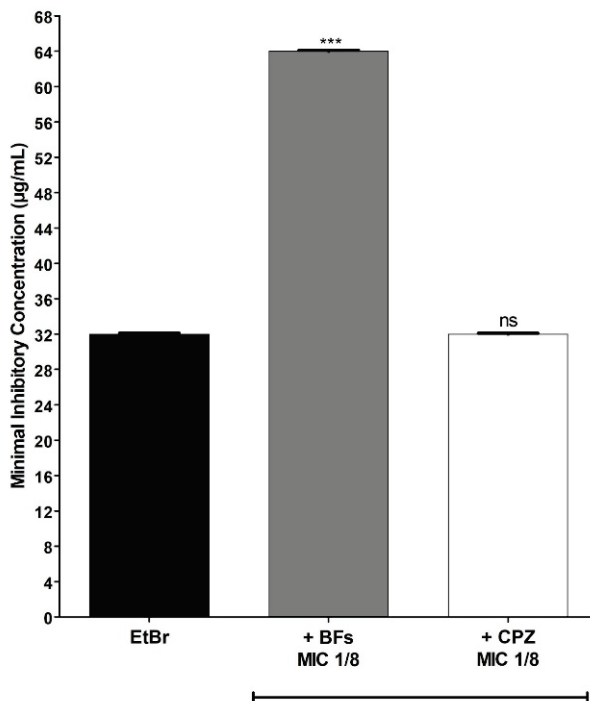


Figure 7. MIC (minimum inhibitory concentration) values of ethidium bromide (EtBr) against *S. aureus* RN4220 (MsrA) in the absence or presence of biflavones (BFs, 128 µg/mL) or chlorpromazine (CPZ, 8 µg/mL). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$). ns: not significant.

To test the hypothesis that the protective effect exerted by the BF_s could be due to a possible interaction between these compounds and EtBr, the spectra in the infrared region of EtBr, the BF_s and the mixture of EtBr + BF_s (1:1, m/m) were analyzed (Figure 8).

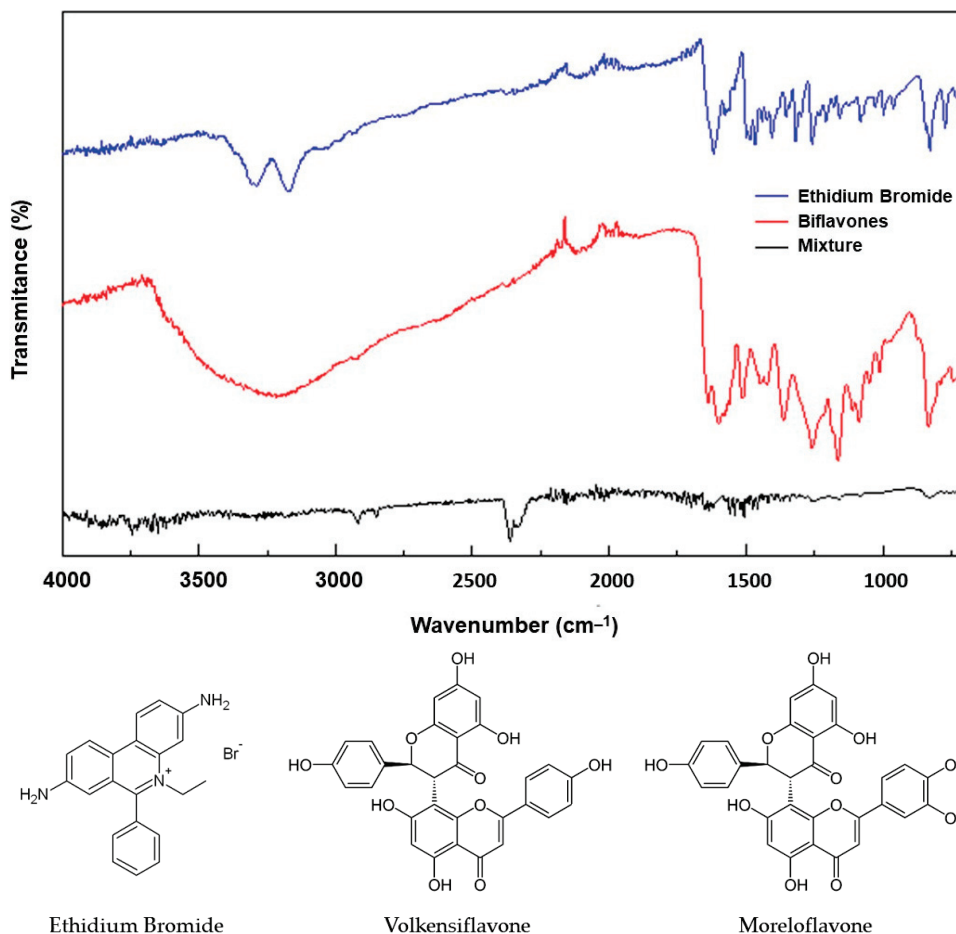


Figure 8. Infrared spectra of ethidium bromide (EtBr), biflavones (BF_s) and EtBr + BF_s mixture.

In the spectrum of EtBr, it can be seen that NH stretching of primary amines occurred in the region between 3540–3380 cm^{−1}. OH stretching of phenols occurred between 3676–3584 cm^{−1}, and both bands had varying intensities. Observing the spectra of ethidium bromide isolated and the spectra of the mixture of biflavones, these bands appear well-defined; however, in the spectrum of the mixture of biflavones with ethidium bromide, these absorptions disappear, and two new absorption bands appear at 2368 cm^{−1} and 2335 cm^{−1}, characteristics of $\text{C}=\text{N} + \text{H}$ (stretch $=\text{CN} + \text{H}$).

The disappearance of the 1600 cm^{−1} peak, which corresponds to carbonyls (C=O), when ethidium bromide is mixed with biflavones may be related to a possible complexation between the two compounds, indicating that there was a preferential interaction between the H₂N– groups of primary amines of ethidium bromide with the carbonyls of biflavones that formed imines $\text{C}=\text{NH}$ (Figure 9). These imines can be protonated by acidic phenolic hydrogens, causing a change in the carbonyl peak.

These results suggest that the protective effect exerted by the BF_s could be related to their ability to covalently bind EtBr, forming complexes of higher molecular weight in the extracellular medium that would not be assimilated by bacterial cells. In this way, the BF_s would be reducing the entry of isolated EtBr molecules into the cell, which would reduce the cell damage induced by this compound.

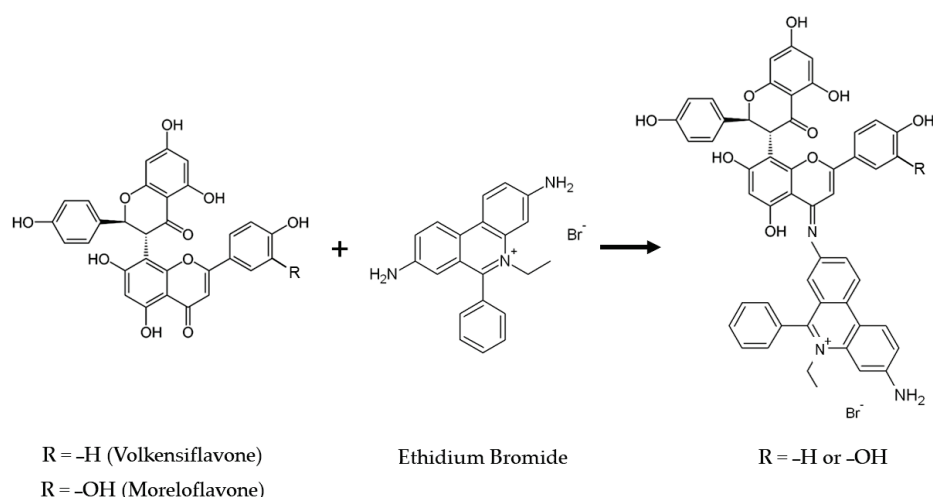


Figure 9. Proposed reaction of the NH_2 (amine) group of ethidium bromide on the $C=O$ (carbonyl) group of biflavones.

A previous study with the same *S. aureus* strains tested in the present study verified that the BFs volkensiflavone and moreloflavone inhibited the NorA, QacA/B, TetK and MsrA efflux pumps, promoting greater accumulation of EtBr in these strains [18]. Therefore, despite the observed complexation, isolated molecules of EtBr and the BFs can be absorbed by bacterial cells. However, the complexes formed in the intracellular medium, due to their higher molecular weight and structure incompatible with the diameter of the DNA, would not be able to intercalate into the double helix, which would also contribute to reducing the cell damage induced by EtBr.

The cytoprotective activity of flavonoids was previously reported in eukaryotic cells. The dietary flavonoid quercetin showed a cytoprotective effect against benzo[a]pyrene-induced toxicity [31] and a cytoprotective effect in epithelial cells against *Pseudomonas aeruginosa* virulence factors [32]. Dihydromyricetin, a natural flavonoid extracted from *Ampelopsis grossedentata*, showed a cytoprotective effect on dairy cow mammary epithelial cells by suppressing mitochondrial membrane depolarization caused by heat stress and mitochondrial dysfunction as well as by modulating oxidative enzymes; thus, it prevented the production of oxygen-reactive species [33]. Hesperetin, a flavonoid derived from citrus fruits, exhibited cytoprotective action, protecting against hypoxia/reoxygenation injuries and doxorubicin-induced cardiotoxicity [34]. Because EtBr is a mutagenic and carcinogenic agent, the results obtained in the present study with prokaryotic cells are promising and open the horizon for future investigations of the protective effect of these BFs against the damage induced by EtBr in eukaryotic cells.

4. Conclusions

The results obtained indicate that the mixture of biflavones volkensiflavone and moreloflavone obtained from the *P. insignis* flowers shows a protective effect against EtBr-induced damage in *S. aureus*, suggesting a possible application of these compounds as cytoprotective agents. Future studies will be needed to investigate whether these compounds exert this same protective effect in eukaryotic cells.

Author Contributions: Conceptualization, H.M.B., and H.D.M.C.; methodology, A.S.G.M., J.d.A.F.B., A.K.Fe.S., M.L.L.B.d.N., E.E.A.P., A.K.S.M. and J.d.S.L.-N.; validation, H.M.B., J.M.d.C.e.S. and A.M.d.G.L.C.; resources, H.M.B., J.M.d.C.e.S. and A.M.d.G.L.C.; writing—original draft preparation, A.S.G.M.; writing—review and editing, H.M.B. and H.D.M.C.; supervision, A.M.d.G.L.C. and H.M.B.; project administration, H.M.B.; funding acquisition, H.M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Fundação de Amparo à Pesquisa do Estado do Piauí (grant number: 050/2019) and Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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Article

In Vitro Schistosomicidal Activity and Molecular Modeling of Quercitrin and Afzelin Isolated from the Leaves of *Copaifera oblongifolia*

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Abstract: Neglected diseases significantly impact the world, and there is a lack of effective treatments, requiring therapeutic alternatives. Thus, the study of the phytochemical and schistosomicidal activity evaluation of *Copaifera oblongifolia* leaves' crude extract was conducted. The quercitrin (**1**) and afzelin (**2**) were isolated from the crude extract. In the in vitro schistosomicidal activity test, the isolated compounds demonstrated promising results, with 75% mortality at a concentration of 12.5 μ M after 72 h. Molecular docking calculations indicated that compounds **1** and **2** could potentially interact with the amino acids of the FAD binding site in the TGR enzyme, a crucial enzyme for the survival of *Schistosoma mansoni*. These interactions could have binding energies comparable to praziquantel, a preferred drug for treating schistosomiasis. Therefore, in silico and in vitro investigations are crucial for developing new studies that can reveal the antiparasitic potential of compounds of plant origin.

Keywords: parasitic activity; *Copaifera oblongifolia*; neglected diseases; in silico

1. Introduction

Neglected tropical diseases are a significant concern in developing countries, affecting millions [1]. These diseases lack innovative treatments and are not profitable for pharmaceutical companies, resulting in a lack of investment in research and development. They are responsible for over 35,000 daily deaths and generate more disability than mortality [2]. In Brazil, 19 tropical diseases are reported, including schistosomiasis. Schistosomiasis affects over 250 million people worldwide and is a significant cause of death [3,4]. The disease is caused by *Schistosoma mansoni* and is treated with oxamniquine or praziquantel (PZQ).

Praziquantel is preferred due to its low cost; however, the development of new drugs is justified due to the potential emergence of resistance [5,6].

In *Schistosoma*, the redox systems depend on the thioredoxin glutathione reductase (TGR) enzyme [7]. Two schistosomiasis drugs used in the past, oltipraz and antimonyl potassium tartrate, promoted the inhibition of the TGR activity, indicating the importance of this enzyme to *S. mansoni* [8]. The fact that the thioredoxin glutathione reductase (TGR) enzyme appears to be an important protein in protecting *S. mansoni* against oxidative stress makes the TGR enzyme a relevant target for potential drugs [9].

Recent advances in the pharmacological targeting of thioredoxin glutathione reductase (TGR) have expanded the scope of schistosomiasis treatment. Notably, non-covalent inhibitors with demonstrated schistosomicidal activity in vivo have been identified, highlighting effective strategies for enzyme inhibition without permanent enzyme modification [10]. Structure-based discovery approaches have revealed phytocompounds from *Azadirachta indica* as promising TGR inhibitors, expanding the chemical diversity of potential therapeutic agents [11]. Additionally, the mechanism of ectopic suicide inhibition of TGR has been characterized, providing insights into irreversible enzyme inactivation [12]. Lead compounds targeting this selenoenzyme have also been extensively characterized, underscoring their potential for drug development [13]. Furthermore, fragment library screening combined with X-ray crystallography has elucidated detailed binding site features of TGR from *Schistosoma mansoni*, facilitating rational drug design [14]. Together, these recent studies demonstrate the dynamic progress in identifying and optimizing TGR inhibitors, supporting its validation as a critical drug target for schistosomiasis.

Thus, searching for new potential drugs from natural sources is urgently needed, as large pharmaceutical industries have neglected these diseases. Among the numerous medicinal plants used by the Brazilian population, the genus *Copaifera*, commonly known as “copaibas”, stands out due to its long-established pharmacological applications in traditional medicine [15–17].

Therefore, this work aims to investigate the Schistosomicidal activity in vitro of quercitrin (**1**) and afzelin (**2**) isolated from the leaves of *Copaifera oblongifolia*. In addition, molecular docking calculations were performed to investigate the binding modes of these molecules to the TGR enzyme. The interactions between praziquantel (PZQ), a broad-spectrum anthelmintic drug employed to treat schistosomiasis, and the TGR enzyme were used as a reference in this study [18].

2. Results and Discussion

2.1. Compounds Isolated

The phytochemical analysis of the FD fraction obtained from *Copaifera oblongifolia* hydroalcoholic extract, using chromatographic techniques, allowed for us to isolate compounds **1** and **2** (Figures 1, 2 and S1–S12). The structures were identified as quercitrin (**1**) and afzelin (**2**). All isolated compounds were identified based on their structures using spectroscopic approaches (¹H NMR, ¹³C NMR, and ESI-MS) and comparisons to published data [19].

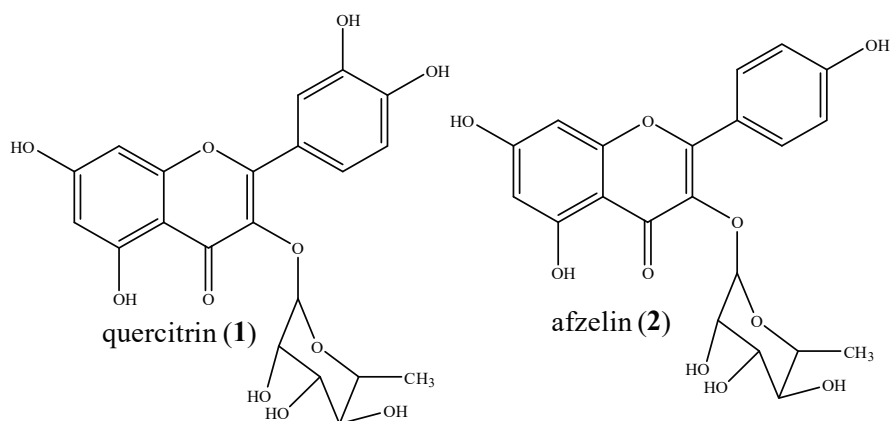


Figure 1. Chemical structures of compounds 1 and 2.

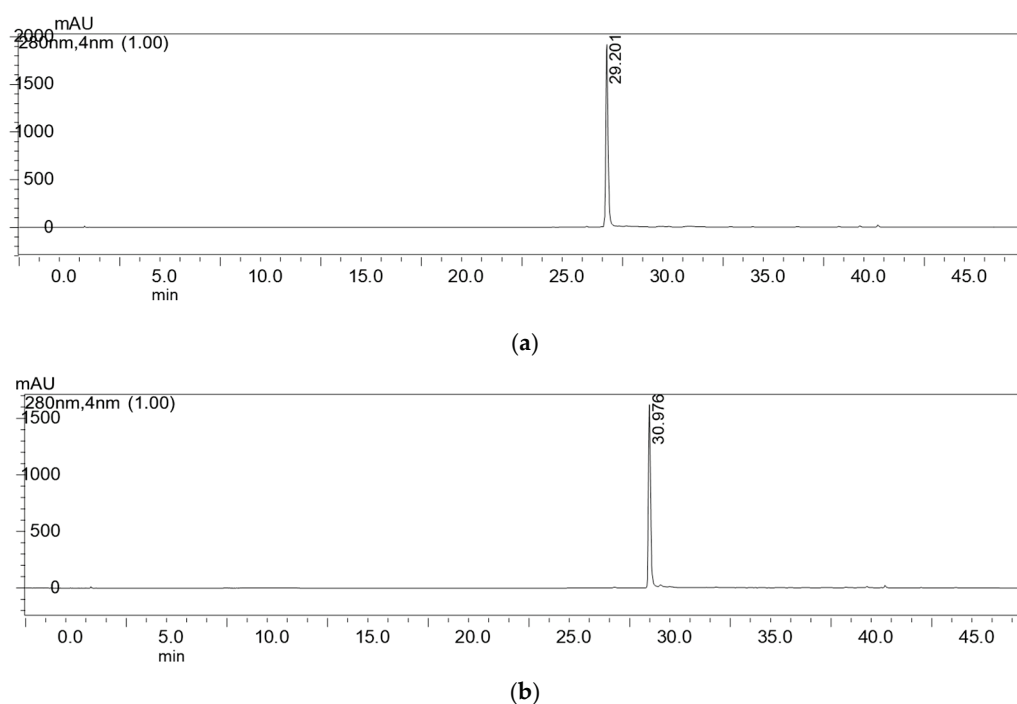


Figure 2. Chromatograms of compounds 1 (a) and 2 (b) after isolation by preparative HPLC.

2.2. Schistosomicidal Activity

Table 1 presents the in vitro evaluation of the schistosomicidal activity of the crude extract of *C. oblongifolia* leaves against adult *S. mansoni* worms at 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ concentrations. The results indicate that there was no change at lower concentrations. However, the crude extract showed 75% mortality at a concentration of 100 $\mu\text{g/mL}$. Tables 2 and 3 show the in vitro evaluation of the schistosomicidal activity of quercitrin (1) and afzelin (2) against adult *S. mansoni* worms. Compound 1 showed 75% mortality at concentrations of 12.5 and 25 μM after 48 h of incubation, with the most active concentration being 50 μM , which resulted in 100% mortality after 72 h. Compound 2 exhibited 75% mortality at concentrations of 12.5, 25, 50, and 100 μM after 72 h of incubation, with the most active concentration being 200 μM , which caused 75% mortality in 24 and 48 h and 100% mortality in 72 h.

Table 1. Results of the in vitro schistosomicide assay of crude extract and fractions.

Samples	Incubation Time (h)	% Dead Worms *	Motor Activity	
			Not Significant (%)	Significant (%)
Negative control	24	0	0	0
	48	0	0	0
	72	0	0	0
Positive Control	24	100	0	0
	48	100	-	-
	72	100	0	0
Crude extract (50 µg/mL)	24	0	0	0
	48	0	0	0
	72	25	0	0
Crude extract (100 µg/mL)	24	25	0	0
	48	50	25	25
	72	75	0	25

* The percentage results were calculated based on a total of 24 adult worms per concentration, obtained from three independent experiments with 8 adult worms each.

Table 2. Results of the in vitro schistosomicide assay of compound 1.

Concentration	Time in Incubation (H)	% Dead Worms *	% Reduction in Motor Activity	
			Not Significant	Significant
Control *	24	-	-	-
	48	-	-	-
	72	-	-	-
12.5 µM	24	-	75	25
	48	75	-	25
	72	75	-	25
25 µM	24	25	50	25
	48	75	25	-
	72	75	25	-
50 µM	24	25	25	50
	48	75	-	25
	72	100	-	-
100 µM	24	25	25	50
	48	50	50	-
	72	50	50	-
200 µM	24	50	25	25
	48	75	-	25
	72	75	-	25

* The percentage results were calculated based on a total of 24 adult worms per concentration, obtained from three independent experiments with 8 adult worms each.

Reports on the schistosomicidal activity of compounds **1** and **2** are scarce. However, numerous reports have been published on various biological activities, primarily in investigations involving different types of cancer. Zhang et al. [20] explored the impact of afzelin, identified in *Nymphaea odorata*, on prostate cancer. Their investigation covered both androgen-sensitive LNCaP cells and androgen-independent PC-3 cells.

Table 3. Results of the in vitro schistosomicide assay of compound 2.

Concentration	Time in Incubation (H)	Dead Worms (%) *	% Reduction in Motor Activity	
			Not Significant	Significant
Control *	24	-	-	-
	48	-	-	-
	72	-	-	-
12.5 µM	24	50	25	25
	48	50	25	25
	72	75	-	25
25 µM	24	50	25	25
	48	50	-	50
	72	75	-	25
50 µM	24	50	-	50
	48	50	-	50
	72	75	-	25
100 µM	24	50	-	50
	48	50	-	50
	72	75	-	25
200 µM	24	75	-	25
	48	75	-	25
	72	100	-	-

* The percentage results were calculated based on a total of 24 adult worms per concentration, obtained from three independent experiments with eight adult worms each.

Furthermore, Shin et al. [21] highlighted afzelin's antioxidant, DNA-protective, UV-absorbing, and anti-inflammatory attributes. Other studies, including that of Diantini et al. [22], corroborated the ability of afzelin to prevent the proliferation of breast cancer cells through the induction of apoptosis. In another study, afzelin demonstrated the potential to mitigate asthma in a murine model, as evidenced by Zhou and Nie [23]. Furthermore, Zhu et al. [24] demonstrated the inhibition of cell proliferation by afzelin in prostate cancer cell lines, primarily targeting the G phase of the cell cycle, as well as its modulation of several kinases involved in maintaining the actin cytoskeleton.

The investigation conducted by Zhang et al. [25] demonstrated that quercitrin extracted from *Toona sinensis* leaves significantly reduced the viability of human colorectal cancer cells, leading to apoptosis. Several studies have documented the inhibitory effects of quercitrin against several protozoan parasites, including *Toxoplasma*, *Babesia*, *Theileria*, *Trypanosoma*, and *Leishmania*. Quercitrin has demonstrated efficacy in inhibiting the growth of various parasite species, including *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania donovani*, both in vitro and in vivo [26]. In addition, compounds 1 and 2 have been reported in the literature to inhibit, through interactions with amino acids, enzymes responsible for the expression of various diseases [27,28]. Studies carried out by Awang et al. [29] showed that the Quercitrin-Rich fraction from *Melastoma malabathricum* leaves had antidiabetic properties, inhibiting the DPP-IV enzyme at a concentration of 100 µg/mL, where inhibition occurred through hydrogen bonding with the amino acids of the enzyme. Therefore, understanding enzyme inhibition is one of the current strategies for discovering treatments for diverse diseases.

2.3. Molecular Docking

Initially, a molecular docking calculation was performed between the native ligand, flavin adenine dinucleotide (FAD), and the TGR enzyme. Three FAD conformations exhibited root mean square deviation (RMSD) values below 2.0 Å relative to the crystal

structure (1.449, 1.649, and 1.659 Å, respectively), demonstrating the robustness of the computational model employed in this study.

The PZQ and the TGR enzyme interact mainly from the amino acids Cys154, Cys159, Gly432, and Ala445 (Figure 3 and Table 4). The first three amino acids are related to the FAD active site in the TGR enzyme [18]. Structure 1 interacts with the TGR enzyme through the amino acids Ser117, Cys159, Lys162, Val297, Asp433, Thr442, and Pro443. Except for Val297, all other amino acids are associated with the FAD active site in the TGR enzyme [13]. Structure 2 interacts with the TGR enzyme via amino acids: Ser117, Cys154, Cys159, Lys162, Tyr296, Val297, Glu300, Leu441, and Pro443. The first five and the last two amino acids correlate to the FAD active site in the TGR enzyme [18]. Considering the ChemScore fitness dG, which represents the total free energy change that occurs on ligand binding (Table 5), it is observed that compounds 1 and 2 show values (-23.44 and -28.90 kcal mol $^{-1}$, respectively) comparable to, or even more negative (thus more stable), than those obtained for PZQ (-22.03 kcal mol $^{-1}$). It can be explained because compounds 1 and 2 establish a larger number of hydrophobic interactions (three and four, respectively) and hydrogen bonds (five to both) with the amino acids of the TGR enzyme than the PZQ structure (three hydrophobic interactions and two hydrogen bonds). In this sense, molecular docking results suggest that compounds 1 and 2 potentially inhibit the TGR, an important enzyme for the survival of *Schistosoma* worms [7]. Furthermore, Kuhn et al. [30] reported quercitrin as a selective inhibitor of the NAD $^{+}$ catabolic enzyme of *S. mansoni* (SmNACE), located on the adult parasite's outer surface (tegument). This enzyme is presumably crucial for the survival of *S. mansoni*, as it can influence the host's immune regulatory pathways [30].

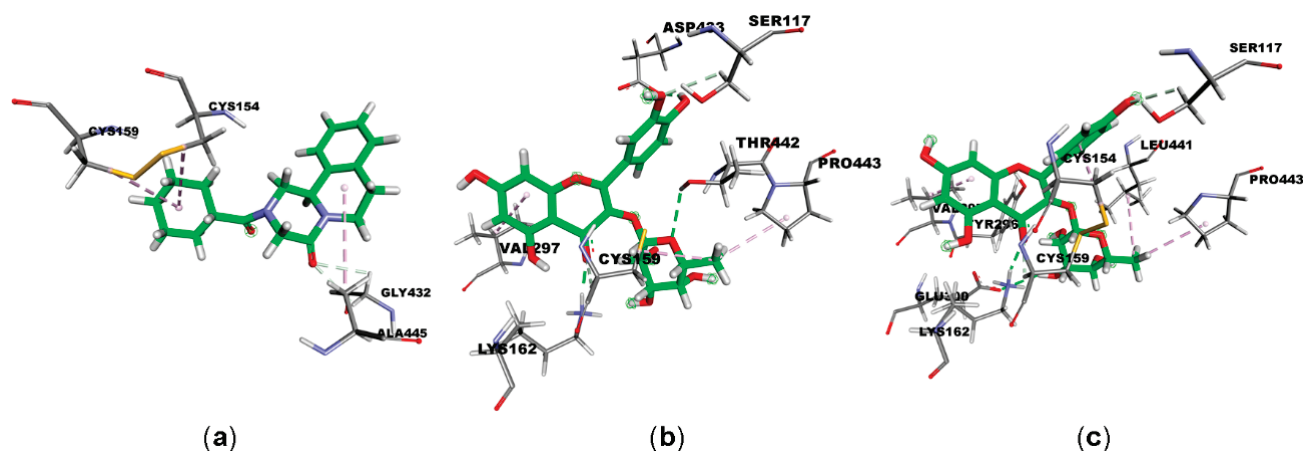


Figure 3. Main interactions between the: (a) PZQ, (b) compound 1, and (c) compound 2; compounds and the amino acids present in the TGR enzyme. Ligands are shown in green; carbon (gray), oxygen (red), nitrogen (blue), and sulfur (yellow) atoms; hydrogen bonds are represented by purple dashed lines.

Thus, the results presented in this work further highlight the potential of compounds 1 and 2 as promising candidates for developing new therapeutic strategies against parasitic diseases, such as Schistosomiasis.

Table 4. Main interactions between the compounds: PZQ, **1**, and **2**, and the amino acids present in the TGR enzyme.

Amino Acid	PZQ-TGR
Cys154	Hydrophobic Interaction (C-S $\cdots$ Ring Center)
Cys159	Hydrophobic Interaction (C-S $\cdots$ Ring Center)
Gly432	Hydrogen Bonds (2x HC-H $\cdots$ O=C)
Ala445	Hydrophobic Interaction (H ₃ C $\cdots$ Ring Center)
Amino Acid	A3-TGR
Ser117	Hydrogen Bond (HC-H $\cdots$ O-H)
Cys159	Hydrogen Bond (C-H $\cdots$ O=C) and Hydrophobic Interaction (C-S $\cdots$ CH ₃)
Lys162	Hydrogen Bond (H ₂ N-H $\cdots$ O=C)
Val297	Hydrophobic Interaction (HC $\cdots$ Aromatic Ring)
Asp433	Hydrogen Bond (C=O $\cdots$ H-O)
Thr442	Hydrogen Bond (O-H $\cdots$ OCC)
Pro443	Hydrophobic Interaction (H ₂ C $\cdots$ CH ₃)
Amino Acid	A4-TGR
Ser117	Hydrogen Bond (HC-H $\cdots$ O-H)
Cys154	Hydrophobic Interaction (C-S $\cdots$ Aromatic Ring)
Cys159	Hydrogen Bond (C-H $\cdots$ O=C)
Lys162	Hydrogen Bond (H ₂ N-H $\cdots$ O=C)
Tyr296	Hydrogen Bond (O-H $\cdots$ OCC)
Val297	Hydrophobic Interaction (HC $\cdots$ Aromatic Ring)
Glu300	Hydrogen Bond (C=O $\cdots$ H-O)
Leu441	Hydrophobic Interaction (HC $\cdots$ CH ₃)
Pro443	Hydrophobic Interaction (H ₂ C $\cdots$ CH ₃)

Table 5. Total free energy change that occurs on ligand (**1**, **2** or PZQ) binding to TGR enzyme.

Enzyme $\cdots$ Ligand (dG kcal mol ^{−1})	1	2	PZQ
TGR	−23.44	−28.90	−22.03

3. Experimental

3.1. Instrument Specifications

Preparative analytical HPLC was carried out on a Shimadzu LC-6A chromatograph with UV–visible detector model SPD-6A, with SCL6B controller and C-R6A integrator. The NMR spectra were obtained on a Bruker spectrometer (400 MHz for ¹H and 100 MHz for ¹³C). The samples were dissolved in methanol-*d*4 and the mass spectra were obtained using an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QTOF/MS, Waters Corp., Milford, CT, USA).

3.2. Collection and Extraction of Plant Material

Copaifera oblongifolia leaves were collected from a specimen located at Usina Santo Ângelo in Pirajuba-Minas Gerais (19°56.833' S 48°33.504' W). A copy was sent to Prof. Dr.

Milton Groppo Júnior, responsible for the SPFR Herbarium, at the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto–USP (FFCLR-USP), being provisionally named Plant 3 pending final deposit. The collected leaves were dried in a circulating air oven (40 °C) and pulverized in a knife mill, producing 913.7 g of powder. This material was subjected to five successive extractions with ethanol/water (75:25 *v/v*), which occurred at an average interval of eight days. After drying, 64.84g of crude extract was obtained.

3.3. Isolation of Compounds 1 and 2 of *Copaifera oblongifolia* Hydroalcoholic Extract

A liquid–liquid partition was performed using 40 g of hydroalcoholic extract. The volumes of solvents used, the order in which they were applied, and the masses of each resulting fraction are shown in Table 6. The mass was dissolved in 300 mL of an ethanol/water solution (80:20 *v/v*) and placed in a separation flask. It was then partitioned with 1 L of dichloromethane, yielding 8.9 g of the FD fraction. The dichloromethane fraction was fractionated using a glass chromatographic column. Fractionation in a classic chromatographic column (CC) was performed in a glass column (5 cm × 60 cm). Silica gel 60 (0.063–0.200 mm, Merck, Darmstadt, Germany) was used as the stationary phase at a proportion of 20 g/g of sample. Organic solvents were used as eluents in an increasing polarity gradient, yielding 110 fractions of 45 mL each. The fractions obtained and the solvents used are summarized in Table 7.

Table 6. Result of the partitioning of the crude extract of *C. oblongifolia*.

Fraction	Solvents	Volume (L)	Mass Obtained (g)
FH	n-hexane	1.5	2.7464
FD	Dichloromethane	0.9	5.6294
FA	Ethyl acetate	1.2	8.9900
FB	n-butanol	1.2	7.0400

Table 7. Fractionation of the dichloromethane fraction from the hydroalcoholic extract of *C. oblongifolia*.

Fraction	Eluent	Volume (L)
1–15	n-Hexane	0.6
16–21	n-Hexane/AcOEt (9:1)	0.3
22–35	n-Hexane/AcOEt (7:3)	1.1
36–59	n-Hexane/AcOEt (1:1)	1.2
60–76	AcOEt	0.9
77–95	AcOEt/EtOH (8:2)	0.9
96–102	AcOEt/EtOH (1:1)	0.3
103–110	EtOH	0.3
111	EtOH	0.9

Fractions 8 to 11 show chromatographic similarity with the presence of two constituents. The fractions were pooled and subjected to preparative HPLC using a Phenomenex[®] column, Gemini C-18 (250 × 21.20 mm equipped with pre-column), particle diameter equal to 5 µm, pore diameter 100 Å, being used as phase mobile CH₃OH (B) and H₂O +0.01% HAc (A) in the ratio 57:43 *v/v* in 50 min; the injection volume was 1mL, flow: 10.0 mL/min. It is possible to isolate the two constituents, compounds **1** (70 mg) and **2** (30 mg).

3.4. HPLC Analysis of the Compounds Obtained

The compounds obtained were analyzed by high-performance liquid chromatography (HPLC). For analytical HPLC, a Phenomenex[®] column, Gemini C-18 (250 × 4.6 mm equipped with pre-column), particle diameter equal to 5 µm, pore diameter 100 Å, was

used as mobile phase CH₃OH (B) and H₂O + 0.01% HAc (A) in linear gradient, 5 min at 5% (B), (5%→100%) in 35 min, 10 min at 100% (B), 3 min of equilibrium, and 15 min to return to initial condition. Injection volume: 20 µL; flow: 1.0 mL/min.

3.5. Assessment of Schistosomicidal Activity

The biological cycle of *Schistosoma mansoni*, strain LE (Luiz Evangelista) is routinely maintained by serial passage in *Biomphalaria glabrata* molluscs, an invertebrate host, and in BALB/c mice as a vertebrate host at the Parasitology Research Laboratory of the University of Franca in the state of São Paulo, Brazil.

S. mansoni eggs present in the feces of mice previously infected with the parasite were collected using the Hoffmann method [31]. Spontaneous sedimentation in the mixture of feces with water was exposed for approximately 1 h under light to release the miracidia. Miracidia were used to infect the intermediate host, which after 38 to 43 days released the infective form of the parasite, the Cercariae, which in turn infected the vertebrate host. Approximately 200 Cercariae are inoculated subcutaneously into mice, and after 21 or 58 days, young liver flukes or adult flukes are recovered from the hepatic portal system and mesenteric veins by perfusion [32]. After collection, the parasites were maintained in RPMI 1640 medium (Invitrogen) until use.

Adult worm pairs were recovered from BALB/c mice via perfusion of the hepatic portal system under aseptic conditions after 58 days of infection, as previously described. Then, the parasites were washed in RPMI 1640 buffered with HEPES20µM, pH 7.5, supplemented with penicillin (100U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum. Subsequently, one adult worm pair was transferred per well into a 24-well culture plate containing the same medium described previously and incubated in a humidifying atmosphere at 37 °C in the presence of 5% CO₂. After 24 h of incubation, the crude extract and fractions were previously dissolved in dimethyl sulfoxide (DMSO) and added to the RPMI 1640 medium in a concentration range ranging 12.5, 25, 50, 100, and 200 µg/mL. The parasites were incubated under the same conditions described previously for 72 h. They monitored the parasites every 24 h using an inverted microscope (Leitz Diavert) to evaluate their general conditions, including mortality rates and motor activity. To confirm mortality, after the absence of movement for more than 2 min, the parasites were washed with RPMI medium and transferred to culture plates containing the same medium without the sample and monitored as previously described [33]. Adult worms maintained in RPMI 1640 medium served as negative controls, while adult worms incubated with 12.5 µg/mL of Praziquantel (Sigma Aldrich) were used as a positive control. Three independent experiments evaluated eight culture wells per concentration [34].

3.6. Theoretical Methods

The target used in the present study, based on molecular docking calculations, was the TGR enzyme. The coordinates of this enzyme were obtained from the protein data bank (PDB) ID: 2V6O [35]. The binding site was identified using the Discovery Studio 2020 software package [36] considering a 10 Å docking sphere from the centroid of the ligands associated with the enzyme found in the protein data bank.

The enzyme and ligand were prepared, and the docking simulations were realized using the GOLD 2020.2.0 software package [37,38]. The ranking of the best poses was achieved from the GoldScore fitness function, where the selection of the pose is accompanied by the use of a scoring function, which includes the following components: hydrogen bond energy of the complex, internal energy of the ligand, and torsional energy [39,40]. The rescore was made using the ChemScore fitness function for the calculation of the parameter dG, which represents the total free energy change that occurs on ligand binding. Keeping

the conformations of the enzyme fixed, the conformations of the compound were allowed to change during the docking. The best poses and their respective molecular interactions were analyzed using the Discovery Studio 2020 software package.

4. Conclusions

Molecular docking calculations revealed that compounds 1 and 2 could interact with the amino acids of the FAD binding site in the TGR enzyme, a vital enzyme for *S. mansoni*, with binding energies comparable to those exhibited by PZQ, a drug of choice for treating schistosomiasis. Thus, this study suggests that flavonoids may have a potential impact on the treatment of Schistosomiasis. However, conducting comprehensive studies to validate the in vitro and molecular docking pharmacological claims for pharmaceutical applications is necessary.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/compounds5030030/s1>, Figure S1: ¹H-NMR spectrum of quercitrin (methanol-*d*₄, 400 MHz), Figure S2: ¹H NMR spectrum expansion of quercitrin, Figure S3: ¹³C NMR spectrum of quercitrin (methanol-*d*₄, 100 MHz), Figure S4: Expansion of the ¹³C NMR spectrum of quercitrin, Figure S5: Expansion of the ¹³C NMR spectrum of quercitrin, Figure S6: Mass spectrum of quercitrin with MS/MS 447, Figure S7: ¹H-NMR spectrum of afzelin (methanol-*d*₄, 400 MHz), Figure S8: ¹H NMR spectrum expansion of afzelin, Figure S9: ¹³C NMR spectrum of afzelin (methanol-*d*₄, 100 MHz), Figure S10: Expansion of the ¹³C NMR spectrum of afzelin, Figure S11: Expansion of the ¹³C NMR spectrum of afzelin, Figure S12: Mass spectrum of afzelin with MS/MS 431.

Author Contributions: Conceptualization, M.F.C.S. and L.G.M.; methodology, R.C.R.; software, R.C.S.V.; validation, S.R.A., R.P.O. and R.L.T.P.; formal analysis, M.L.A.e.S.; investigation, J.K.B.; resources, M.d.O.S.; data curation, H.O.G.C.; writing—original draft preparation, A.C.R.R.; writing—review and editing, W.Z.C.; visualization, M.F.C.S.; project administration, R.C.R.; funding acquisition, supervision, W.R.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: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee of UNIFRAN (permit 6242260122, 10 August2023).

Informed Consent Statement: Not applicable.

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

Acknowledgments: The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant # 2011/13630-7), Coordenadoria de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES, Finance Code 001), to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the fellowships and the FAPES.

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

Abbreviations

The following abbreviations are used in this manuscript:

PZQ	Oxamniquine or praziquantel
TGR	Thioredoxin glutathione
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
DMSO	Dimethyl sulfoxide

RPMI	Roswell Park Memorial Institute medium
FAD	Flavin adenine dinucleotide

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Article

Novel Butyrylcholinesterase Inhibitor Alkaloids from *Cannabis sativa* Roots: Bioguided Isolation and In Silico Study [†]

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[†] In Memoriam: Francisco Cecati (1984–2025), esteemed researcher and professor at the Universidad Nacional de San Luis. His passionate dedication to the study of medicinal cannabis and his unwavering commitment to teaching will continue to inspire future generations of scholars and researchers.

Abstract: Neurodegenerative diseases, including Alzheimer’s disease (AD), represent one of the main global health challenges. *Cannabis sativa* synthesizes spermidine-type alkaloids, whose potential biological activities have been little studied. This study aimed to isolate bioactive alkaloids from an alkaloid-enriched extract (AEE) of *C. sativa* roots throughout a bioguided approach using conventional chromatographic techniques based on AChE and BuChE inhibitory activities. A qualitative and semiquantitative analysis by UPLC-ESI-MS/MS as well as molecular modeling simulations were performed. In addition, predictive in silico analyses were conducted to assess toxicity properties. The alkaloids cannabissativine (CS) and anhydrocannabissativine (ACS) were isolated, and showed highly selective BuChE inhibitory activity. The molecular modeling study revealed a conserved interaction profile across both alkaloids, indicating the amino acids TRP82, GLU197, TYR440, and HIS438 as the major contributors involved in the complex formation. Finally, CS and ACS exhibited low in silico predictive toxicity values. In conclusion, CS and ACS alkaloids emerge as new selective BuChE inhibitors with therapeutic potential that deserves the attention from the field of pharmacology in neurodegenerative disease research. Additionally, this approach promotes innovation and environmental sustainability through the use of *C. sativa* roots.

Keywords: *Cannabis sativa* roots; spermidine alkaloids; butyrylcholinesterase; cannabissativine; anhydrocannabissativine

1. Introduction

Cannabis sativa L., a plant known since ancient times, has been recognized for its diverse uses as a source of fiber, food, oil, and for both recreational and religious purposes. Their therapeutic potential was emphasized, particularly in the management of chronic pain, depression, and inflammation [1]. Other studies reported antioxidant, antibacterial, anti-inflammatory, anti-acetylcholinesterase [2], anticoagulant, insecticide, anticancer, anti-aflatoxigenic, antifungal, cytotoxic, anti-elastase, anti-collagenase, neuroprotective (anti-Alzheimer’s, anti-epilepsy, and anti-Parkinson’s, to treat substance- and alcohol-use

disorders), and dermo cosmetic (anti-tyrosinase, anti-collagenase, and anti-elastase) properties [1,3–5]. Additionally, the synergistic relationship between terpenoids and cannabinoids was highlighted, showcasing their complementary roles in enhancing therapeutic efficacy for treating pain, psychiatric disorders, cancer, and various other conditions [6].

The secondary metabolite profile of *Cannabis sativa* has been extensively investigated, with approximately 750 compounds including cannabinoids and non-cannabinoids, and a wide range of biological activities have been documented, particularly those linked to central nervous system modulation [7,8]. A diverse spectrum of lesser-known phytocannabinoids, alongside terpenes, stilbenoids, lignanamides, carotenoids, flavonoids, and alkaloids types, exhibit a wide range of pharmacological activities by modulating the endocannabinoid system and engaging additional biochemical pathways [9–11]. The multifunctional spanning antioxidative, anti-inflammatory, and neural regulation actions of the mentioned compounds highlight their therapeutic relevance in neurodegeneration research [4].

Although the *C. sativa* exploitations are focused on the unfertilized female inflorescences, as the principal source of pharmacologically active constituents, other plant tissues also harbor unique unexplored biochemical profiles. For instance, the cannabis roots are rich in other compounds, including triterpenoids, friedelin, epifriedelane, alkaloids, cannabissativine (CS), and anhydrocannabissativine (ACS). Likewise, the ethnopharmacological data of decoctions, juices, and teas of the *Cannabis* roots indicate that these preparations could be used for the treatment of inflammation, fever, infection, and pain, as well as relief of symptoms related to labor and postpartum hemorrhages [1,2,5,12]. Furthermore, Ferrini et al. [13] proposed utilizing cannabis roots as a natural source of epi-friedelanol and friedelin, which are antioxidants and anti-inflammatory bioactive compounds with highly appealing properties. The leaves contain terpenes, polyphenols, cannabinoids, and alkaloids [1], and Fasakin et al. [14] explored this in cannabis leaves CS, cannabimine C, and ACS. The first identification of a non-quaternary alkaloid from the roots of *C. sativa* L. was reported as CS [15], while ACS was reported for first time by Elsohly et al. [16]

Considering the diverse pharmacological applications of the *C. sativa*, it becomes evident that plant components commonly discarded during processing, such as stems, leaves, and roots, may contain bioactive alkaloids with complementary properties. These underexplored tissues represent a promising reservoir of therapeutic compounds, particularly relevant to neurodegenerative diseases like Alzheimer's disease (AD), which pose one of the most pressing global health challenges today.

AD is a multifactorial neurodegenerative condition characterized by severe degeneration of cholinergic neurons and a marked reduction in acetylcholine levels, which contribute to synaptic dysfunction. Its onset involves the accumulation of amyloid- β (A β) peptides forming senile plaques and hyperphosphorylated Tau protein, leading to neurofibrillary tangles (NFTs). The pathological processes include neuroinflammation, mitochondrial and bioenergetic impairments, oxidative stress, and vascular abnormalities. Additional contributing factors include metal ion dysregulation, glutamate-induced excitotoxicity, microbiota–gut–brain axis disruptions, and defective autophagic pathways [17]. Alterations in the cholinesterases levels led to a cholinergic deficit in the brain, marked by reduced levels of acetylcholine. Understanding the interplay between AChE and BuChE in both normal and pathological conditions offers promising compelling therapeutic opportunities [18]. BuChE inhibition may also mitigate abnormal A β aggregation, further justifying the targeting of BuChE in AD treatment. Pharmacologically, BuChE and its modulators are linked to therapies for AD, multiple sclerosis, cocaine addiction, and organophosphate poisoning [19]. On the other hand, in vitro studies demonstrated that BuChE inhibitors reduce pro-inflammatory cytokines levels such as IL-1 β and TNF- α

in peripheral blood mononuclear cells, highlighting their immunomodulatory role [20]. Complementary findings reinforce this anti-inflammatory potential, showing that altered BuChE activity correlates with neuroinflammation markers in cerebrospinal fluid [19,21]. Selective inhibition of BuChE has emerged as a promising strategy for neurodegenerative and metabolic disorders, owing to its dual role in preserving acetylcholine levels and attenuating neuroinflammation [21,22]. Novel BuChE-selective compounds further demonstrated dual effect in cholinergic restoration and cytokine suppression [23,24], underscoring BuChE's evolving role from a peripheral biomarker to a prime therapeutic target in AD intervention strategies. To date, no studies have been reported regarding the biological activity of CS and ACS alkaloids isolated from Cannabis leaves or roots, leaving their potential therapeutic effects unexplored.

This work aimed to isolate bioactive alkaloids from *C. sativa* roots using a bioguided fractionation strategy based on AChE and BuChE inhibitory activity. To further investigate the molecular characteristics of the isolated compounds, *in silico* analyses were conducted to assess their binding interactions with the target cholinesterase enzymes.

The alkaloids CS and ACS were isolated through a bioactivity-guided approach from the alkaloid extract by conventional chromatographic techniques, and the AChE and BuChE-inhibitory activities are reported. The inhibitory effect of CS and ACS against BuChE were highly selective, and for further insight into the experimental results, an *in silico* study was performed employing docking simulations and molecular dynamics, revealing a conserved interaction profile across both alkaloids, especially on ACS.

2. Materials and Methods

2.1. Chemicals

Methanol (MeOH), sulfuric acid (H₂SO₄), ethyl ether (Et₂O), ammonium hydroxide (NH₄OH), ethyl acetate (EtOAc), and anhydrous sodium sulfate (Na₂SO₄) were purchased from Laboratorios Cicarelli (Buenos Aires, Argentina). Silica gel 60 0.063–0.2 mm from Macherey-Nagel GmbH & Co. KG (Düren, Germany) and TLC Silica gel 60 F₂₅₄ plates (20 × 20 cm) Merck KGaA were used. AChE from *Electrophorus electricus* (electric eel), BuChE from equine serum, dipotassium hydrogen phosphate (K₂HPO₄), sodium dihydrogen phosphate (NaH₂PO₄), sodium chloride (NaCl), disodium hydrogen phosphate (Na₂HPO₄), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), Galanthamine (Gal), and Donepezil (Don) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Plant Material and Extraction

Roots of *Cannabis sativa* var. *ultraviolet* were kindly provided by CANME, a public institution in San Juan province, Argentina (Programa de Investigación Plataformas Estratégicas (PIPE) N°1400-000091-2022). Fresh roots of *C. sativa* var. *ultraviolet* (512 g) were collected in March 2024, after flowering period, and were dried under air current at 40 °C until constant weight. The dried material was milled, resulting in 250 g of dried roots. It was subjected to maceration with MeOH (2 × 2500 mL, for 72 h each time), kept in darkness at room temperature, filtered, combined, and concentrated under reduced pressure, to give the methanolic extract (ME) 6.25 g, yielding 2.5% *w/w* referred to dry starting material. The ME was dissolved in a 2% (*v/v*) H₂SO₄ to pH 3.5–4. Three extractions with Et₂O (3 × 100 mL) were performed to remove fats and nonpolar compounds. The remaining aqueous phase was alkalized to pH 9–10 with 20% (*w/v*) NH₄OH and subjected to extractions with AcOEt (3 × 100 mL) to recover the alkaloids. The AcOEt fraction was separated, and anhydrous sodium sulphate was added to remove any remaining water.

Finally, the solvent was evaporated to dryness using a rotary evaporator (Waltham, MA, USA) in order to obtain the alkaloid-enriched extract (AEE; 45.9 mg) yielding 0.0198%.

2.3. Bioguided Isolation

The alkaloids were isolated through a bioactivity-guided approach from the AEE by conventional chromatographic techniques, evaluating in each separation instance, their inhibitory effect against AChE and BuChE by a modified Ellman's method, and selecting the active fractions for further isolation. Consequently, the AEE was chromatographed on a silica gel flash column (6 cm length, 2.5 cm i.d.), eluted with a gradient from EtOAc 100% to EtOAc: MeOH (8:2) giving three fractions; F1 (29 mg), F2 (3.7 mg), and F3 (14 mg). After being assayed, F3 showed selective BuChE inhibitory activity. Then, F3 was subjected to semi-preparative TLC (mobile phase EtOAc/MeOH – 9:1) to afford Cannabisativine (CS) (3.2 mg) and anhydrocannabisativine (ACS) (2.5 mg), which were evaluated against BuChE.

2.4. Ultra Performance Liquid Chromatography (UPLC) Analysis

The LC-MS analysis was performed in an ACQUITY H-Class UPLC instrument equipped with a XEVO TQ-S micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) with electrospray ionization (ESI). An UPLC ACQUITY BEH C18 (1.7 μ m, 2.1 mm $\times$ 50 mm) column was used for separation at 35 °C.

The mobile phase for semiquantitation analysis consisted of A (0.1% formic acid), B (acetonitrile), and C (methanol) with a flow rate of 0.2 mL/min. The gradient conditions were as follows: initially, 90%A—5%B—5%C; 3 min, 70%A—15%B—15%C; 3.10 min, 90%A—5%B—5%C and hold for 2 min; completing a total of 5 min. The AEE was prepared at 65 ppm. Caffeine was used as standard for calibration curve at the concentrations 10, 15, 20, 25, and 30 ppm. The samples were dissolved in a mixture of methanol/water (50:50) and filtered through a membrane filter (0.22 μ m). The injection volume was 10 μ L. The capillary, cone, and collision energies were 3 kV, 34 V, and 20 eV, respectively. The data were acquired in ESI positive mode using MS2 scan (60–1000Da) and daughters (m/z 195, 364, 382) function. The data were processed using MassLynx Software V4.2 (Waters, Milford, MA, USA).

2.5. Microplate Assay for AChE and BuChE Inhibitory Activities

Cholinesterases inhibitory activities were performed according to Ellman et al. [25] with modifications [26]. In each well of a 96-well microplate, 50 μ L of AChE or BuChE enzyme solution (0.25 U/mL, in phosphate-buffer saline (PBS): 8 mM K₂HPO₄, 2.3 mM NaH₂PO₄, 0.15 M NaCl, pH 7.5) and 50 μ L of each sample dissolved in the same buffer were added. The plates were incubated at room temperature for 30 min. Subsequently, 100 μ L of the substrate solution, containing DTNB and ATC or BTC (0.6 mM), prepared in a saline solution with Na₂HPO₄ (pH 7.5), was added. The absorbance at 405 nm was recorded using a Thermo Scientific Multiskan FC spectrophotometer (Waltham, MA, USA), 5 min after the reaction began. To calculate the IC₅₀ values, the concentrations of the AEE, fractions, and the alkaloids assayed ranged from 1 to 200 (μ g/mL or μ M). Gal and Don were used as positive controls and PBS was used as a negative control. The enzyme-inhibitory activity was calculated as a percentage using PBS without inhibitor.

2.6. Molecular Docking Study

2.6.1. Automated Docking Setup

The X-ray crystal structure corresponding to HsBuChE (PDB code: 1P0I) [27] was obtained from the Protein Data Bank (<http://www.rcsb.org> (accessed on 22 July 2025)) and used as the receptor. Before the calculations, any non-amino acid residues were removed.

Both ligands, CS and ACS (compounds CID: 442846 and 14106160, respectively), were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/> (accessed on 22 July 2025)). Molecular docking was performed with AutoDock4 [28], treating the receptor structure as rigid. Grid box dimensions were defined as $50 \times 50 \times 50$ in the X, Y, and Z directions, with a spacing resolution of $0.375 \text{ \AA}$, centered at the enzyme's active site. Gasteiger charges were assigned to the ligands, and all non-polar hydrogen atoms were merged. All possible torsions of the ligand were allowed to rotate, and 200 docking poses were generated. The maximum number of energy evaluations was set to 2.5×10^6 , and the maximum number of generations to 2.7×10^4 , with all remaining parameters left at their default values. The resulting poses were grouped into clusters based on the root-mean-square deviation (RMSD) of their backbones and then ranked according to their predicted binding free energies. The conformation from the most populated cluster displaying the lowest relative free energy was selected for MD simulations.

2.6.2. Molecular Dynamics Simulations

MD simulations were carried out using the AMBER22 [29] software package with the ff14SB [30] and GAFF [31] force fields. Complex assembly was performed with the Leap module, and ligand parameters were generated using the antechamber module. Each selected docked conformation was solvated in an octahedral periodic box filled with TIP3P water molecules [32], ensuring a $10 \text{ \AA}$ buffer from the solute in all directions. To neutralize the systems, Na^+ or Cl^- ions were added as required. The Sander module was then used to minimize the potential energy of each system for 5000 steps with the steepest-descent algorithm. Next, each system was equilibrated under constant volume conditions for 2500 ps, during which the temperature was gradually raised from 0 to 300 K using a Langevin thermostat [33] at a collision frequency of 1.0 ps^{-1} . The SHAKE algorithm [34] was applied to constrain all bonds involving hydrogen, allowing an integration time step of 2 fs. Production MD simulations were executed in triplicate at 298 K for a total of 50 ns each, resulting in 150 ns of simulated time per complex. The Particle Mesh Ewald (PME) method [35] was used with a grid spacing of $1.2 \text{ \AA}$, a spline interpolation order of 4, and a real-space cutoff of $10 \text{ \AA}$. Analyses of the simulation trajectories were conducted using CPPTRAJ [36].

2.6.3. MM-GBSA Free Energy Decomposition

The relative binding free energies for each complex were evaluated using the MM_PBSA module in Amber version 22, followed by a decomposition of the total free energy to identify the critical BuChE active-site residues interacting with the ligands. Snapshots corresponding to the last 30 ns of each trajectory were collected, and explicit water molecules along with counter ions were removed before analysis [29].

2.7. Preliminary Toxicological Assessment

The ProTox-3.0 web tool (<https://comptox.charite.de/protox3/> (accessed on 22 July 2025)) [37] was employed to predict the toxicological profiles of cannabissativine and anhydrocannabissativine, including acute toxicity, organ-specific effects, genotoxicity, and potential cholinesterase inhibition. In parallel, the SwissADME web tool (<http://www.swissadme.ch> (accessed on 22 July 2025)) [38] was used to predict ADME-related properties (Absorption, Distribution, Metabolism, and Excretion), including gastrointestinal absorption, topological polar surface area (TPSA), lipophilicity (cLogP), hydrogen-bonding capacity, drug-likeness, blood–brain barrier permeability, and cytochrome P450 inhibition.

2.8. Statistical Data Analysis

All measurements from the AChE and BuChE inhibition assays were performed in triplicate, and IC_{50} values were expressed as the mean $\pm$ standard deviation (SD) of three independent experiments. Data were analyzed using Prism 10.4.1 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. UPLC-MS/MS Analysis

For the qualitative and semiquantitative analysis, several UPLC-ESI-MS/MS and DI-ESI-MS/MS measurements were carried out for the *C. sativa* alkaloids-enriched extract (AEE), fractions, and the isolated compounds. The alkaloids were identified based on the m/z species and their fragmentation patterns, comparing the results obtained with those in the literature. The AEE DI-MS analysis revealed the m/z ions; 382, 364 as well as the m/z ions; 182.6 and 191.6, which were tentatively assigned to cannabissativine (CS) and anhydrocannabissativine (ACS) spermidine alkaloids and would correspond for the species $[M+H]^+$ and $[M+2H]^{2+}$, respectively. In order to confirm the alkaloids identity, UPLC-ESI-MS/MS and DI-MS/MS analysis were performed for the AEE, F3 fraction, ACS, and CS (daughters analysis of m/z 364 and 382) (Figures S1–S3). Thus, in all samples, the results for ACS $[M+H]^+ = 364(7)$ displayed the following fragments: 208(100), 171(20), 250(12), 129(9), 112(5), 198(4), and 181(1) which were in agreement with those of ACS found in the literature [16,39–41]. Regarding CS $[M+H]^+ = 382(44)$ the fragments observed were m/z 96(100), 226(62), 198(27), 112(9), 126(8), 157(7), 364(5), 129(5), 143(5), 264(4), 171(3), and 208(3), confirming the identity for CS [15,39–42]. The proposed ESI(+)-MS/MS main fragments structures for ACS and CS, are available in Supplementary Materials (Table S1 and S2).

In regard to the alkaloid content analysis, CS and ACS were semiquantified by UHPLC-ESI-MS/MS related to dry weight of *C. sativa* roots, obtaining concentration values of 0.002% (20.45 mg/kg dw) and 0.0042% (42.88 mg/kg dw) (w/w), respectively. The linear correlation curve was $r^2 = 0.988735$ and the linear equation was $y = 199931x - 62742.5$ (Figure S4). The structures of the compounds are shown in Figure 1. Although the structural differences among caffeine, CS, and ACS are considerable, these results provide an approximation that allows us to estimate the potential levels of these natural products in *C. sativa* roots. A quantitative analysis using a closely related derivative will be performed in the future.

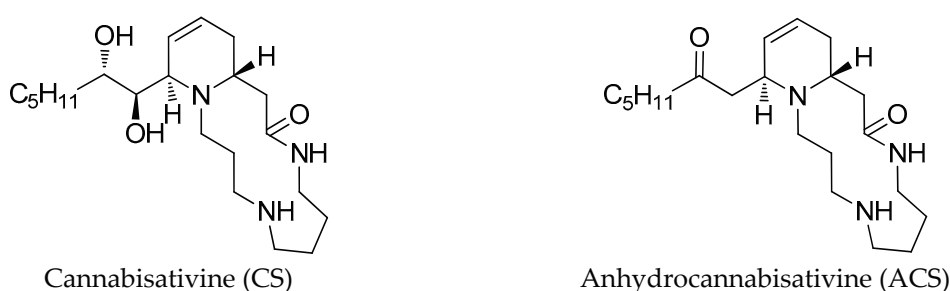


Figure 1. Chemical structures of the identified alkaloids from *C. sativa* roots.

3.2. Cholinesterases Inhibitory Bioassay

The AEE, along with its fractions and isolated compounds (CS and ACS), were evaluated for in vitro inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The results are summarized in Table 1, expressed as half-maximal inhibitory concentration (IC_{50}) values, and selectivity indices (SI) as IC_{50} AChE/ IC_{50} BuChE. Under the experimental conditions, the standard compounds galanthamine (Gal)

and donepezil (Don) exhibited IC_{50} values of $0.97 \pm 1.33 \mu\text{M}$ and $0.06 \pm 0.01 \mu\text{M}$ against AChE, and $15.60 \pm 1.25 \mu\text{M}$ and $4.71 \pm 0.11 \mu\text{M}$ against BuChE, respectively. The AEE showed inhibition against BuChE ($IC_{50} = 51.52 \pm 2.90 \mu\text{g/mL}$), while for AChE it was inactive ($IC_{50} = 311.24 \pm 13.02 \mu\text{g/mL}$). After flash chromatography, three fractions (F1–F3) were obtained and assessed for cholinesterase inhibition. Notably, F3 exhibited strong inhibitory activity only for BuChE ($IC_{50} = 13.40 \pm 1.04 \mu\text{g/mL}$), whereas F1 and F2 were inactive against both enzymes. The bioguided isolation yielded the alkaloids CS and ACS (Figure 1), showing selective activity against BuChE IC_{50} $26.38 \pm 0.95 \mu\text{M}$; SI ≥ 7.58 , and IC_{50} 24.97 ± 1.08 ; SI ≥ 8.00 , respectively.

Table 1. Acetylcholinesterase and butyrylcholinesterase inhibitory activities of the alkaloid-enriched extract, fractions, cannabistatine, and anhydrocannabistatine from *Cannabis sativa* roots.

Samples	^a IC_{50} [$\mu\text{g/mL}$]		^b SI
	AChE	BuChE	
AAE	311.24 ± 13.02	51.52 ± 2.90	6.04
F1	>200	>200	-
F2	>200	>200	-
F3	>200	13.40 ± 1.04	≥ 14.92
^a IC_{50} [μM]			
CS	>200	26.38 ± 0.95	≥ 7.58
ACS	>200	24.97 ± 1.08	≥ 8.00
^c Gal	0.97 ± 1.33	15.60 ± 1.25	0.062
^c Don	0.06 ± 0.01	4.71 ± 0.11	0.012

^a IC_{50} values are expressed as the means $\pm$ SD of three replicate determinations. ^b SI is the BuChE selectivity index calculated as IC_{50} AChE/ IC_{50} BuChE. ^c Reference compounds.

3.3. In Silico Toxicological and ADME Profiling

Both alkaloids, CS and ACS, were subjected to in silico toxicological evaluation using the ProTox-3.0 platform [37]. These compounds were classified as toxicity class 4, with a predicted LD_{50} of 418 mg/kg^{-1} . While the neuro- and respiratory toxicity were estimated to have moderate probability, the hepatotoxicity, nephrotoxicity, cardiotoxicity, cytotoxicity, mutagenicity, and carcinogenicity were predicted inactive (Table 2). A subtle difference emerged in immunotoxicity classifying CS as marginal, while ACS remained inactive.

Regarding oral bioavailability, both alkaloids satisfied Lipinski's rule-of-five and were predicted to have high gastrointestinal absorption, according to SwissADME [38]. Their physicochemical properties, including topological polar surface area (TPSA), calculated partition coefficient (cLogP), and hydrogen-bonding capacity, were in accordance with this prediction. The blood–brain barrier (BBB) properties for both compounds were predicted to be permeable by ProTox-3.0; however, SwissADME predicted permeability only for ACS (Table 2).

Table 2. In silico toxicity and pharmacokinetic parameters predicted for CS and ACS.

Descriptor	Cannabistatine	Anhydrocannabistatine
	ProTox-3.0	
LD_{50} (mg/kg^{-1})	418	418
Toxicity class	4	4
Neuro-toxicity (probability)	0.62	0.73
Respiratory-toxicity (probability)	0.78	0.72
Immunotoxicity (probability)	0.54	0.56
Mutagenicity/Carcinogenicity	Inactive	Inactive

Table 2. Cont.

Descriptor	Cannabisativine	Anhydrocannabisativine
Hepatotoxicity	Inactive	Inactive
BBB permeation (probability)	0.51	0.85
Acetylcholinesterase (AChE)	Inactive	Inactive
<i>SwissADME</i>		
BBB permeation (qualitative)	No	Yes
<i>P</i> -gp substrate	Yes	Yes
GI absorption	High	High
cLog <i>P</i>	2.16	3.40
TPSA (Å ²)	84.8	61.4
H-bond donors / acceptors	4/6	2/5
Bioavailability score	0.55	0.55
CYP inhibition	None	CYP2D6 *

* ProTox-3.0 flags a low-confidence CYP2D6 interaction for ACS (probability 0.52); SwissADME predicts “non-inhibitor”.

3.4. Molecular Modeling

Molecular docking studies were applied to obtain accurate predictions of protein–ligand interaction for CS and ACS. To evaluate the dynamic behavior of the compounds over time, molecular dynamics simulations were conducted. These analyses provided insight into the stability and adaptability of the ligand–enzyme complexes. The final stage involved calculating the binding free energies based on the trajectories obtained from MD simulations. A per-residue decomposition analysis was also performed to identify key amino acids in the active site of BuChE that contribute to the intermolecular interactions within each complex. Both ligands, CS and ACS, bind to the catalytic gorge of human BuChE, interacting with key amino acids commonly associated with ligand binding in this enzyme. A comparative breakdown of key observations, overall binding position, and orientation are shown in Figures 2 and 3 (panels A and B).

In both complexes, the ligands occupy the same general region in the BuChE catalytic site, suggesting they interact with the same set of critical residues: TRP82, GLU197, TYR440, and HIS438. The close-up views (panels B) confirm that each ligand establishes contact via aromatic stacking (notably with TRP82 and TYR440) and hydrogen bonds (with GLU197 and HIS438). The bar charts display how each residue in the BuChE sequence contributes to the total interaction energy with the ligand. Negative values indicate favorable (stabilizing) contributions; larger negative peaks suggest more significant binding interactions. Regarding CS, the amino acids TRP82 and GLU197 emerge as major contributors, reinforcing their role as key anchoring residues. However, the magnitude of these peaks might differ compared to ACS. Likewise, TYR440, HIS438, and PHE329 show notable negative energy contributions, indicating aromatic and/or hydrogen-bond interactions that stabilize the ligand. Some additional residues, such as LEU125 and TYR128, would also contribute to binding, although these interactions slightly differ from those observed with ACS.

For ACS, the residues TRP82 and GLU197 show particularly large negative energy contributions, indicating strong stabilizing interactions. To a lesser extent, TYR440 and HIS438 also contribute to the stabilization of the complex. Additional residues such as TYR128, THR120, and GLY116 provide moderate yet favorable contributions, suggesting ancillary interactions that help position or stabilize the ligand in the binding pocket.

Both ligands rely on the same fundamental set of anchoring residues, but subtle variations in the contribution of individual residues may explain any differences in binding affinity or specificity. Comparison of the key peaks, TRP82 vs. GLU197, in each system may provide insights into how each ligand orients within the active site and whether it engages in stronger hydrogen bonding, π – π stacking, or electrostatic interactions.

In summary, both CS and ACS interact with key BuChE residues, especially TRP82 and GLU197. Variations in peak magnitudes from the per-residue energy decomposition suggest that, while both ligands bind in a similar region, their interaction patterns vary, which may influence overall binding affinity and inhibitory activity.

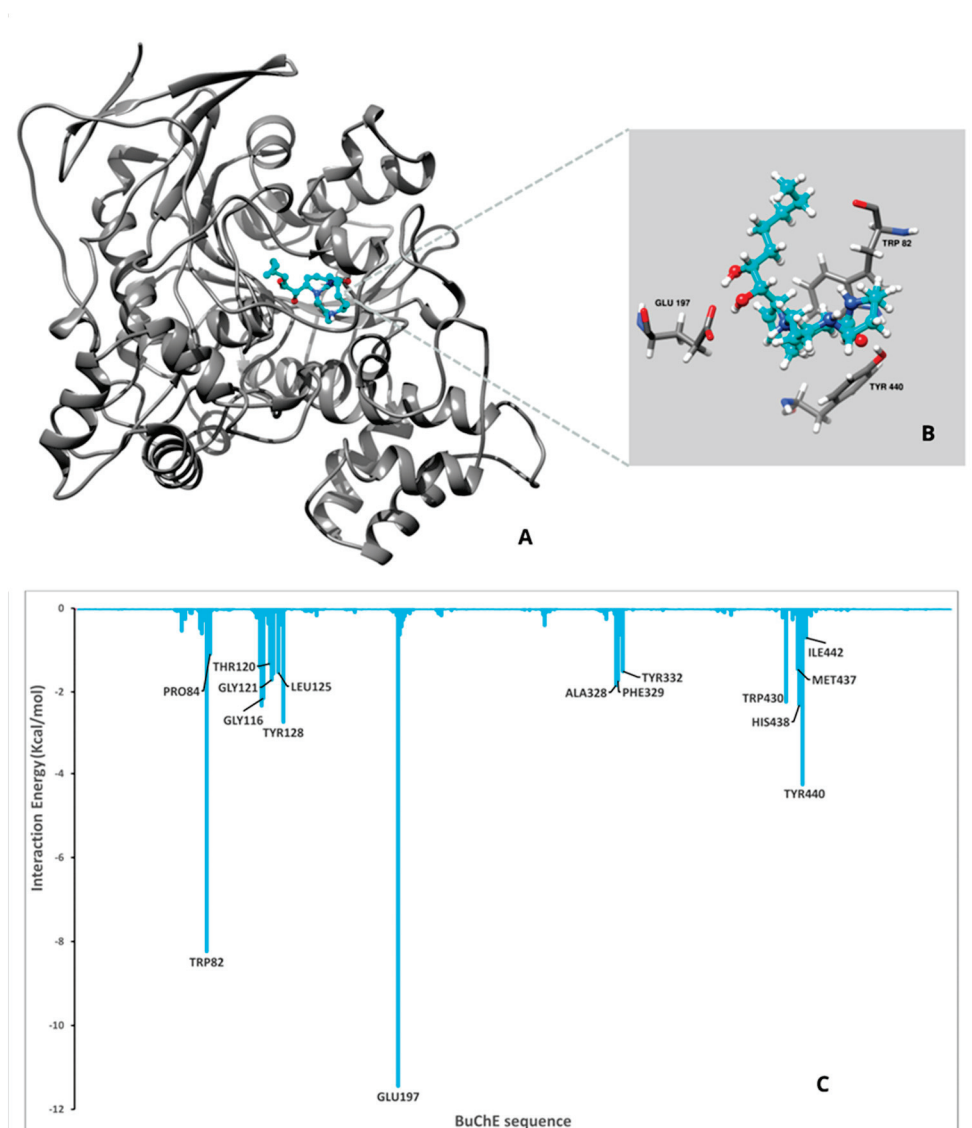


Figure 2. Results for CS Molecular Dynamics (MD) analysis. **(A)** The complex between ligand and enzyme obtained after the MD: BuChE is shown in gray and the ligand in cyan. **(B)** Active site zoom showing the strongest interaction between the ligand and the amino acids. **(C)** per residues analysis for the complex, the bars indicate the interaction energy between an amino acid and the ligand. “-” represents negative values in Y-axis numbers.

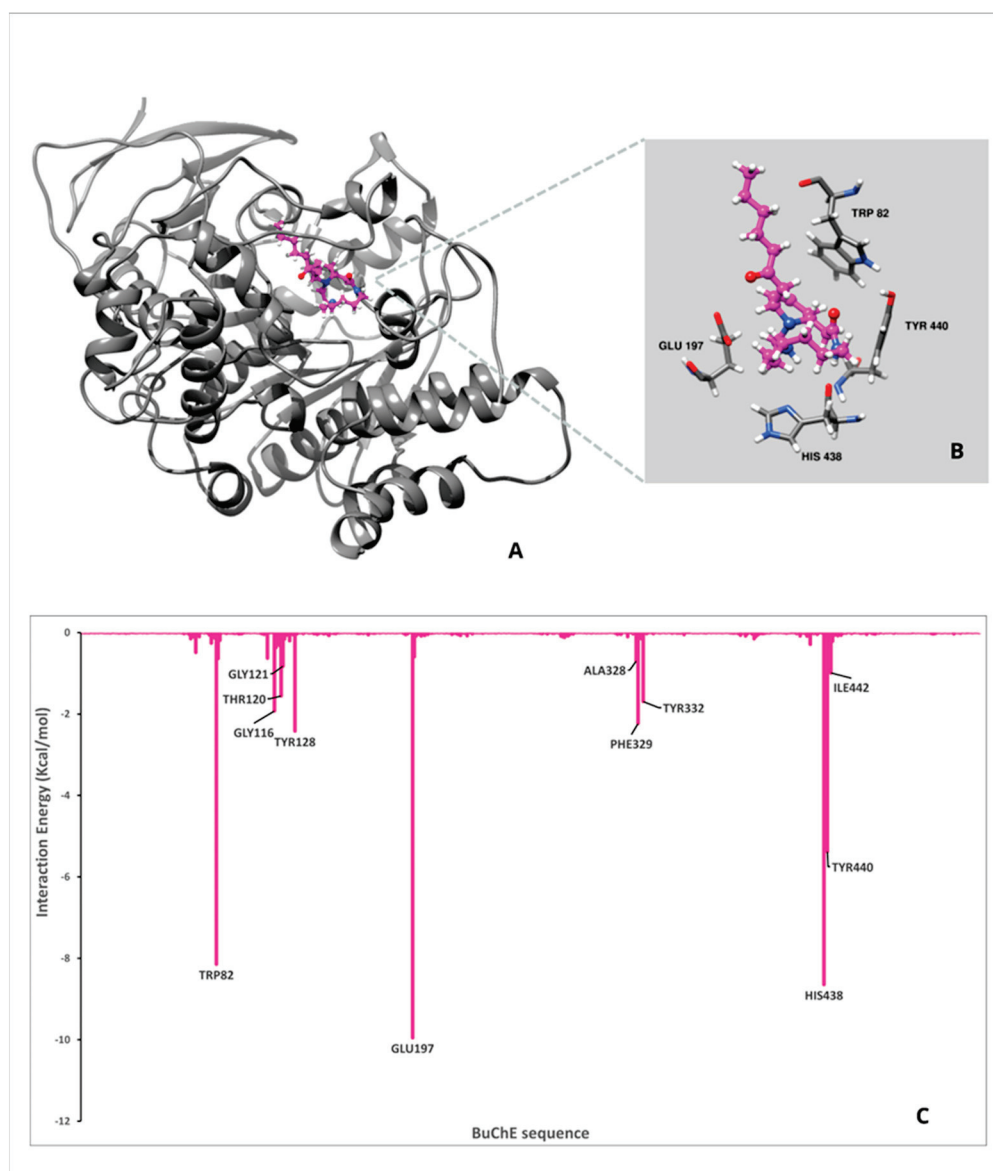


Figure 3. Results for ACS Molecular Dynamics (MD) analysis. (A) The complex between ligand and enzyme obtained after the MD: BuChE is shown in gray and the ligand in magenta. (B) Active site zoom showing the strongest interaction between the ligand and the amino acids. (C) Per-residue analysis for the complex, the bars indicate the interaction energy between an amino acid and the ligand. “-” represents negative values in Y-axis numbers.

4. Discussion

The *Cannabis* roots have been comparatively overlooked, receiving far less scientific attention than the aerial parts, despite their traditional use in treating fever, inflammation, infections, and arthritis [7]. This work investigates the neuroprotective potential of cannabissativine (CS) and anhydrocannabissativine (ACS) isolated from *C. sativa* roots through a bioguided fractionation strategy centered on BuChE inhibition. To further characterize the molecular features of both alkaloids, *in silico* docking analyses were performed, revealing favorable binding interactions with the BuChE active site.

The alkaloids in *C. sativa* are mainly in the roots and leaves [42,43]. Nowadays, the cannabis roots are discarded in the production of medicinal cannabis, and represent between 30% and 50% of the plant biomass. To the best of our knowledge, the spermidine-type alkaloids, CS and ACS, have not been investigated for their potential biological activities [5]. CS and ACS are polyamines subclassified as dihydroperiphylline type alkaloids [43].

The semiquantitative analysis of the isolated alkaloids revealed that CS accounted for 0.002% and ACS for 0.004% related to dry weight of *C. sativa* roots. These values were notably higher than those previously reported, approximately two-fold higher, suggesting that this extraction method may be more efficient. Considering that under acidic thermal conditions CS may undergo dehydration, it is plausible that during the extraction process, partial conversion of CS to ACS occurs [15,16].

The bioguided isolation process revealed that the inhibitory activities were BuChE-selective for AEE, F3, and the isolated alkaloids. Likewise, the inhibitory potency progressively increased during the bioguided isolation, with F3 showing four-fold and CS and ACS five-fold higher activity than AEE, indicating that these alkaloids are responsible for the inhibitory effect in the AEE. In this regard, Gagné et al. [2] reported antimicrobial, anti-inflammatory, anticholinesterase, and antioxidant activities for different cannabis roots extracts (aqueous, alcohol, and acid-base), indicating the presence of several phenolic compounds, cannabinoids, terpenoids, amino acids, and nitrogen-containing compounds as responsible for the studied biological activities. Likewise, aqueous extracts from cannabis roots with antitussive, expectorant, anti-inflammatory, antiasthmatic, and slight toxic effects were reported [39,44]. Additionally, de Lima Araújo et al. [41] described its antidysmenorrheic and nociceptive effects, recognizing CS, ACS, *p*-coumaroyltyramine, and feruloyltyramine as the active compounds. However, all the reported bioactivities were only evaluated for the *C. sativa* roots extracts, while the isolated compounds were not tested.

In order to gain deeper insight into the observed experimental outcomes, a molecular modeling investigation was carried out over BuChE. Molecular docking and molecular dynamics simulations were performed to evaluate the interactions of CS and ACS with BuChE to identify the most favorable conformations.

The dynamic simulations indicated that CS and ACS are capable of binding within the BuChE catalytic site in a manner analogous to Gal and Don interaction on CAS and PAS, respectively [45]. These findings suggest a conserved interaction pattern across all these alkaloids, supporting their inhibitory activity. In summary, a comparative study of two similar alkaloids, which differ primarily in their interaction with GLU197, revealed that ACS interacts with nearly three times the intensity of CS. Perhaps the absence of hydroxyl groups in ACS allows it to penetrate more deeply into the BuChE active-site gorge, thereby promoting stronger interactions with CAS and influencing overall binding strength and potential inhibitory activity.

Regarding *in silico* toxicity and pharmacokinetic parameters, the predictions for CS and ACS indicate that these compounds are not active against AChE (Table 2). These results are in agreement with those of the AChE *in vitro* assay. In terms of metabolic interactions, neither CS nor ACS were predicted to significantly inhibit any major cytochrome P450 (CYP450) isoforms. However, ProTox-3.0 predictions identified a low-confidence potential interaction between CS and CYP2D6.

ProTox-3.0 prediction indicated favorable BBB permeability for both alkaloids, while SwissADME predicted permeability only for ACS; this difference may be attributed to its lower polarity and fewer hydrogen bond donors relative to CS [46]. In addition, the CS and ACS alkaloids were also identified as *P*-glycoprotein (*P*-gp) substrates, which may reduce their effective accumulation in the central nervous system despite predicted permeability. Regarding to metabolism, no relevant inhibition of major CYP450 isoforms was predicted. However, a low-confidence interaction with CYP2D6 was indicated for ACS by ProTox-3.0. These results suggest that both alkaloids display acceptable toxicological profiles, promising pharmacokinetic characteristics, and potential for central nervous system accessibility, particularly ACS. The moderate neuro- and respiratory toxicity predictions, along with the potential CYP2D6 interaction, highlight aspects requiring further experimental validation.

Considering that BuChE has recently gained relevance as a target in neurodegenerative diseases research [47], CS and ACS alkaloids would emerge as promising compounds in the research for future potential treatments. In the early stages of AD, AChE inhibitors are employed to enhance synaptic acetylcholine (ACh) levels. AChE activity declines by approximately 50% as the disease advances, while BuChE activity increases up to 900% in late-stage of the AD [19,21]. Under these conditions, BuChE becomes the predominant enzyme responsible for ACh hydrolysis, thereby exacerbating cholinergic deficits and highlighting its compensatory substitution for AChE activity [47]. This abnormal upregulation reinforces its potential as an emerging therapeutic target in AD treatment strategies [19,21,47]. Inhibition of BuChE is generally accompanied by fewer adverse effects [47]. In this regard, the ProTox 3.0 results predicted that CS and ACS exhibited moderate toxicity with equal values of LD50 of 418 [mg/kg, classified as Class4, being less toxic at higher doses compared to Gal (LD50 = 85 mg/kg, Class 3), and similar to Don (LD50 = 505 mg/kg, Class 4), making it an attractive target for therapeutic intervention as BuChE inhibitors.

5. Conclusions

The bioguided phytochemical study from *C. sativa* roots led to the identification of cannabissativine (CS) and anhydrocannabissativine (ACS) spermidine-type alkaloids showing low in silico predictive toxicity values and highly selective inhibitory activity, as well as favorable binding affinities and interaction patterns against BuChE.

Cannabis roots, typically discarded as waste during medicinal cannabis production, can be transformed into a valuable source of bioactive compounds with potential applications in neurodegenerative disorders. In conclusion, CS and ACS alkaloids emerge as new selective BuChE inhibitors with therapeutic potential that deserves attention from the field of pharmacology and medicinal chemistry for its potential to drive global therapeutic innovation while reinforcing environmental sustainability.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/compounds5030035/s1>, Figure S1: Cannabissativine (CS) MS/MS spectrum (daughters of 382); Figure S2: Anhydrocannabissativine (ACS) MS/MS spectrum (daughters of 364); Figure S3: MS spectrum of the alkaloid-enriched extract from *C. sativa* roots (DI-ESI-MS). Red arrows indicate $[M+H]^+$ species of CS and ACS, while blue arrows their $[M+2H]^{2+}$ species.; Figure S4: Caffeine calibration curve for CS and ACS quantification.; Table S1: Proposed ESI(+)-MS/MS main fragments structures for cannabissativine.; Table S2: Proposed ESI(+)-MS/MS main fragments structures for anhydrocannabissativine.

Author Contributions: Conceptualization, J.E.O. and G.E.F.; methodology, D.C., J.E.O., C.W.A.-F., and O.L.-C.; software, C.W.A.-F., A.G., and O.L.-C.; formal analysis, J.E.O., C.W.A.-F., and G.E.F.; investigation J.E.O., C.W.A.-F., and G.E.F.; resources, J.E.O. and G.E.F.; writing—original draft preparation, J.E.O., C.W.A.-F., and G.E.F.; writing—review and editing J.E.O. and G.E.F.; supervision G.E.F.; project administration, J.E.O. and G.E.F. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially supported by Programa de Investigación Plataformas Estratégicas (PIPE) N°1400-000091. 2022 SECITI Gobierno de la Provincia de San Juan, CICITCA- UNSJ.

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

Acknowledgments: We would like to express our gratitude to Julian Moreno and Mariano Distefano for their technical assistance. C.W.A.-F. and O.L.-C. hold a fellowship from CONICET. G.E.F., which belongs to CONICET. Generative AI tools were not utilized. The authors independently conceptu-

alized, integrated, and finalized the graphical abstract using Canva (www.canva.com, accessed on 22 July 2025).

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

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Review

Therapeutic Capabilities of Triterpenes and Triterpenoids in Immune and Inflammatory Processes: A Review

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Abstract: Plant-derived secondary metabolites such as triterpenes and triterpenoids are present in a wide range of plant species. These compounds are particularly attractive due to their extensive range of biological properties and potential applications as intermediates in the synthesis of novel pharmacologically promising medications. Saponins, which are glycosylated triterpenoids found in nature, exhibit the same properties. At this point, the effectiveness of saponins as an anti-inflammatory medication has been verified. This review article examines the primary connections between immune responses and anti-inflammatory activity, focusing specifically on the correlation between triterpenes and triterpenoids. These connections have been investigated in various cell models, as well as *in vitro* and *in vivo* studies. The present research provides a comprehensive overview of the current understanding of the therapeutic capabilities of triterpenes and triterpenoids in immune and inflammatory processes. It also highlights emerging standards and their potential utilization in pharmaceutical and clinical settings.

Keywords: bioactive compounds; leaves; saponins; therapeutic agents; extraction; α -amyrenone; ursane; oleanane; squalene

1. Introduction

Bioactive compounds are present in various plant materials and are categorized into several classes, including terpenoids, alkaloids and other nitrogen-containing compounds, organosulfur compounds, and phenolics [1,2]. Bioactive compounds are natural primary and secondary metabolites obtained from different plant parts, including leaves, stems, roots, seeds, flowers, and fruits. These compounds can be recovered through various extraction methods [3,4]. The demand for these compounds has risen due to their perception as natural and safe for use in various industries, including cosmetics, food, agriculture, and pharmaceuticals [5]. Bioactive compounds exhibit a broad range of health-promoting properties in humans and animals. These properties include antibacterial, antimicrobial, anti-inflammatory, anti-aging, and anticancer effects [1,6].

Triterpenes, along with sterols, are isoprenoids that are synthesized via the mevalonate pathway [7]. Triterpenes are a type of secondary metabolite comprising 30 carbon atoms that are generated through the conjunction of six isoprene units. Through the process of cyclization and oxidation of two units with 15 carbon atoms each, squalene or similar acyclic 30-carbon precursors can be produced [8]. Approximately 20,000 triterpenes have been identified in nature, with a significant number existing in their unbound state. Other compounds could be found as glycosides (saponins) or in other forms. The main natural triterpenes are cyclic derivatives and can be classified based on the number of rings in

their structures. Figure 1 illustrates typical structures of triterpenes and triterpenoids. In this regard, the majority of C₃₀ derivatives fall into two categories: tetracyclic (lanostanes, euphanes, tirucallanes, cucurbitanes, dammaranes, and baccharenes) or pentacyclic (cycloartanes, ursanes, oleananes, lupanes, friedelanes, hopanes, and serratanes) derivatives, as also stated by Thimmappa et al. [7]. Cyclic triterpenes have the ability to undergo additional conversion into a range of metabolites such as steroids, saponins, limonoids, and other chemically related compounds [9]. However, triterpenic molecules are highly hydrophobic, which severely limits their use as effective pharmacological agents. Currently, one of the most common ways to increase triterpenic potency and bioavailability is chemical modification. Typically, this necessitates elevated temperature and pH levels, the utilization of expensive reagents, and the introduction of protective groups to the reactive centers of the molecule [10–13]. Due to the preceding factors, there is a search for plant-derived compounds that can be used as an alternative or complementary approach to antibiotic treatment. These compounds may also have the ability to prevent the formation of biofilms or aid in the complete removal of existing biofilms. Recently, there has been a growing fascination with pentacyclic triterpenes. Polycyclic organic compounds derived from plants are utilized in traditional phytotherapy for their diverse range of beneficial therapeutic qualities [14]. Preclinical investigations have demonstrated that triterpenes exhibit a diverse array of pharmacological actions such as anticancer, antioxidant, anti-inflammatory, anti-atherosclerotic, antiviral, hepatoprotective, and immunomodulatory activity [8,15].

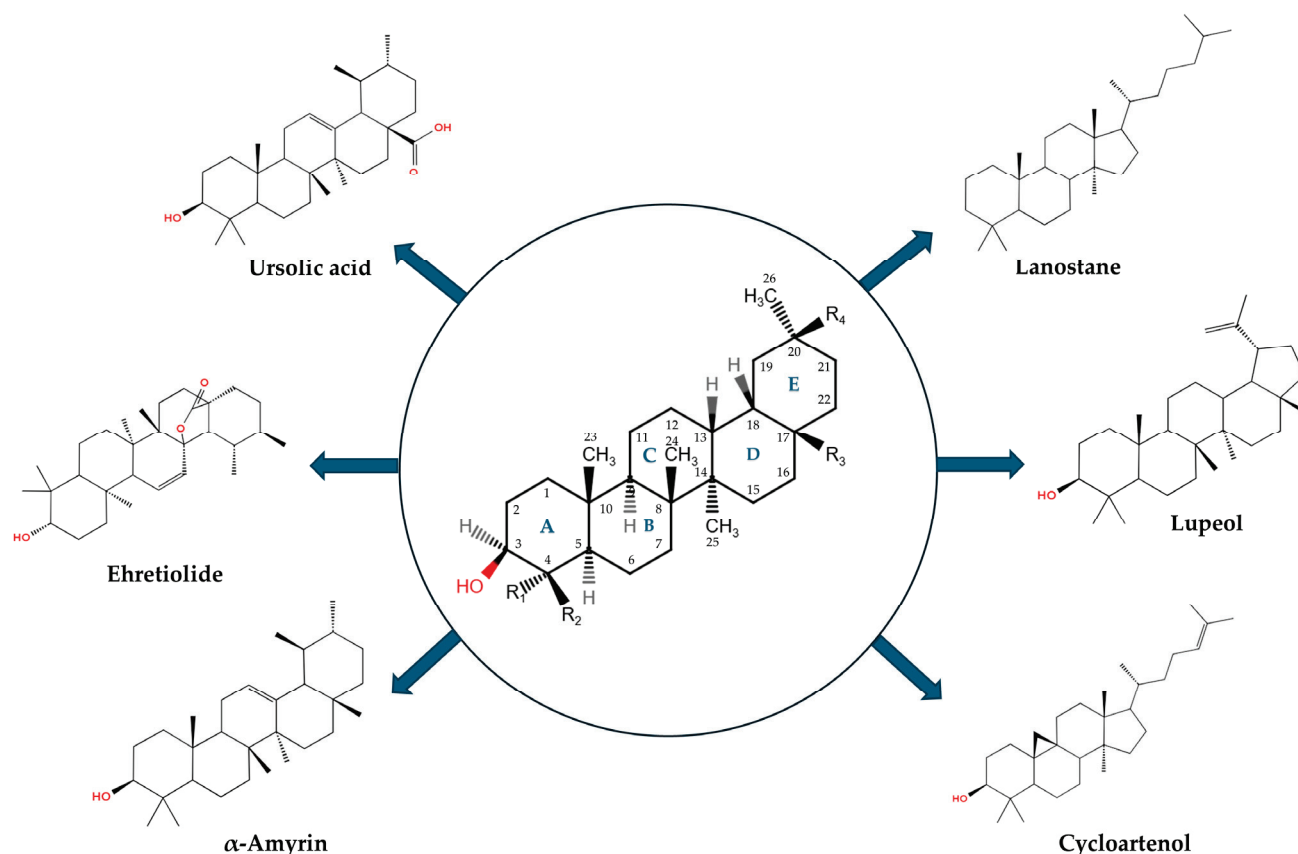


Figure 1. Structures of different triterpenes and triterpenoids derived from the same biosynthetic pathway. A typical triterpenoid structure is depicted in the center.

Terpenoids, commonly referred to as terpenes, are widely recognized for their antioxidant properties [1]. Out of the terpene-based group, triterpenes are considered to possess neuroprotective properties [16]. Triterpenoids are triterpenes that contain heteroatoms, typically oxygen, and they are complex organic compounds composed of 30 carbon atoms

arranged in five 6-carbon rings, each with various substituents. This triterpene group is produced through the cytosolic mevalonate route from acetyl-CoA, which is transformed into active isoprene units. Terpenoids are formed by the condensation of six molecules of activated isoprene [17]. They are most frequently found in the peels of fruits, leaves, and the bark of plants [18]. Their primary physiological function is to protect against the detrimental impacts of bacteria and insects [18]. Triterpenoids represent a class of chemicals that is found in many different plants, and they have a wide range of structures. They have attracted interest due to their pharmacological properties [19]. The biosynthesis of isoprenoids/terpenoids is important for living organisms, agriculture, and industry [20]. Natural products are crucial for the identification of numerous potentially bioactive medications due to a substantial body of research that showcases their analgesic, hepatoprotective, anti-inflammatory, antibacterial, antioxidant, and antiallodynic properties.

Natural compounds, such as polyphenols and triterpenic compounds, have been utilized in traditional medicine and continue to be employed today because of their bioactive properties [21]. The pharmacological actions of many triterpenoids have been linked to the significant functional groups present in the ring structure, such as the hydroxyl group, which has been attributed to the numerous pharmacological effects found for certain pentacyclic triterpenoids, such as their anticancer properties [22]. Additional research revealed that the functions of hydroxylated pentacyclic triterpenoids are greatly influenced by the quantity of hydroxyl groups present in rings C1–C6. This was observed in chemically altered 18 β -glycyrrhetic acid, which is an oleanane-type triterpenoid [23]. Terpenes obtained from plants have also been linked to therapeutic benefits such as anticancer and anti-inflammatory properties [24], along with the capacity to overcome treatment resistance and inhibit cancer migration [22,25]. Triterpenoids possess a diverse array of biological properties, such as anticancer, antiangiogenic, anti-inflammatory, antiviral, antioxidant, antidiabetic, antihyperlipidemic, antibacterial, hepatoprotective, and cardioprotective effects, among others. Thus, the majority of triterpenoids have significant biological activity while maintaining a low level of toxicity, suggesting their potential as a substitute for conventional chemotherapeutic agents [18,26]. Despite the recent reviews of Biswas et al. [27] and Bildziukevich et al. [28], in which the biosynthesis and pharmacological properties of triterpenoid saponins were presented, the authors believe that a further in-depth examination of the anti-inflammatory effects of terpenes and triterpenoids from leaf extracts could shed more light on their value for the pharmaceutical sector.

2. Biosynthetic Pathways of Triterpenes and Triterpenoids

Plants synthesize terpenes through complex and intricate biosynthetic pathways, as seen in Figure 2. The terpenes that have been identified and studied are all constructed using a triterpene core, which is formed by six isopentenyl pyrophosphate (IPP) precursors derived from the mevalonate pathway [29]. IPP is partially converted into the allylic isomer dimethylallyl pyrophosphate by IPP isomerase. Then, two IPP molecules and one allylic isomer dimethylallyl pyrophosphate molecule combine to create farnesyl pyrophosphate by prenyltransferase, which serves as the fundamental unit for various terpene pathways dependent on the mevalonate pathway. These pathways include the production of tri- and sesquiterpenes [30]. The initial and crucial stage in the process of triterpene biosynthesis involves the merging of two farnesyl pyrophosphate molecules through the action of squalene synthase, resulting in the formation of the first triterpene compound, known as squalene. Subsequently, this molecule undergoes activation through epoxidation by a squalene epoxidase, leading to the synthesis of 2,3-oxidosqualene [29,31].

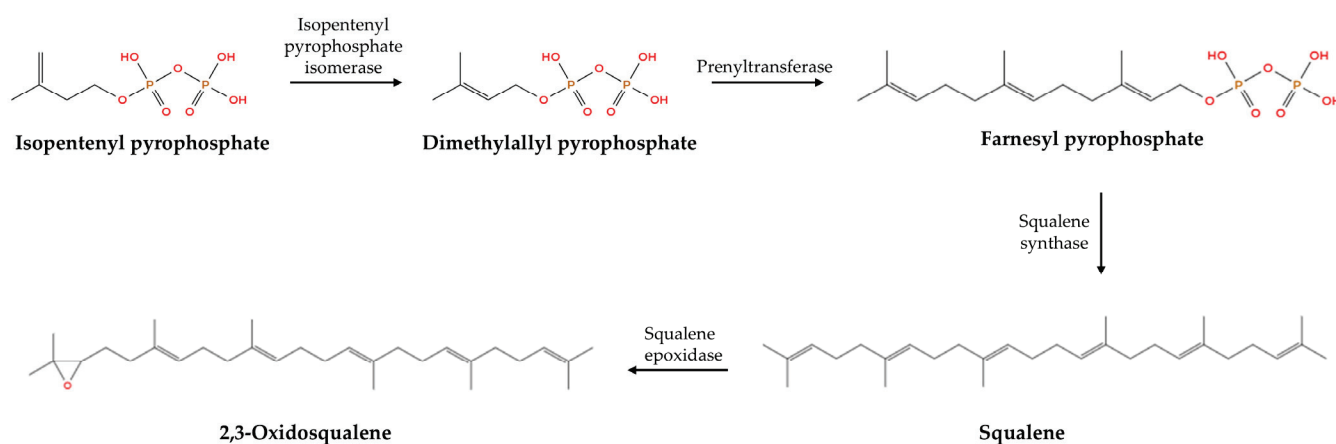


Figure 2. Biosynthesis of triterpenes and triterpenoids through the mevalonate pathway.

The initial branch in triterpene biosynthesis occurs when 2,3-oxidosqualene is converted into various triterpene backbones through cyclization by oxidosqualene cyclase enzymes. This process results in over a hundred different triterpene structures, including cycloartenol for main sterol synthesis and α -amyrin for specialized triterpenes (Figure 1). The cyclization process is initiated by the protonation of an epoxy group, which is then followed by the cyclization and rearrangement of carbocation species [32]. The process is ultimately halted either by deprotonation or the addition of water [29]. The structures produced are then modified through oxidations facilitated by cytochrome P450 enzymes, thereby increasing their variability in both structure and biological function [29,33,34]. Ultimately, triterpenes have the potential to undergo modifications by the addition of sugars, sugar chains, or other chemical groups to the activated carbons. Glycosylated triterpenes are commonly known as triterpene saponins.

Triterpenoids are synthesized from plants through two different pathways, the methylerythritol 4-phosphate/deoxyxylulose 5-phosphate pathway and the mevalonate pathway [35]. This pathway has been studied by examining the genes involved in the biosynthesis of terpenoids [20,36]. Isoprenoids are generated from IPP and its isomer, allylic dimethylallyl pyrophosphate, which are C5 building blocks synthesized through the isoprenoid route. This pathway produces both primary and secondary metabolites. IPP and dimethylallyl pyrophosphate are produced through the mevalonate and methylerythritol 4-phosphate/deoxyxylulose 5-phosphate pathways [37].

3. Methodology

The methodology included the general approach of the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA, Berlin, Germany) 2020 statement [38], as illustrated in Figure 3. This study employed electronic databases, including Science Direct and Scopus, to perform an extensive search for research studies related to the anti-inflammatory properties of triterpenes and triterpenoids derived from plant leaf extracts. The search for articles published between 2019 and 2024 that satisfied the review criteria was conducted using the following terms: ("plant" OR "plants") AND ("leaf" OR "leaves") AND ("extract" OR "extracts" OR "extraction") AND ("triterpene" OR "triterpenoid") AND ("anti-inflammatory" OR "antiinflammatory"). Publications were included by using the above inclusion criteria, with limitation only to research papers, from which the full text and references that were recognized as possibly relevant were collected. The number of papers retrieved was 200, of which 159 were removed due to not being relevant to the topic. For instance, studies including other plant parts or materials (i.e., roots, seeds, pomace, and pulp) or conducting experiments other than inflammation-related assays were excluded

from the present review. In addition, review papers that were mistakenly classified as research papers were been excluded.

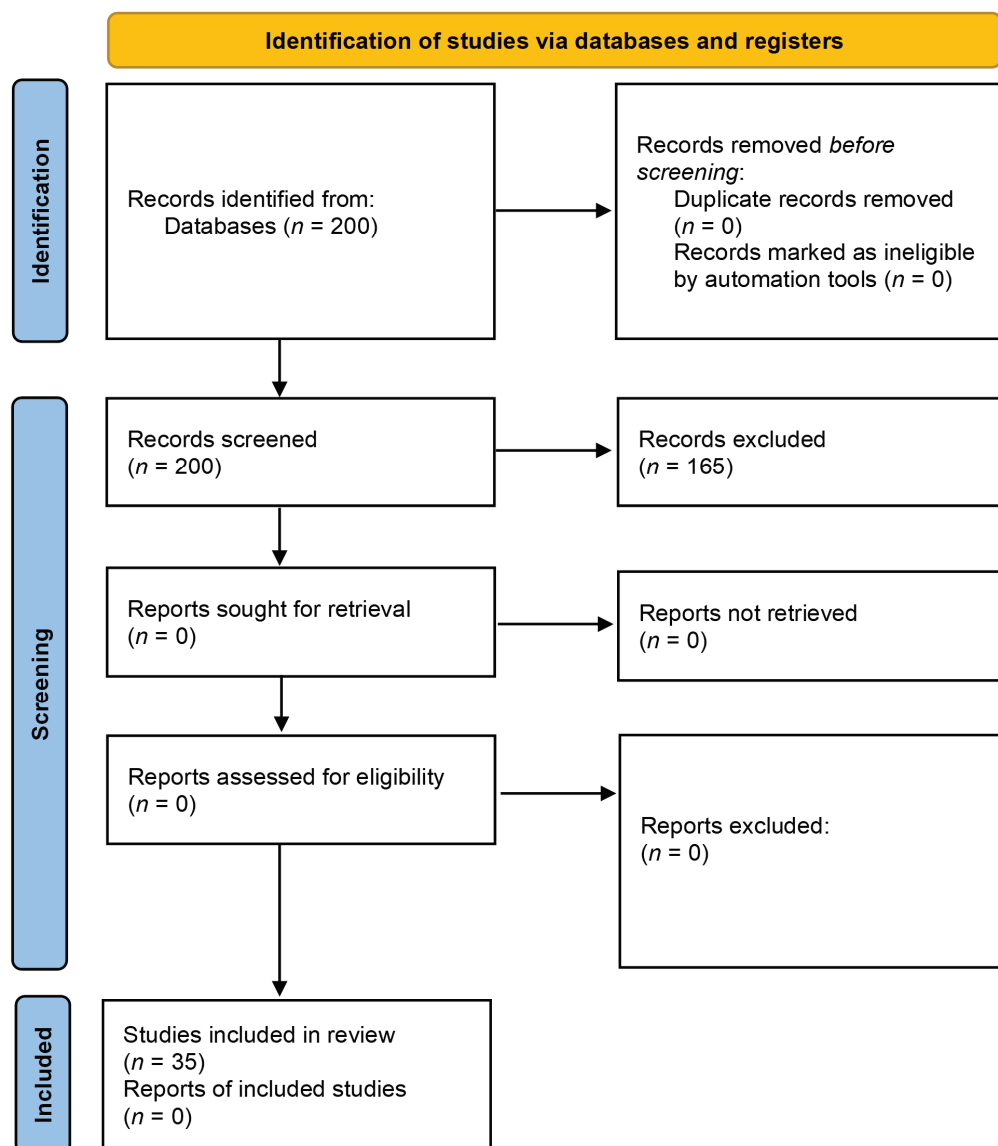


Figure 3. PRISMA methodology flowchart for literature search.

4. Extraction of Targeted Compounds

The initial stage in obtaining targeted natural compounds from their original sources is extraction. Solvent extraction methods such as maceration, hydrodistillation, and Soxhlet extraction are all procedures that are based on the same principle, which involves the diffusion of natural compounds from a solid plant matrix into a liquid phase. A number of cycles carried out successively within these laborious protocols ensure the thoroughness of the extractions [1]. However, a large amount of energy is required for the release of the preferred compound in some methods, which involve using greater temperatures or mechanical forces. It should be noted that several green techniques could also be employed for that reason. When extracting compounds of interest from their natural sources, “green” extraction methods aim to minimize negative impacts on the environment and the use of harmful solvents. Compared to conventional extraction techniques, these approaches offer several benefits, such as a shorter extraction times, smaller volumes of harmful organic solvents, a greater extraction yield, and the ability to automate the process for greater reproducibility while consuming less energy [39]. These techniques include accelerated

solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, pulsed electric field extraction, and enzyme-assisted extraction [40]. The extraction process can be made more effective with any solvent that increases solubility and diffusivity in the aforementioned processes. Solvents with polarity values close to those of the solute are more likely to dissolve the natural compounds effectively, according to the law of miscibility and similarity. When it comes to solvent extraction for phytochemical research, alcohols, acetone, water, and ethyl acetate are some of the frequently used solvents [41].

5. Anti-Inflammatory Activity of Triterpenes and Triterpenoids

Inflammation is a normal response of the human body to several detrimental stimuli, including physical injury, noxious chemicals, and microorganisms. The inflammatory response is triggered by a sequence of external and internal signals that arise from mechanical, chemical, or biological tissue injury [42]. Inflammation is strongly linked to the initiation and progression of numerous significant illnesses. It is primarily characterized by local redness, swelling, pain, edema, and an increase in temperature due to reactions in the vascular system. If recurrent acute inflammatory events are not resolved, they can result in chronic inflammatory diseases such as arthritis [43], colitis [44], or asthma [45]. These conditions are linked to irreversible tissue damage and an increased risk of developing cardiovascular disease [46], cancer [47], rheumatoid arthritis [48], and osteoporosis [49]. Chronic inflammation encompasses debilitating autoimmune disorders characterized by intense inflammation [50] and resulting in significant tissue impairment. Pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α) [51], interleukin (IL)-6 [52], and IL-1 beta [53], are present for extended durations in chronic inflammatory diseases. Inflammation arises from a persistent immunological response involving pro-inflammatory macrophages, microglia, and neutrophils [54], as well as cytokines secreted by several cell types, including endothelial cells, oligodendrocytes, and fibroblasts [55]. The mechanism by which inflammatory disorders occur is an intricate network of interrelated processes. Numerous enzymes and components participate in cellular signaling networks. Drug treatment can often improve inflammatory responses by inhibiting the generation or release of inflammatory mediators [42]. The clinical management of inflammation involves the use of two primary categories of medications, nonsteroidal anti-inflammatory drugs and steroidal anti-inflammatory drugs [42,56,57].

Anti-inflammatory activity is a frequently attributed biological activity of natural triterpenes [9]. Recently, several studies have highlighted the anti-inflammatory activity of ursane-type triterpenes, especially ursolic acid [58–60]. Therefore, ursane-type triterpenes can be investigated as candidates for the treatment of diseases associated with neuroinflammation. From this viewpoint, interesting results of experimental models have been obtained for the treatment of multiple sclerosis through the effect of ursolic acid on immunomodulation and neuronal repair by direct remyelination in the chronic progressive stage of the disease, for which there is currently no effective therapy [61]. However, recent data suggest that ursolic acid may have negative consequences, including toxicity, in certain situations [62]. This raises concerns about the concentration and resolution of its toxicity.

Triterpenes possessing an oleanane structure are believed to have much higher activity compared to those with an ursane backbone [63]. Moreover, the inclusion of a keto group at C3 and carboxyl groups at C17 in the oleanane skeleton, along with the augmentation of the quantity of oxygenated functional groups in other locations, amplifies the anti-inflammatory activity [9,63]. Furthermore, a comparison of anti-inflammatory evidence for both oleanolic and moronic acids indicated that the latter demonstrates greater promise. In accordance with a preliminary study on the association between structure and activity, it is

suggested that the presence of a Δ^{18} double bond at the C13–C18 ring may be the cause of this activity.

5.1. Leaf Extracts Containing Triterpenes

5.1.1. Membrane Stability Assay on Triterpenes

Bolaji et al. [64] investigated leaf extracts from *Euphorbia graminea* plants for the presence of phytochemical components. The ground leaves were first macerated in methanol and then vacuum treated. Among other compounds, triterpenes were identified in the phytochemical analysis. A membrane stabilization assay was employed to assess anti-inflammatory activity. Membrane stabilization has been recognized as a mechanism through which specific medicinal plants confer anti-inflammatory effects. Due to the stability of the membrane, after inflammation, serum proteins are prevented from leaking into the tissues during the period of increased permeability, as stated by Fujiati et al. [65]. The mechanism of a plant extract's anti-inflammatory activity can be measured by its ability to inhibit hypotonicity impact via membrane stabilization in red blood cells. To this end, after incubation at 56 °C for 30 min, stressed bovine red blood cells exhibited enhanced protection after treatment with leaf extracts, achieving 100% stability at lower concentrations (0.05–0.15 µg/mL), in contrast to the positive control treated with the anti-inflammatory drug diclofenac, which reached a plateau at 20% stability. At a higher concentration of 1.0 µg/mL, a plateau of 30% membrane stability was observed, whereas diclofenac yielded approximately 80% membrane stability. Studies indicate that saponins (i.e., triterpene glycosides) can bind to macromolecules on cell membranes, obstructing the interaction of inflammatory agents with these regions. This intriguing discovery warrants further investigation using fractions of leaf extract to isolate the triterpenes exhibiting anti-inflammatory properties.

5.1.2. Protein Inhibition Assay on Triterpenes

An aromatic tree native to North America and adapted to Brazil, *Liquidambar styraciflua* L. is commonly called the sweet gum or alligator tree. Its leaves are often employed to alleviate gastrointestinal issues, scratches, and coughs. Mancarz et al. [66] evaluated the chemical composition and biological efficacy of extracts obtained from the aerial portions of *L. styraciflua* L. A hydroalcoholic mixture was employed for the extraction of ground leaves. The authors identified triterpenes only by phytochemical analysis in several fractions. To assess anti-inflammatory activity, a hyaluronidase enzyme inhibition assay was used. One type of inflammatory process involves the hydrolysis of hyaluronic acid by the enzyme hyaluronidase, which causes edema and other inflammatory symptoms by degrading the extracellular matrix and increasing tissue permeability. Many pathophysiological processes require hyaluronidase, and inflammation is linked to numerous chronic diseases. Various fractions of the extract were acquired using chloroform, *n*-hexane, butanol, ethyl acetate, and water. A commercial propolis extract (positive control) exhibited a 50% inhibition concentration (IC₅₀) value of 32.13 mg/mL in hyaluronidase enzyme. The IC₅₀ values for the aqueous extract (75.32 mg/mL), ethyl acetate fraction (59.57 mg/mL), butanol fraction (51.29 mg/mL), and hydroalcoholic fraction (49.13 mg/mL) indicated that the hydroalcoholic fraction displayed the closest value to propolis extract and was the most desirable fraction. The remaining fractions of *n*-hexane and chloroform were deemed to lack significant inhibitory activity and were excluded from subsequent investigations. Consequently, additional anti-inflammatory investigations on the percentage of inhibition activity were conducted. At a concentration of 100 mg/mL, propolis extract attained approximately 98% inhibition, while the hydroalcoholic fraction reached around 83%, indicating its potential as an anti-inflammatory agent for possible application in the pharmaceutical industry.

Nonetheless, the terpene composition must be elucidated alongside the *in vivo* activity to enhance understanding of the anti-inflammatory properties of this chemical class.

5.1.3. *In Vivo* Assays on Triterpenes

Regarding the treatment of snakebite envenomation, only the use of antivenom has been approved in Colombia. Since the antivenom effectively neutralizes systemic effects but is ineffective against local effects, complications may arise. In contrast, for basic medical needs, people living in rural areas often turn to plants that are both common and easy to reach. *Piper auritum* is one of these plants that has long been revered by the indigenous peoples of Chocó and Antioquia. An alcoholic extract from the leaves of *P. auritum* was tested for its anti-edema effects on mice as part of the research conducted by Rengifo-Rios et al. [67]. Soxhlet extraction was conducted for 48 h to prepare the extract. The right leg of each mice was injected with venom, followed by the administration of the alcoholic extract in the same leg. The National Institute of Health anti-edema serum was utilized as the positive control, with the effective dose 50 (ED₅₀) assessed. The findings indicated that the National Institute of Health serum exhibited an ED₅₀ value of 1.36 mL/mg of venom, while the alcoholic *P. auritum* extract demonstrated an ED₅₀ value of 1.78 mL/mg of venom, suggesting a value comparable to the positive control. The authors assert that the triterpenes present in the plant may underlie the extract's anti-edematous properties, as these compounds exhibit anti-myonecrotic activity by forming covalent bonds with toxins, particularly phospholipases A₂. However, they emphasize that to gain a comprehensive understanding of the extract's efficacy, it is essential to isolate and analyze its constituents.

Most African countries have a long history of using *Ficus asperifolia* to treat inflammatory diseases. The exploration of the anti-inflammatory properties of a methanolic leaf extract from *F. asperifolia* was the aim of the study by Abdullahi et al. [68]. Phytochemical and acute toxicity tests were performed on the extract. The extract was evaluated at doses of 250–1000 mg/kg for its anti-inflammatory properties through carrageenan-induced paw edema and acetic acid-induced writhing in mouse models. The median lethal dose (LD₅₀) of *F. asperifolia*, when administered intraperitoneally, was established at 3800 mg/kg. In comparison to the control group, the group administered piroxicam, the positive control, at 1000 mg/kg exhibited reduced writhing behavior by ~21%, achieving a plateau of ~35% after 6 h. Similarly, at a dosage of 1000 mg/kg of the extract, the outcomes were comparable to those of the anti-inflammatory medication, exhibiting a peak activity level above 50% inhibition of pain. The methanolic leaf extract of *F. asperifolia* demonstrated potential anti-inflammatory properties, with the authors identifying the presence of triterpenes; however, further elucidation of their anti-inflammatory activity could be achieved through isolation.

Traditional Peruvian medicine involves the use of the plant *Encelia canescens* Lam, which has anti-inflammatory activity. The study by Fernández-Flores et al. [69] was aimed to determine whether an ethanolic extract of *E. canescens* leaves could protect levels of mouse albumin, total protein, and malondialdehyde (MDA) in a 1% carrageenan-induced air pouch model. Three different concentrations of *E. canescens* were tested (100, 250, and 500 mg/kg) compared to 2 mg/kg of dexamethasone, a National Institute of Health (NIH) serum. The results showed that the extract had dose-dependent effects on the three biochemical parameters, all of which exhibited significant differences ($p < 0.05$) compared to the control group. In addition, 500 mg/kg of extract was more effective than 2 mg/kg of dexamethasone in the albumin denaturation assay (achieving 1.01 compared to 1.21 g/dL) and in the MDA assay (1.24 versus 1.44 nmol/L). Comparable values were observed in the total protein assay, with both anti-inflammatory agents achieving ~3.6 g/dL. Terpenes may reduce inflammation by stabilizing lysosomes. These lysosomes contain proteases and hydrolytic enzymes that cause inflammatory chemical mediators. When inflammation

occurs, these lysosomes disintegrate, causing damage and other inflammatory phenomena. The histological analysis of *E. canescens*'s anti-inflammatory effects revealed that, in healthy tissue, inflammation did not appear as a layer of flattened cells overlaying loose vascular connective tissue. Upon observing inflammatory changes such as leukocyte infiltration and edema, the lining layer of flattened cells in the carrageenan 1% group showed an increase in macrophages and fibroblasts. Reduced leukocyte migration and MDA concentration were associated with the anti-inflammatory efficacy of the 500 mg/kg extract. Nevertheless, it is still not known how chemical composition relates to anti-inflammatory activity, so such investigations could shed more light on this aspect.

Research into the compounds and anti-inflammatory properties of *Morinda citrifolia* (noni) leaf extract was conducted in a study by Ly et al. [70]. Dried leaves were extracted with 70% ethanol at 60 °C, and a protein denaturation assay was utilized for anti-inflammatory activity measurement. The results demonstrated that noni leaves contained coumarins, triterpenes, tannins, alkaloids, saponins, and flavonoids. In terms of concentration-dependent anti-inflammatory activity, the noni leaf extract showed the following values: 12.18% (at 20 mg/L), 34.06% (at 40 mg/L), 45.79% (at 60 mg/L), 52.75% (at 80 mg/L), and 60.90% (at 100 mg/L). In comparison to that for ascorbic acid (168.81 mg/L), the IC₅₀ value for inhibiting protein denaturation with noni leaf extract was lower, at 70.21 mg/L. After eleven days of treatment, noni leaf extract aided in wound healing in mice by decreasing wound area and increasing histological regeneration. When applied to rabbit skin, noni leaf extract produced negligible skin swelling, suggesting a promising anti-inflammatory effect. However, further *in vivo* studies are required to support this evidence.

The *Ammoides pusilla* plant is a medicinally significant species with a long history of use in traditional medicinal practices. A study by Belkhodja et al. [71] included an *in vivo* anti-inflammatory potential assessment of *A. pusilla* leaves and the determination of its value in the treatment of a severe disease that affects the stability of articular cartilage and bone structure. Dried leaves were extracted with boiling water for 15 min. The phytochemical analysis of the extract revealed that it was abundant in polyphenols (~22 mg/g of extract), including flavonoids, tannins, coumarins, anthocyanins, and triterpenes. Rheumatoid arthritis led to unique swelling in the paw. The measurement of this edema was performed in mice to assess anti-inflammatory activity. When tested on mice with Freund's adjuvant-induced rheumatoid arthritis, the anti-inflammatory activity data demonstrated that the aqueous *A. pusilla*-infused extract may have promising antiarthritic effects. The treated mice (preventive or curative) had stable body weights compared to arthritic mice, which lost 5 g from an initial weight of ~35 g. In addition, the thickness of edema decreased from 5.56 to 2.59 mm for the curative group and from 4.76 to 2.51 mm for the preventive group when 250 mg/kg of the aqueous extract of *A. pusilla* was administered orally, as compared to the reference medicine (diclofenac). Edema, a sign of inflammation, decreased in volume with 20 mg/kg of diclofenac, with the average thickness decreasing from 5.67 mm to 2.71 mm. The authors attributed these anti-inflammatory effects to triterpenes that were present in the extract as inflammation-reducing mediators. A class of medications known as nonsteroidal anti-inflammatory drugs has shown promise in the treatment of edema and pain by blocking cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2). As an alternative chemical for inflammation prevention, the aqueous extract of *A. pusilla* is thus of great therapeutic interest, as it provides a cure for edema. Table 1 provides a brief review of non-specific triterpenes and their anti-inflammatory activity. The authors employed a non-specific colorimetric approach to detect the presence of triterpenes in the mentioned investigations. Identification techniques for the chemical composition of the extracts could clarify their anti-inflammatory properties.

Table 1. Anti-inflammatory effects of plant leaf extracts and identification of the main triterpenes.

Sample	Triterpene	Anti-Inflammatory Effect	Ref.
<i>E. graminea</i>		30% membrane stability from the extract, ~80% membrane stability from diclofenac	[64]
<i>L. styraciflua</i> L.		Aqueous extract IC ₅₀ values (75.32 mg/mL), ethyl acetate fraction (59.57 mg/mL), butanol fraction (51.29 mg/mL), and hydroalcoholic fraction (49.13 mg/mL), compared to propolis IC ₅₀ value of 32.13 mg/mL in hyaluronidase inhibition assay	[66]
<i>P. auritum</i>	Triterpenes determined through non-specific colorimetric techniques	Antivenom and anti-inflammatory activities at 500 mg/kg better than those of NIH serum dexamethasone when used at 2 mg/kg in an albumin denaturation assay	[67]
<i>F. asperifolia</i>		Comparable activity to piroxicam, achieving >50% pain inhibition	[68]
<i>E. canescens</i> Lam		Albumin denaturation assay: 500 mg/kg of extract achieved 1.01 compared to 1.21 g/dL that 2 mg/kg for dexamethasone	[69]
<i>M. citrifolia</i>		IC ₅₀ : higher protein denaturation activity (70.21 mg/L) than ascorbic acid (168.81 mg/L)	[70]
<i>A. pusilla</i>		Edema reduced from 5.56 to 2.59 mm in the curative group and from 4.76 to 2.51 mm in the preventive group at 250 mg/kg of the aqueous extract of <i>A. pusilla</i>	[71]

IC₅₀: 50% inhibitory concentration; NIH: National Institute of Health.

5.2. Leaf Extracts Containing Triterpenoids and Saponins

5.2.1. Protein and NO Inhibition Assays on Triterpenoids and Saponins

An investigation by Anjum et al. [72] aimed to assess the biological activities and qualitative and quantitative chemical composition of an ethyl acetate extract of *Ardisia solanacea* Roxb. from the Tarai region of Uttarakhand. The Soxhlet method was employed to obtain extracts from the plant, utilizing ethyl acetate as the solvent. Oils, resins, diterpenes, triterpenoids, carbohydrates, and alkaloids were among the many bioactive components identified by qualitative GC-MS analysis. It was also possible to quantify the triterpenoid α -amyrenone. Anti-inflammatory activity was assessed through a protein denaturation assay. The leaf extract's anti-inflammatory activity was assessed in comparison to that of diclofenac, which served as the positive control. Diclofenac had an IB₅₀ value of 2.80 μ g/mL in the protein denaturation assay (i.e., meaning 50% inhibition of the denaturation of proteins), whereas the ethyl acetate leaf extract of *A. solanacea* had strong anti-inflammatory properties, with an IB₅₀ value of 3.13 μ g/mL. Further *in vivo* studies could provide evidence of whether the plant is safe for use in the pharmaceutical industry.

Fractionation is a highly effective method for assessing the anti-inflammatory properties of specific chemical compounds, as it is highly effective for their separation and, thus, for their separate study. The process is typically conducted using the column chromatography technique with solvents of appropriate polarity. Aro et al. [73] conducted a study to examine the anti-inflammatory properties of acetone leaf extracts from *Oxyanthus speciosus* subsp. *stenocarpus*. The main objective was to reduce chronic inflammatory diseases, such as tuberculosis. The air-dried leaves were extracted with acetone, and the extract was evaporated *in vacuo*. Hexane, ethyl acetate, and methanol were utilized for the fractionation. The results indicated that the fraction containing the triterpenoid rotundic acid exhibited a significant anti-inflammatory effect. Nitric oxide (NO) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages ranging from 2.39 to 0.48 μ M was achieved within the concentration range of 3.12–25 μ g/mL. In comparison, the fractions of the carotenoid lutein ranged from 1.98 to 0.14 μ M, while the fraction of the flavonoid quercetin ranged from 2.50 to 0.35 μ M. This research explored the anti-inflammatory efficacy of certain component fractions as regulators of cellular redox potential. Rotundic acid showed encouraging outcomes as a therapeutic agent against tuberculosis, as evidenced by its efficacy both *in vitro* and intracellularly in macrophages. However, further investigation is required to analyze its bioavailability, its effects *in vivo*, and its mechanism of action.

The leaves of *Nyctanthes arbor-tristis* contain betulinic acid, a lupane-type triterpenoid that had never been reported before but was studied by Karan et al. and exhibited strong anti-inflammatory properties [74]. The ground leaf powder was extracted with methanol and fractionated with water, *n*-butanol, and ethyl acetate. The bioactivity-guided fractionation method was used for the first time to isolate betulinic acid from an ethyl acetate leaf extract of *N. arbor-tristis*. The betulinic acid structure was determined through several spectroscopic analyses, including nuclear magnetic resonance, Fourier-transform infrared spectroscopy, and high-resolution mass spectrometry. It demonstrated excellent anti-inflammatory properties, with IC₅₀ values of 10.34 µg/mL for COX-1, which had two levels of enzyme (i.e., 10 µL of enzyme referred to as COX-1). The other IC₅₀ values were 12.92 µg/mL for COX-2, 15.53 µg/mL for 5-lipoxygenase assay (5-LOX), 15.21 µg/mL for NO production, and 16.65 µg/mL for TNF-α in RAW 264.7 cells. As a positive control for the inhibition of COX-1, COX-2, and 5-LOX, boswellic acid was used at a concentration of 50 µg/mL. It achieved 97.36, 94.32, and 92.64 µg/mL values for COX-1, COX-2, and 5-LOX, respectively. For NO production and TNF-α, dexamethasone was used at a concentration of 15 µg/mL. Both drugs achieved higher than 95% inhibition in each assay (i.e., 97.99 and 98.34%, respectively), whereas several subfractions of ethyl acetate extract achieved 3.35–92.20% in all assays, with subfraction 7 being the most preferable. It could be concluded that betulinic acid acts as a promising anti-inflammatory agent; however, *in vivo* assays would confirm this outcome.

Globally, *Ziziphus* plants are renowned for their medicinal and nutritional value, although their complete chemical composition has not yet been disclosed. A study by Sakna et al. [75] examined the anti-inflammatory properties of *Ziziphus mauritiana*, *Z. spina-christi*, and *Z. jujuba* methanolic leaf extracts. To this end, 80% methanol was used for the extraction process. Several triterpenoids were identified through the liquid chromatography–mass spectrometry method, such as ceanothic acid, isoceanothic acid, apiceanothic acid, and epigouanic acid. However, the total triterpenoids were expressed photometrically as ursolic acid equivalents (UAE). A COX-1 assay was used to measure anti-inflammatory activity, with a commercially available selective cyclooxygenase inhibitor (SC-560) achieving 65.71% inhibition at the same concentration as the extracts (10 mg/mL). The results showed promising anti-inflammatory activity determined through % COX-1 inhibition of *Z. mauritiana* (90.34), *Z. spina-christi* (89.31), and *Z. jujuba* (76.87). It was observed that the first two extracts exhibited the highest inhibition, with no statistical differences ($p > 0.05$). However, it should be highlighted that *Z. mauritiana* had 30% less triterpenoid content when compared to *Z. spina-christi* (224.15 mg UAE/g extract) but had comparable anti-inflammatory activity. The authors report that this activity was probably caused by the presence of triterpenoids. *Z. jujuba* also recorded the lowest amount of triterpenoids (146.12 mg UAE/g extract). However, the authors state that these findings were merely suggestive. Subsequent comprehensive investigations of *Ziziphus* leaf extracts in living organisms or, preferably, studies focusing on individual, isolated compounds, are necessary to establish more definitive conclusions. In addition, studies could include more cultivars of the *Ziziphus* plant to shed more light on the anti-inflammatory properties of this plant.

A methanolic extract from the leaves of *Calophyllum inophyllum* with substantial anti-inflammatory effects *in vitro* was the subject that Van Than et al. [76] investigated. Dried leaves were extracted with methanol and fractionated with dichloromethane, ethyl acetate, and *n*-hexane through column chromatography. The authors investigated the inhibition of NO production and cytokine production in RAW 264.7 cells when stimulated with lipopolysaccharide from these compounds. From the total of twelve compounds, two new compounds with coumaroyl ester structures were identified as terpenoids and had comparable IC₅₀ values to the positive control dexamethasone (0.012 µM). Compounds

1 and 2 achieved 2.44 and 7.00 μM , respectively. Compound 9 achieved 15.46 μM , and all remaining compounds exhibited $>50 \mu\text{M}$ IC_{50} values. The two specific compounds also revealed effectiveness in deteriorating abnormal inflammation responses, as they lowered concentrations of interleukin (IL)-1 β and TNF- α at concentrations of 5 and 10 μM in a dose-dependent way. For instance, IL-1 β concentration was reduced from >300 to ~ 200 and ~ 100 pg/mL at concentrations of 5 and 10 μM of compound 1, respectively. A similar reduction was observed for compound 2. Regarding TNF- α , a reduction from 250 to 200 and 150 pg/mL was noticed when concentrations of 5 and 10 μM of both compounds were administered. In the case of prostaglandin E_2 (PGE2) and IL-6, neither compound affected the concentration of these two substances. As for NO production, it was significantly reduced to a comparable level to that achieved with dexamethasone. It was also revealed that compound 1 inhibited the phosphorylation of I κ B, which led to a decrease in the nuclear translocation of nuclear factor κ light-chain enhancer of activated B cells (NF- κ B) when treated at 10 μM , whereas compound 2 did not show any significant effects at this concentration. Hence, the *E*-coumaroyl ester triterpenoid, rather than the *Z*-coumaroyl ester triterpenoid, may be responsible for NF- κ B inhibition activation by CIL, which is in line with its NO inhibitory effect. The anti-inflammatory effects of *C. inophyllum* extract may be attributed to these compounds, which are active phytochemical components. Further *in vivo* studies could support these findings and provide evidence for a novel anti-inflammatory extract for the pharmaceutical industry.

Some interesting research studies involve the discovery of novel triterpenic compounds (*vide infra*). To start with, Tai et al. [77] explored the anti-inflammatory activity of *Syzygium bullockii* leaf extract by using a NO production inhibition assay. The methanolic extract was further partitioned with solvents of ranging polarities (i.e., ethyl acetate, dichloromethane, and *n*-hexane). Seventeen compounds in total were identified in the methanolic *S. bullockii* extract, wherein terpenoids syzygiumursanolide C, chebuloside II, and three undescribed compounds (i.e., syzybullosides A–C) were isolated. The results showed that the identified compounds had an IC_{50} range of 1.30–13.70 μM in NO production inhibition using lipopolysaccharide-activated RAW 264.7 cells. The three unknown compounds showed values of 6.93–13.61 μM , whereas the positive control N^G-monomethyl-L-arginine (L-NMMA) had a value of 33.8 μM . The compounds showed promising anti-inflammatory activity, which could be further supported with additional experiments.

Vinh et al. [78] explored a total of 23 triterpenoid saponins, including two novel compounds (12 and 15), that were isolated from an ethanol extract of *Stauntonia hexaphylla* leaves. NO production and cytokine production assays were used to determine the anti-inflammatory activities of the isolated saponins. With an IC_{50} value of 0.59 μM in the NO production assay, compound 13 showed the strongest inhibitory effect when compared to that of the positive control dexamethasone at 0.13 μM . Compounds 1 and 3 also achieved values of 1.82 and 16.65 μM , while the remaining compounds had values $>50 \mu\text{M}$. The two compounds were also used at a concentration of 1–10 μM to examine the inhibitory effects on LPS-stimulated RAW 264.7 cells. Both compounds led to the concentration-related inhibition of pro-inflammatory cytokines. Specifically, compound 13 inhibited the protein expression of NO synthase and COX-2 in LPS-activated RAW 264.7 cells, along with NO, IL-1 β , IL-6, and PGE2 but not TNF- α secretion. The authors used structure–activity relationships to investigate the chemical byproducts of these isolated chemicals. The findings raised the possibility that compound 13, derived from *S. hexaphylla*, could have anti-inflammatory properties. This study presented the first in-depth analysis of saponins derived from *S. hexaphylla* leaves. Applying screening criteria for anti-inflammatory extracts and performing *in vivo* experiments could lead to more comprehensive results about saponins' mechanism of action.

Capparis L. (Capparaceae) is cultivated globally. Traditional medicine practitioners have long relied on capers to alleviate a wide range of symptoms, including rheumatism, kidney, and liver issues. The goal of Gazioglu et al. [79] was to identify the anti-inflammatory activity of *Capparis ovata* extracts. *C. ovata* leaves were extracted with dichloromethane/hexane (1:1), and the structure of eight identified compounds was studied with spectroscopic methods. The compounds were tested at concentrations ranging from 12 to 26 μM against the production of inflammatory cytokines in SH-SY5Y human cell lines. Well-known triterpenoids such as oleanolic acid, β -sitosterol, ursolic acid, bismethyloctylphthalate, and stigmast-5,22-dien-3- β -myristate were identified in this extract. Interestingly, the treatment of SH-SY5Y cells with 26 μM of compound 1 (i.e., a novel triterpenoid ester named olean-12-en-28-ol, 3 β -pentacosanoate), as the lethal dose to 10% of cells treated (i.e., EC_{10}), reduced mRNA expression levels of chemokine (C-X-C motif) ligand 9 (i.e., CXCL9) (19.36-fold), CXCL10 (8.14-fold), and TNF (18.69) compared to those in the control group. This research showed that triterpenoids extracted from *C. ovata* have anti-inflammatory properties. It was discovered that compounds 1 and 3 (i.e., oleanolic acid) showed great promise as therapeutic agents for the treatment of inflammatory diseases.

Menopausal discomfort and intestinal hemorrhage have both been alleviated with the use of *Osmanthus fragrans* var. *aurantiacus*. A methanolic leaf extract was generated by Jeong et al. [80], and the extracted compounds were isolated through a fractionation procedure utilizing water, *n*-hexane, ethyl acetate, and *n*-butanol. The authors identified four triterpenoids, namely, 3-*O*-*trans*-*p*-coumaroyltormentic acid (1), 3 β -*trans*-*p*-coumaroyloxy-2 α -hydroxyl-urs-12-en-28-oic acid (2), 3- β -*cis*-*p*-coumaroyloxy-2 α -hydroxyl-urs-12-en-28-oic acid (3), and 3-*O*-*cis*-coumaroylmaslinic acid (4). The anti-inflammatory activity was examined through NO production inhibition in RAW 264.7 cells with the ethyl acetate fraction, which was found to be the most preferable. The four compounds showed dose-dependent NO production inhibition using concentrations of 1–10 μM and were compared with the positive control curcumin (10–100 μM). The results revealed that compound 2 at 10 μM exhibited the greatest inhibition (>80%), while curcumin at 100 μM led to ~50% inhibition. Moreover, the levels of LPS-stimulated protein, COX-2, NF- κB , and phosphorylated extracellular regulated kinase (pERK) 1/2 were significantly decreased by compounds 2–4 according to Western blotting. Specifically, compound 2 at 10 μM had the greatest inhibitory effect (~50%) on the phosphorylation of ERK 1/2 at a 0.5 relative density of pERK/ERK through Western blot analysis, whereas the highest value of LPS-stimulated cells was ~1.2. Interestingly, levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 were reduced in LPS-induced macrophages and colonic epithelial cells under exposure to compounds 2–4. The study suggested that these compounds extracted from *O. fragrans* var. *aurantiacus* leaves could be useful in preventing and treating inflammatory bowel disease. However, further evidence for the identified triterpenoids could be provided by prospective *in vivo* experiments. The medicinal application of *O. fragrans* var. *aurantiacus* in the treatment of inflammation-related disorders has made it an increasingly popular commodity in China and Korea [80]. Meanwhile, new research has shown that *O. fragrans* var. *aurantiacus* possesses anti-inflammatory properties, which could make it an effective anticancer agent. This is particularly true in colorectal cancer, where inflammation plays a key role in the progression and evolution of the disease [81]. Considering the above information, Han et al. [82] aimed to study *O. fragrans* var. *aurantiacus* for its possible effects on colorectal cancer by examining its anti-proliferative and pro-apoptotic characteristics. Using two sonication cycles of ~2 h each, the dried leaves were immersed in ethanol for the extraction process. Following the procedure previously outlined by Jeong et al. [80], the extracts were fractionated using *n*-hexane, ethyl acetate, *n*-butanol, and water. Ethyl acetate fractions were selected to be reanalyzed. Maslinic acid and corosolic acid are two

triterpenoids that the authors identified, and they are thought to be anticancer compounds for colorectal cancer. In two colorectal cancer cell lines (HCT 116 and HT29), the ethyl acetate fraction at a concentration of 100 µg/mL considerably decreased p65 and ERK phosphorylation levels without impacting protein expression levels. However, at a lower concentration of 50 µg/mL, the fraction primarily decreased inhibitor of nuclear factor kappa α (i.e., I κ -B α) phosphorylation in HT29 cells. Furthermore, the COX-2 gene, which is implicated in the mediation of inflammation-related cancer pathways, was shown to have its expression reduced in colorectal cancer cells by the ethyl acetate leaf fraction. The COX-2 gene is a downstream target of NF- κ B and ERK1/2 that mediates inflammation-related carcinogenic pathways. Taken together, these findings point to the fact that the ethyl acetate fraction of *O. fragrans* leaves inhibits the NF- κ B and mitogen-activated protein kinase (MAPK)-ERK pathways, which in turn cause colorectal cancer cells to undergo apoptosis. The above results were validated since the measured IC₅₀ values from the two specific compounds were ~30 µg/mL, and no cytotoxic effects were observed. In addition, normal human colon cells showed a ~30% decrease in cell viability at 20 µg/mL. The findings highlight that the two triterpenoids induce colorectal cancer cell death, with corosolic acid being more potent in this activity and demonstrating the anti-inflammatory and anticancer characteristics of *O. fragrans* var. *aurantiacus* leaves.

Cecropia pachystachya, one of the species of the Urticaceae family, is widely used in South America as a folk medicine due to its pain-relieving properties. Its bioactive compounds can exert anti-inflammatory activity by halting the production of inflammatory mediators from immune cells. To this end, Daga et al. [83] investigated an extract obtained with the supercritical CO₂ technique as a medication for Chagas disease, a zoonotic infection with which millions of people are affected every year. NO inhibition is an essential assay to assess the inflammation process [84], wherein lipopolysaccharide is administered as a pro-inflammatory stimulus control to RAW 264.7 macrophages. The results showed that NO production was reduced from ~20 to ~5 µM when a 300 µg/mL *C. pachystachya* extract was used. Ursolic acid was proposed to be responsible for the anti-inflammatory activity of *C. pachystachya* extract, a trend that has also been supported by Ahmad et al. [85].

In South China, *Cyclocarya paliurus* is often used for the treatment and prevention of inflammatory disorders. However, no active anti-inflammatory compounds have been identified, despite the fact that research on the anti-inflammatory effects of *C. paliurus* leaves has been reported. To this end, Liu et al. [86] explored the anti-inflammatory effects of an ethanolic *C. paliurus* leaf extract and identified its anti-inflammatory compounds by using RAW 264.7 cells to build a bioactivity-guided identification model. The extract was fractionated with polyethylene, ethyl acetate, water, and *n*-butanol. The structures of 18 dammarane triterpenoid saponins were identified with spectroscopic methods and determined after isolating the active fraction. Among these, 11 were newly discovered 3,4-*seco*-dammarane triterpenoid saponins (1–11). The IC₅₀ values for compounds 7, 8, 10, and 11 varied from 8.23–11.23 µM, indicating a strong inhibition of NO production. The most preferable fraction was that of ethyl acetate at 80 µg/mL, which achieved comparable inhibition to the positive control dexamethasone (~90%). The release of TNF- α , PGE₂, and IL-6 in RAW 264.7 cells activated by lipopolysaccharide was considerably reduced by these four compounds in a concentration-dependent way. Indeed, the highest concentration of the above compounds found in the extract (i.e., 40 µM) achieved a substantial decrease compared to values in untreated cells in TNF- α expression (~60 from ~375 pg/mL), in PGE₂ (~60 from ~140 pg/mL), and in IL-6 (~120 from ~410 pg/mL) cytokine expression. In addition, the activities of COX-2 (from ~0.7 to ~0.15 COX-2- β -actin ratio), iNOS (from ~0.55 to ~0.25 iNOS- β -actin ratio), and NF- κ B/p65 (from ~0.7 to ~0.3 NF- κ B/p65- β -actin ratio) were reduced by compound 7 at a 20 µM concentration. Further *in vivo* investigation

is warranted because the results indicate that 3,4-*seco*-dammarane triterpenoid saponins have the potential to be used as anti-inflammatory drugs.

Luo et al. [87] isolated four new tetracyclic triterpenoids from *Jatropha gossypifolia* ethanolic leaf extract (1–4) through thorough spectroscopic analyses. The extracts were suspended in water and fractionated with petroleum ether. Compounds named jagabeoeuphols A–C (1–3) are uncommon triterpenoids of the 19 (10 → 9)*abeo*-euphane type that have a $\Delta^{5(10)}$ group and a 7,8-epoxide moiety, while jagoseuphone A (4) is a common euphane-type triterpenoid. NO production in RAW 264.7 cells stimulated by LPS assistance was examined to study the inhibitory effects of terpenoid compounds. Only compound 4 showed a substantial inhibitory effect, with an IC_{50} value of 20.1 μ M, whereas the positive control quercetin had a value of 16.8 μ M. The other three compounds had significantly higher ($p < 0.05$) IC_{50} values ($>50 \mu$ M). Further *in vitro* and preferably *in vivo* assays could lead to clearer insights into the relationship between the structure and anti-inflammatory activity of these triterpenoids, especially the uncommon ones (1–3).

Three known triterpenoids (ternstroenols) were isolated from the leaves of *Ternstroemia cherryi* from the Australian rainforest via chromatographic separation in a study by Singh et al. [88]. The dried leaves were extracted with *n*-hexane, ethyl acetate, methanol, ethanol, and water. The ethanolic extract was found to be the most preferable for the anti-inflammatory assay. Extensive spectroscopic analysis, chemical derivatization, and degradation assays were used to determine the ternstroenol structures. The extracts showed an IC_{50} value of 0.72–3.70 μ M for NO production in RAW 264.7 macrophages activated by LPS and interferon- γ (IFN- γ). Further *in vivo* studies could shed more light on the anti-inflammatory activity of this leaf extract, as the authors mainly described the structure of five novel compounds in the *T. cherryi* fruit extract.

A wide variety of Southeast Asian countries produce *Citrus hystrix*. The anti-inflammatory properties of this leaf extract have been examined by Buakaew et al. [89]. Few details are known about the leaves' anti-inflammatory and anti-inflammasome capabilities, however. The purpose of this research was to examine how *C. hystrix* leaves influence the nucleotide-binding domain, leucine-rich repeat-containing family, pyrin domain-containing-3, and nuclear factor κ light-chain enhancer of activated B cells (i.e., NLRP3 and NF- κ B, respectively) signaling pathways. Three crude extracts were obtained from the *C. hystrix* leaves through a sequential extraction process using hexane, ethyl acetate, and 95% ethanol. The extract was fractionated with organic solvents of varying polarities, generating six fractions. Spectroscopic methods were used to identify and confirm the structure of the active compound lupeol, which was fractionated from the ethanolic extract using chromatographic techniques. The lupeol fraction, along with the ethanolic extract, both suppressed the expression of inflammasome genes and NF- κ B proteins and considerably decreased the secretion of pro-inflammatory cytokines. Indeed, IL-1 β , IL-6, and TNF- α were significantly reduced with either the lupeol fraction at 25 μ g/mL or the ethanolic *C. hystrix* extract at 1.5 or 2.5 μ g/mL in a dose-dependent way. The positive control, dexamethasone, was administered at 0.1 nM and was the most effective anti-inflammatory agent of all, except for the NF- κ B1 cytokine and nitric oxide synthase 2 (NOS2). In both cases, dexamethasone and the two extracts achieved a two- and one-and-a-half-fold decrease in these cytokines compared to the control group. The results suggested that the triterpenoid lupeol found in *C. hystrix* leaves could lead to the discovery of new anti-inflammatory and anti-inflammasome agents.

A novel ursane-type triterpenoid that decreases nitrite levels in LPS-stimulated macrophages was examined in an additional investigation of *Centella asiatica* leaf extracts by Chianese et al. [90]. Using 90% ethanol at 70 °C, the authors extracted dried *C. asiatica* leaves. Multiple fractionations utilizing a 50% hydroacetic solution, hexane, *n*-butanol,

and methanol were performed to isolate compounds identified as madecassoside (1), terminolide (2), and isomadecassoside (3). The compounds were tested for the inhibition of NO production in the J774A1 cell medium. At the highest concentration of 50 μ M, the first two compounds induced a decrease from $\sim$ 19 to 15 μ M in nitrites. Isomadecassoside was also efficient, as it lowered nitrates from $\sim$ 18 to 14 μ M. Many phytochemical analyses on *C. asiatica* leaves were performed; however, none of them included isomadecassoside, despite its similarity in molecular formula and chromatographic behavior to compounds (1) and (2). It appears that all three of these compounds have nearly the same nitrite reduction potential, with isomadecassoside displaying comparable or even superior activity to madecassoside.

The anti-inflammatory activity and accumulation of corosolic (CA), ursolic (UA), and oleanolic (OA) acids were examined in chloroform extracts from *Prunus padus* leaves in a study by Magiera et al. [91]. The compounds were identified and measured through chromatographic methods. The total levels of these compounds at different times of harvest ranged from 1.41 to 4.54 mg/g DW in leaves. The concentration-dependent inhibition of pro-inflammatory enzymes (lipoxygenase and hyaluronidase) by the plant extracts was correlated with the levels and activity of pure triterpenoid acids, particularly corosolic and ursolic acids, according to their activity parameters. The comparison of the results with positive controls (heparin, indomethacin, dexamethasone) may lend credence to traditional medicine's claims about *P. padus*'s use in anti-inflammatory treatments. The pure triterpenoid fractions, including the most active CA, showed similar potency toward both enzymes (IC₅₀: 14.3–22.2 μ g/U for LOX and 12.6–18.1 μ g/U for HYAL), but the extracts were more effective LOX inhibitors (IC₅₀: 10.5–12.8 μ g/U) than HYAL inhibitors (IC₅₀: 19.3–22.0 μ g/U). It is worth noting that the LOX assay showed lower activities of the triterpenoids compared to those of the extracts. This finding could indicate a potential synergy between the triterpenoids and other components of the extracts. To fully understand the plant's beneficial effects on reducing inflammation progression and any potential toxicity, additional *in vivo* research is needed.

Inflammatory disorder, epilepsy, diarrhea, pain, and anemia treatments are some of the traditional uses of *Waltheria indica* L. in Africa, South America, and Hawaii. The purpose of the research by Termer et al. [92] was to quantify the secondary metabolite composition of *W. indica*. There was a correlation between the type and quantity of secondary metabolites and the extract's activity when tested for COX-2 inhibition. The authors observed that the extract composition and the COX-2 enzymatic inhibitory activity were affected by the extraction parameters. Water, ethanol, methanol, and ethyl acetate were used as solvents at temperatures ranging from 30 to 90 °C in a procedure including accelerated solvent extraction, where high temperatures and pressures (i.e., 1460 psi) were applied. Triterpenoid saponins ranged from 47.3 to 214.8 mg of oleanolic acid equivalents per gram of dried weight, based on the solvent used. The COX-2 enzyme was inhibited to a lesser extent by the ethanolic extract at 30 °C and the ethyl acetate extract at 90 °C, with a 37.7% and 38.9% inhibition rate, respectively. In contrast, the methanolic and aqueous extracts exhibited a 21.9% and 9.2% inhibition rate, respectively. The amount of triterpenoid saponins in the extracts was proportional to their COX-2 inhibitory activity. The saponin concentration was directly proportional to the increase in COX-2 inhibition. The study showed that extracts of *W. indica* inhibit the key inflammatory enzyme COX effectively; however, *in vivo* studies are required.

The Brazilian Unified Public Health System strongly favors the anti-inflammatory agent *Varronia curassavica*, a vital medicinal plant in Brazil. At the same time, essential oil extracted from *V. curassavica* leaves is among the most marketed products in Brazil. To this end, Moro et al. [93] investigated the anti-inflammatory activity of ethanolic extracts of *V. curassavica* leaves with inhibition assays (phospholipase A₂, COX-1, and COX-2) after

further fractionation using several solvents and mixtures (i.e., hexane, ethyl acetate, and methanol). The isolated triterpenoid cordialin A was evaluated for its effectiveness by comparing it to two controls: diclofenac (a positive control) and thioetheramide (a negative control); all three substances were tested at the same concentration (10.0–100.0 µg/mL). The results showed that the inhibition of phospholipase A₂ (2.8–10.3%), COX-1 (8.3–27.2%), and COX-2 (3.3–22.2%) was observed with ascending concentrations of cordialin A. This compound had greater anti-inflammatory activity than flavonoid brickellin; however, the authors stated that neither compound exhibited anti-inflammatory activity.

Over twenty unknown isolated triterpenoids identified as brujavanoids A–U from a methanolic extract of *Brucea javanica* leaves (1–21) were investigated by Hu et al. [94]. An LPS-activated RAW 264.7 cell model was used to assess the anti-inflammatory effects of all the isolated compounds. In RAW 264.7 cells activated by LPS, the most active one, brujavanoid E (5), inhibited the nuclear translocation of NF-κB p65 and suppressed the transcriptional expression of common pro-inflammatory mediators. This compound had the lowest IC₅₀ value of all 27 identified at 4.1 µM, lower than the positive control dexamethasone (9.2 µM). *B. javanica* offers a fresh opportunity to discover anti-inflammatory agents thanks to its structural diversity and bioactivity. However, further *in vivo* assays could shed more light on the safety of this extract.

Camellia japonica is among the most valuable species of the Theaceae family, as it provides great economic value and has high-added-value bioactive compounds. Triterpenoid saponins, or tea saponins, are one kind of bioactive compound found in *Camellia* species at ~10% *w/w* of dry weight. Phytochemical research on *C. japonica* is necessary since its chemical profile is the building block of its industrial use. To this end, Liu et al. [95] provided aqueous *C. japonica* leaf extracts, which were fractionated in order to comprehensively identify the triterpenoid compounds of the specific plant. The fractions were evaluated for their anti-inflammatory activity through NO production inhibition assays in RAW 264.7 cells. The authors annotated thirty-eight triterpenoids through the use of molecular networking, half of them being potentially novel compounds. They identified 13 compounds through spectroscopic techniques, 6 of them being the known camellioside A, camellioside B, camellioside E, camellioside G, 3β,17β-dihydroxy-16-oxo-28-norolean-12-en-3-O-β-D-glucopyranosyl-(1→2)-O-β-D-galactopyranosyl-(1→3)-O-[6-O-acetyl-β-D galactopyranosyl-(1→3)]-β-D-glucopyranosiduronic acid, and 3β,16α,17β-trihydroxy-28-norolean-12-en-3-O-β-D-glucopyranosyl-(1→2)-O-β-D-galactopyranosyl-(1→3)-O-[β-D-galactopyranosyl-(1→2)]-β-D-glucopyranosiduronic acid. The six known compounds showed substantial anti-inflammatory activity, since at 25 µM, they achieved inhibition rates of 3.21–37.99%, whereas the positive control L-NMMA achieved 57.04% at 50 µM. Specifically, an unknown compound reached the highest inhibition rate, while camellioside B (31.31%) and camellioside A (28.96%) showed promising results.

5.2.2. *In Vivo* Assays on Triterpenoids and Saponins

One traditional remedy for skin inflammatory disorders is *Tabernaemontana catharinensis*, commonly described as snakeskin. The leaves were macerated in 70% ethanol for 10 days at room temperature. To validate the topical anti-inflammatory properties of *T. catharinensis* leaves, the study conducted by Camponogara et al. [96] examined the therapeutic efficacy of a crude extract and its various constituents using a mouse model of irritant contact dermatitis using the ear edema assay through Croton oil exposure. A qualitative phytochemical study of snakeskin crude extract and *n*-butanol, dichloromethane, and ethyl acetate fractions was performed using a chromatographic technique, with the triterpenoids ehretioidide, oleanolic acid, and oleanonic acid being identified in ethyl acetate and dichloromethane fractions. Applying croton oil to the skin caused a significant

increase in ear thickness in mice, with a maximum effect of ~144 μm noticed 6 h after treatment. In contrast, applying the crude extract did not cause any change in the ear thickness of mice (~23 μm). The extract and its fractions were applied at concentrations of 0.001–10 $\mu\text{g}/\text{ear}$ and showed a dose-dependent reduction in acute ear edema caused by croton oil. The inhibitory dose 50% (ID_{50}) values for the snakeskin extract and its fractions were 0.006 $\mu\text{g}/\text{ear}$ for crude extract and 0.061, 0.002, and 0.001 $\mu\text{g}/\text{ear}$ for dichloromethane, *n*-butanol, and ethyl acetate fractions, respectively. By applying 10 $\mu\text{g}/\text{ear}$, maximum inhibition (100%) was achieved by crude extract and the positive control dexamethasone. Fractions of dichloromethane (~85%), ethyl acetate (~86%), and *n*-butanol (~83%) indicated promising anti-inflammatory activities. Inflammatory illnesses may be amenable to treatment with formulations containing snakeskin leaf extracts. Since snakeskin leaves decreased inflammatory parameters in a croton oil-induced irritating contact dermatitis model, it is feasible for them to be used in the pharmaceutical industry.

A study of another *Miconia* species was conducted by Gatis-Carrazzoni et al. [97] to examine *Miconia minutiflora* (Bonpl.) leaf extract's anti-inflammatory properties. Ground powder was extracted through maceration with methanol for 48 h, with the extract being finally evaporated. The phytochemical profile was determined through the chromatographic technique. Anti-inflammatory activity was examined through carrageenan and acetic acid-induced vascular permeability in male and female mice. All tested concentrations of *M. minutiflora* methanol extract (i.e., 50–200 mg/kg) demonstrated anti-inflammatory activity. Myrianthric acid and arjunolic acid were identified in the extract. With a 70% inhibitory effect on leukocyte migration, the methanolic extract at a dose of 100 mg/kg demonstrated the highest level of activity at 5.36×10^6 polymorphonuclear leukocytes (PMNLs) per milliliter. The positive control indomethacin was administered at 5 mg/kg and had an activity of 6.54×10^6 PMNL/mL. The effects of the extracts were not dependent on dosage. Regarding carrageenan-induced edema, carrageenan was injected subplantarically and induced paw edema promptly. At concentrations of 100 and 200 mg/kg, *M. minutiflora* extract decreased the edema and had similar anti-inflammatory activity to the positive control indomethacin, reaching ~80 $\mu\text{g}/\text{mL}$ by Evans blue staining (1%). Compared to that of the control group, all the above injections significantly decreased the volume of edema at all timepoints evaluated. A myeloperoxidase activity assay was performed to assess the anti-inflammatory activity associated with neutrophil abundance in the subplantar tissue. Methanolic extracts and indomethacin decreased the activity of myeloperoxidase (MPO) in the plantar region of animals displaying edema, with a mean optical density (mOD) of 0.256 and 0.866 mOD/tissue, respectively, in comparison to the control value of 1.197 mOD/tissue. After inducing peritoneal capillary permeability with acetic acid in mice, methanolic extract inhibited the effect statistically significantly ($p < 0.001$) and did not demonstrate a dose-dependent inhibitory effect. The results were similar to those for the carrageenan-induced edema, as the peritoneal capillary permeability was significantly inhibited by indomethacin at a dose of 5 mg/kg and by methanolic extracts of 100 and 200 mg/kg at ~90 $\mu\text{g}/\text{mL}$ based on Evans blue staining (1%). In contrast to the control treatment (vehicle), methanolic *M. minutiflora* extract demonstrated efficacy in impeding cell migration into the pleural cavity in carrageenan-induced pleurisy. At a dose of 100 mg/kg, the methanol extract of *M. minutiflora* inhibited polymorphonuclear cell migration by 62%. In conclusion, the anti-inflammatory activity of *M. minutiflora* leaf extract was evidenced through multiple assays. However, fractionation to examine the impact of triterpenoids could shed more light on the investigation.

Immune dysregulation is the primary cause of many disorders. Atopic dermatitis (AD) is a chronic inflammatory skin disease that may occur from immune dysregulation. This disorder was the focus of an investigation by Lee et al. [98], who explored the

anti-inflammatory properties of *C. asiatica* (CA) ethanol leaf extract. The main bioactive triterpenoids in the plant, which are asiatic acid, asiaticoside, madecassic acid, and madecassoside, are responsible for its medicinal properties. This study was the first to use both *in vitro* and *in vivo* models to show that CA had pharmacological effects on skin inflammation caused by DNCB. In inflammation-stimulated human epidermal keratinocytes (HaCaT) cells, treatment with the extract inhibited the expression of IL-6 and TNF- α in a dose-dependent manner through interferon- γ (IFN- γ)- and TNF- α -induced inflammation. A mouse model of atopic dermatitis was established with the administration of 2,4-dinitrochlorobenzene. In the AD mouse model, two forms of treatment were used. One was an oral dose of 200 mg/kg/d (AD + CA-200), and the other was a topical application of 80 $\mu\text{g}/\text{cm}^2$ (AD + CA-80). Interestingly, there was a significant decrease in mast cell infiltration in the ear tissue of the groups that were treated with CA. Moreover, in both the AD + CA-80 and AD + CA-200 groups, there was a decrease in the expression of IL-6 in mast cells and in the levels of various pathogenic cytokines like TNF- α , IL-4, IL-5, IL-6, IL-10, IL-17, the inducible isoform of NO synthase (iNOS), COX-2, and CXCL9. The authors found that CA regulates the allergic inflammation of the skin through its pharmacological role and signaling mechanism. This supports the hypothesis that CA could be developed as a therapeutic agent for AD. According to the authors, CA successfully decreases inflammation and restores immunological equilibrium by blocking p38 mitogen-activated protein kinase signaling. However, to confirm CA's anti-atopic dermatitis effects on human skin, additional clinical trials are necessary. In addition, investigations with the isolated compounds madecassoside, asiaticoside, madecassic acid, and asiatic acid could provide strong evidence for CA extract to be made publicly available to the pharmaceutical industry.

A popular species of *Miconia* (*Melastomataceae*) native to South and Central America is *Miconia albicans*. Pain, inflammation, and other infections can all be alleviated with the help of *M. albicans* leaf extracts. Aqueous *M. albicans* leaf extracts were prepared with boiling water by Dembogurski et al. [99] to study their anti-inflammatory activities and components. The triterpenoid *O*-hexosyl was one of several components identified by the authors. To investigate its anti-inflammatory effect, two assays were employed: the carrageenan-induced paw edema assay and the leukocyte influx assay. An hour prior to injecting carrageenan, three groups of mice were treated with different substances: saline water (vehicle), 15 mg/kg of indomethacin (positive control), or 256 mg/kg of *M. albicans* extract. Carrageenan's 55.5% reduction in paw edema after 240 min was comparable to indomethacin's 66.6% reduction. Furthermore, 4 h following the injection of carrageenan, the leukocyte influx in the abdominal cavity was recorded at 4238 cells/mm³. Both indomethacin (1438 cells/mm³) and *M. albicans* aqueous extract (1488 cells/mm³) reduced leukocyte influx, while saline solution (2100 cells/mm³) and indomethacin (66 and 65%, respectively) had little effect. A reduction in leukocyte influx was observed for saline solution (2100 cells/mm³), indomethacin (1438 cells/mm³), and *M. albicans* aqueous extract (1488 cells/mm³), indicating 66 and 65% reductions for the latter two medications, respectively.

Fibrosis of the liver develops as a result of chronic liver disease and is marked by an overabundance of fibrillary collagen. A study by Chang et al. [100] explored bitter melon leaf triterpenoid-enriched extract (TEE) and how it could protect mice against carbon tetrachloride-induced liver fibrosis. Dried bitter melon leaves were extracted with ethanol, and the crude extract was fractionated. Among the compounds, 24-*O*-acetyl-cimigenol-3-*O*- β -*D*-xylopyranoside was the most abundant, followed by methyl lucidenate P, 25-*O*-acetyl cimigenol-3-*O*- β -*D*-galactoside, and asperosaponin VI. Five fractions were generated, but the first four were discarded, so that TEE was produced. One week prior to beginning carbon tetrachloride administration and for the duration of the experiment, male mice

were administered 100 or 150 mg/kg of TEE orally once a day. Mice were administered carbon tetrachloride intraperitoneally for nine weeks. The aspartate (AST) and alanine transaminase activities (ALT) induced in the serum by carbon tetrachloride were found to be decreased when TEE supplementation was administered. Specifically, ALT in the control group treated with carbon tetrachloride decreased from ~130 to 60–70 U/L when either TEE at 100 or 150 mg/kg or silymarin at 200 mg/kg was previously delivered to mice. Similar results were obtained for AST, where a decrease from ~80 to ~40 U/L was noticed. Statistically insignificant ($p > 0.05$) values were observed in AST and ALT assays between TEE at both concentrations and silymarin. It was proven that transdermal endothelial growth factor (TEE) could alleviate liver fibrosis by controlling the secretion of inflammatory cytokines and the expression of α -smooth muscle actin (α -SMA) in the liver, thus decreasing the buildup of collagen. The triterpenoid-enriched fraction of wild bitter melon leaf extract may represent a promising candidate for the prevention of liver fibrosis, according to these findings. An acetonetic extract produced from the leaves of *Sapium lateriflorum* contained ten compounds, one of which was a novel acyl triterpenoid with anti-inflammatory properties, 3 β -palmitoyloxy-1 β ,11 α -dihydroxy-olean-12-ene (1). Other triterpenoids were also found, such as 3 β -palmitoyloxy olean-12-ene (2), lupeyl palmitate (3), 3 β -palmitoyloxy-11-oxo-olean-12-ene (5), and 6'-O-palmitoyl 3-O- β -sitosteryl- β -D-glucopyranoside (9). Novillo et al. [101] fractionated the extract, and the compounds showed negligible cytotoxic effects; they were also tested for their anti-inflammatory properties in mouse models of ear and paw edema caused by TPA and carrageenan, respectively. The novel compound (1) had significant anti-inflammatory properties comparable to those of indomethacin and was significantly better than the other compounds, according to the results. Indeed, at a dose of 0.1 μ mol/ear, it achieved 68.76% inhibition, whereas indomethacin achieved 78.76% inhibition (2.88 mg) in TPA-induced ear edema. This activity was attributed to the hydroxyl at C-11. The anti-inflammatory activity could be explained by potential molecular mechanisms, according to docking studies of olean-12-ene's 3 β -palmitoyloxy esters with NF- κ B and COX-2 receptors. In carrageenan-induced ear edema, it was revealed that the results were also comparable to those of indomethacin. At a dose of 31.6 mg/kg, compound (1) achieved 0.040 mL of edema volume, similar to 0.034 mL when 7.5 mg/kg of indomethacin was applied. The research indicates that the novel triterpenoid (1) could be a promising anti-inflammatory agent.

Momordica charantia L. leaves may alleviate conditions associated with inflammation. However, their safety profile is still unknown, and it is not known which components in their extracts have anti-inflammatory or harmful effects. A study by Chou et al. [102] examined the cytotoxicity, anti-inflammatory effects, and underlying mechanisms of the characteristic cucurbitane-type triterpenoid species found in the leaves of *M. charantia* L. Air-dried leaves were extracted with methanol at room temperature. Momordicines I, II, and IV and (23E) 3 β ,7 β ,25-trihydroxycucurbita-5,23-dien-19-al (TCD), four structurally related triterpenoids, were extracted and isolated from the triterpenoid-rich fractions of *M. charantia* leaf extracts. Normal cells were cytotoxically affected by momordicine I, while momordicine II was less toxic, and there were no discernible negative effects on cell growth for momordicine IV or TCD. In both *in vitro* and *in vivo* assays, TCD reduced inflammation. Incorporating 40 μ M of TCD considerably reduced iNOS expression from 1 to ~0.2 regarding relative band intensity of iNOS/actin compared to that of the control group through Western blot analysis. Ear edema improved after TCD administration. All three tested doses of TCD (250, 500, and 750 μ g/ear) considerably decreased the amount of ear edema in mice 4, 16, and 24 h following TPA stimulation in a dose-dependent way from ~45 to 30 μ m of ear thickness compared to ~50 μ m in the TPA control group. Indomethacin had effects similar to those of 500 and 750 μ g/ear TCD on reducing ear inflammation. One

possible anti-inflammatory mechanism is that TCD inhibits the differentiation of RAW 264.7 cells into pro-inflammatory cells resembling macrophages, which is induced by LPS. IKK/NF- κ B, which was activated by LPS, was inhibited by TCD, according to the findings. A future investigation into the extract's safety could be a reasonable goal for developing an extraction protocol that can increase TCD content while decreasing momordicine content. Further information about leaf extracts with anti-inflammatory activity due to triterpenoid compounds is provided in Table 2.

Table 2. Anti-inflammatory effects of plant leaf extracts and identification of the main triterpenoids.

Sample	Triterpenoid	Anti-Inflammatory Effect	Ref.
<i>A. solanacea</i> Roxb.	α -Amyrenone	Ethyl acetate extract had an IB ₅₀ value of 3.13 μ g/mL in the protein denaturation assay compared to 2.80 μ g/mL for diclofenac	[72]
<i>O. speciosus</i> subsp. <i>stenocarpus</i>	Rotundic acid	Extract concentrations of 3.12–25 μ g/mL achieved 2.39–0.48 μ M NO production inhibition	[73]
<i>N. arbor-tristis</i>	Betulinic acid	IC ₅₀ : COX-1 (10.34 μ g/mL), COX-2 (12.92 μ g/mL), 5-LOX (15.53 μ g/mL), NO (15.21 μ g/mL), TNF- α (16.65 μ g/mL)	[74]
<i>Z. mauritana</i> <i>Z. spina-christi</i> <i>Z. jujuba</i>	Ceanothic acid, isoceanothic acid, apiceanothic acid, and epigouanic acid	% COX-1 inhibition of <i>Z. Mauritiana</i> (90.34), <i>Z. Spina-Christi</i> (89.31), and <i>Z. Jujuba</i> (76.87)	[75]
<i>C. inophyllum</i>	Twelve unknown triterpenoids	Compounds (1) and (2) had 2.44 and 7.00 μ M IC ₅₀ values compared to dexamethasone (0.012 μ M) against cytokine production in RAW 264.7 cells	[76]
<i>S. bullockii</i>	Six triterpenoid glycosides, including an unknown compound (syzybulloside A)	IC ₅₀ values of 6.93–11.58 μ M for triterpenoids (1–6) against NO production in RAW 264.7 cells	[77]
<i>S. hexaphylla</i>	Twenty-three triterpenoid saponins, two of them novel	Compound (13) had an IC ₅₀ value of 0.59 μ M compared to 0.13 μ M for dexamethasone against NO production	[78]
<i>Cappars ovata</i>	Oleanolic acid, β -sitosterol, ursolic acid, bismethyl-octylphthalate, stigmast-5,22-dien-3- β -myristate, and the novel compound olean-12-en-28ol, 3b-pentacosanoate (1)	Compound (1) reduced the mRNA Expression of CXCL9 (19.36-fold), CXCL10 (8.14-fold), and TNF (18.69) compared to that in the control group	[79]
<i>O. fragrans</i> var. <i>aurantiacus</i>	3- <i>O-trans-p</i> -coumaroyltormentic acid (1), 3 β - <i>trans-p</i> -coumaroyloxy-2 α -hydroxyl-urs-12-en-28-oic acid (2), 3- β - <i>cis-p</i> -coumaroyloxy-2 α -hydroxyl-urs-12-en-28-oic acid (3), and 3- <i>O-cis</i> -coumaroylmaslinic acid (4)	Compounds (2–4) decreased the levels of LPS-stimulated cytokines COX-2, NF- κ B, and phosphorylated extracellular regulated kinase (pERK)1/2	[80]
	Maslinic acid, corosolic acid	In both HCT116 and HT29 cell lines, the phosphorylation levels of p65, I κ -B α , and ERK were considerably reduced by the ethyl acetate fraction (at 50 and 100 μ g/mL, respectively); however, corosolic acid had stronger anticancer activity	[82]
<i>C. pachystachya</i>	Ursolic acid, pomolic acid	NO production in RAW 246.7 macrophages decreased from ~20 to ~5 μ M using 300 μ g/mL extract	[83]
<i>C. paliurus</i>	Eighteen dammarane triterpenoid saponins	IC ₅₀ values for compounds (7), (8), (10), and (11) ranged from 8.23 to 11.23 μ M for NO inhibition; ethyl acetate extract at 80 μ g/mL decreased TNF- α , PGE2, IL-6, COX-2, iNOS, and NF- κ B/p65 expression	[86]
<i>J. gossypifolia</i>	Unknown jagaboeuphols A–C (1–3) and jagoseuphone A (4), a common euphane-type triterpenoid	Compound (4): lower IC ₅₀ value (20.1 μ M) than the positive control quercetin (16.8 μ M) for NO production inhibition	[87]
<i>T. cherryi</i>	Three ternstroenols	IC ₅₀ value of 0.72–3.70 μ M against NO production in RAW 264.7 macrophages activated with LPS and IFN- γ	[88]
<i>C. hystrix</i>	Lupeol	At 25 μ g/mL, IL-1 β , IL-6, and TNF- α were significantly reduced with either a lupeol fraction or with ethanolic <i>C. hystrix</i> extract at 1.5 or 2.5 μ g/mL	[89]
<i>C. asiatica</i>	Asiatic acid, asiaticoside, madecassic acid, and madecassoside	Decrease in the expression of cytokines TNF- α , IL-4, IL-5, IL-6, IL-10, IL-17, iNOS, COX-2, and CXCL9	[98]
	Madecassoside (1), terminolloside (2), and isomadecassoside (3)	At 50 μ M, compounds (1) and (2) decreased from NO production from ~19 to 15 μ M; compound (3) lowered NO production from ~18 to 14 μ M	[90]
<i>P. padus</i>	Corosolic (CA), ursolic (UA), and oleanolic (OA) acids	CA fraction: IC ₅₀ value 14.3–22.2 μ g/U for LOX and 12.6–18.1 μ g/U for HYAL; the extracts were more effective LOX inhibitors (IC ₅₀ : 10.5–12.8 μ g/U) than HYAL inhibitors (IC ₅₀ : 19.3–22.0 μ g/U)	[91]
<i>W. indica</i>	Triterpenoid saponins expressed as oleanolic acid equivalents	Ethanolic and ethyl acetate extracts achieved 37.7% and 38.9% inhibition of COX-2	[92]
<i>V. curassavica</i>	Cordialin A	Inhibition of phospholipase A ₂ (2.8–10.3%), COX-1 (8.3–27.2%), and COX-2 (3.3–22.2%) at a concentration range of 10.0–100.0 μ g/mL	[93]
<i>B. javanica</i>	27 triterpenoids named brujavanoids A–U	Brujavanoid E had a lower IC ₅₀ value compared to dexamethasone (4.1 and 9.2 μ M, respectively) in the NO production inhibition assay	[94]
<i>C. japonica</i>	Unknown, camellioside B, and camellioside A	The three triterpenoids achieved 37.99, 31.31, and 28.96% NO production inhibition at a 25 μ M concentration	[95]
<i>T. catharinensis</i>	Ehretiolide, oleanolic acid, and oleanonic acid	ID ₅₀ values for ear edema ranged from 0.001 to 0.0061 μ g/ear for fractions	[96]
<i>M. minutiflora</i>	Myrianthnic acid, arjunolic acid	Methanolic extract achieved 5.36×10^6 PMNL/mL compared to indomethacin 5 mg/kg (6.54×10^6 PMNL/mL); ~80 μ g/mL decreased edema at 100 or 200 mg/kg of extract	[97]
<i>M. albicans</i>	O-hexosyl triterpenoid	Reduction in leukocytes by 65% (compared to 66% for indomethacin) and paw edema by 55.5% (compared to 66.6% for indomethacin) from carrageenan induction	[99]

Table 2. Cont.

Sample	Triterpenoid	Anti-Inflammatory Effect	Ref.
Bitter melon	24-O-acetyl-cimigenol-3-O-β-D-xylopyranoside as the most abundant, methyl lucidenate P, 25-O-acetyl cimigenol-3-O-β-D-galactoside, and asperosaponin VI	Triterpenoid-rich extract: ALT decreased from ~130 to 60–70 U/L; AST decreased from ~80 to ~40 U/L	[100]
<i>S. lateriflorum</i>	Ten triterpenoids, one of which is novel (3β-palmitoyloxy-1β,11α-dihydroxy-olean-12-ene) (1)	0.1 μmol/ear of compound (1) achieved 68.76% inhibition, indomethacin 78.76% in TPA-induced ear edema; 31.6 mg/kg of compound (1) achieved a 0.040 mL edema volume (0.034 mL when 7.5 mg/kg of indomethacin was used)	[101]
<i>M. charantia</i>	Momordicines I, II, and IV and (23E) 3β,7β,25-trihydroxycucurbita-5,23-dien-19-al (TCD)	40 μM TCD reduced iNOS expression from 1 to ~0.2 iNOS/actin compared to the control group. Regarding the TPA control group, TCD at 250, 500, or 750 μg/ear decreased ear edema in mice after 4, 16, and 24 h from ~45 to 30 μm of ear thickness compared to ~50 μm in the control group, comparable to indomethacin	[102]

ALT: alanine transaminase; AST: aspartate transaminase; COX: cyclooxygenase; CXCL: chemokine (C-X-C motif) ligand; HYAL: hyaluronidase; IB₅₀: 50% inhibition of the denaturation of protein; IC₅₀: 50% inhibitory concentration; ID₅₀: 50% inhibitory dose; IFN: interferon; IL: interleukin; iNOS: inducible isoform of nitric oxide; LOX: lipoxygenase; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor κ light-chain enhancer of activated B cells; NO: nitric oxide; pERK: phosphorylated extracellular regulated kinase; PMNL: polymorphonuclear leukocyte; TCD: (23E) 3β,7β,25-trihydroxycucurbita-5,23-dien-19-al; TNF: tumor necrosis factor; TPA: tetradecanoylphorbol-13-acetate.

6. Limitations

Extensive research has explored the association between triterpenes and triterpenoids and their potential anti-inflammatory activity. However, there is a lack of fractionation and *in vivo* or *in vitro* laboratory testing to substantiate these findings. Most researchers isolated and identified compounds from extracts and simply attributed their medicinal properties to these compounds based on the literature. In addition, there are studies in which the qualitative identification of extracts has been carried out and medicinal activity is simply attributed to specific groups of compounds. To advance the development of leaf extract preparation from plants of pharmacological interest, it would be advisable to conduct more *in vitro* and *in vivo* tests after the fractionation of the extract and isolation of specific compounds.

7. Conclusions and Future Perspectives

Triterpenes and triterpenoids are secondary metabolites of plant origin. Due to their complex chemical structures, they have attracted interest as potential therapeutic agents against various diseases. Their most studied potential action is anti-inflammatory. Several studies indicate that plant leaf extracts rich in triterpenes or triterpenoids have anti-inflammatory activity both *in vivo* and *in vitro*. So far, the anti-inflammatory activity of triterpenoids appears to be stronger than that of triterpenes.

Given the chemical similarity between many bioactive triterpenes and steroidal anti-inflammatory compounds, these natural products have the potential to serve as a foundation for the identification of novel prototypes in the search for new medication development. However, additional investigations are needed to ascertain the clinical efficacy of these compounds. Triterpenes should be examined more thoroughly for their potential pharmacological activity. These studies should include various *in vivo* experiments, the determination of pharmacokinetic and pharmacodynamic parameters, the assessment of acute toxicity, and the examination of potential adverse effects such as herb–drug interactions. Furthermore, efforts should be made to enhance the biological activity and bioavailability of these chemicals through structural alterations. These modifications could include the development and synthesis of new derivatives, improvement of water solubility, and encapsulation in carriers such as nanostructures.

Author Contributions: Conceptualization, V.A. and S.I.L.; writing—original draft preparation, D.K. and M.M.; writing—review and editing, V.A., D.K., E.B., M.M. and S.I.L.; visualization, D.K. and M.M.; supervision, V.A. and S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: No new data were created or analyzed in this study.

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

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Review

Formation and Characterization of Aroma Compounds in Camembert Cheese

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Abstract: The characterization of volatile compounds in cheese is crucial for understanding sensory properties and consumer acceptance. Camembert cheese, a surface-ripened variety, presents a complex aroma profile shaped by biochemical and microbial interactions. Despite advances in analytical methods such as gas chromatography–mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC–O), the metabolic pathways and microbial interactions defining Camembert’s aroma remain incompletely understood. This review explores the synergistic roles of microbial communities, enzymatic activity, and environmental conditions in volatile compound formation. A systematic literature review was conducted using Scopus, Web of Science, and Google Scholar to analyze the classification of volatile compounds, biochemical pathways of aroma formation, and microbial contributions. The findings highlight the essential role of *Penicillium camemberti* and lactic acid bacteria in aroma modulation, particularly in sulfur compounds, esters, and short-chain fatty acids. Emerging technologies such as solid-phase microextraction (SPME) and metabolomics provide new insights into volatile compound dynamics. Understanding these mechanisms may enhance aroma control in cheese production through microbial engineering and biochemical monitoring. This review underscores the need for integrated approaches to optimize fermentation and ensure sensory standardization, contributing to improved quality and consumer acceptance of Camembert cheese.

Keywords: volatile compounds; camembert cheese; aroma formation; microbial metabolism

1. Introduction

Camembert cheese is a traditional French soft-ripened cheese with a white mold rind and creamy texture, developed in the 18th century in the Normandy region. Recognized for its complex aroma and flavor, Camembert de Normandie AOC is produced exclusively with raw cow’s milk and traditional methods, including surface inoculation with *Penicillium camemberti* and a minimum ripening period of 21 days. This contrasts with industrial versions made using pasteurized milk and standardized starter cultures, which tend to have milder and more uniform aromatic profiles [1,2].

Volatile compounds are essential in shaping the flavor profile of cheeses, directly influencing their sensory perception and commercial acceptance. Most of these compounds result from complex biochemical processes that occur during ripening, involving reactions such as proteolysis, lipolysis, and microbial metabolism [3]. In the case of white mold-ripened cheeses, such as Brie and Camembert, the aroma profile is particularly diverse, being modulated by the activity of specific microorganisms, such as *Penicillium camemberti*

and *Lactococcus lactis*, which contribute to the production of acids, esters, ketones, and alcohols responsible for characteristic notes of mushroom, butter, and ammonia [4,5].

It is also important to consider that the term “Camembert” encompasses a broad range of cheeses with sensory variability influenced by raw material origin, microbial strains, and regional or industrial production practices. The use of different strains of *Penicillium camemberti*, varying milk treatments (raw or pasteurized), and environmental parameters during ripening—such as temperature, humidity, and salinity—all contribute to differences in aroma compound formation [5,6].

Despite significant advances in the characterization of these compounds, gaps remain in understanding the chemical and metabolic interactions that determine the final aroma of Camembert [7]. Additionally, environmental, technological, and microbiological factors directly influence the volatile composition of the cheese, potentially affecting its sensory acceptance and overall quality [8]. While some studies suggest that modifications in the ripening process can be explored to optimize the aroma profile, the variability in volatile compound formation is still not fully understood.

Notably, analytical innovations such as solid-phase microextraction (SPME), gas chromatography–mass spectrometry (GC–MS), and gas chromatography–olfactometry (GC–O) have enhanced the identification of trace-level aroma-active compounds in dairy matrices, enabling a more accurate correlation between chemical composition and sensory relevance [9]. Adjustments in the ripening environment—including temperature, relative humidity, and microbial consortia—have been shown to influence the synthesis of key volatiles like diacetyl, 2-undecanone, and methanethiol [5,10].

Given this context, this review aims to explore the complexity of Camembert cheese aroma by identifying the main classes of volatile compounds and their respective biochemical formation pathways. Furthermore, this review seeks to analyze the microbiological interactions during ripening and discuss the practical implications of these findings within the dairy sector, offering a thorough analysis of the factors that influence the sensory quality of this cheese.

2. Materials and Methods

This study is based on a literature review aimed at identifying and analyzing the volatile compounds responsible for the characteristic aroma of Camembert cheese, as well as the biochemical and microbiological mechanisms involved in its formation. The search for publications was conducted in the Scopus, Web of Science, and Google Scholar databases using keywords such as “volatile compounds”, “aroma”, “Camembert cheese”, “gas chromatography (GC)”, and “solid-phase microextraction (SPME)”. These terms were selected to encompass relevant studies on Camembert’s odor-active volatile profile, the associated biochemical processes, and the analytical techniques used to characterize these compounds.

The selection criteria for articles were based on their relevance to the review’s scope, including: (i) studies addressing the volatile and odor-active composition of Camembert; (ii) articles describing the biochemical and microbiological processes associated with the formation of volatile and odor-active compounds; and (iii) publications utilizing analytical techniques such as gas chromatography (GC), mass spectrometry (MS), and solid-phase microextraction (SPME), in addition to olfactometric techniques such as gas chromatography–olfactometry (GC–O), when available. Although the primary focus was Camembert, studies on other white mold cheeses, such as Brie, were included when they provided directly relevant insights for the review. There was no temporal restriction, as the selection prioritized studies that made significant contributions to the topic. Publications focusing exclusively

on other cheese types, such as washed-rind cheeses (e.g., Munster, Taleggio), blue cheeses, or pressed cheeses (e.g., Gouda, Cheddar), were excluded.

To better illustrate the conceptual structure of the literature included in this review, a co-occurrence analysis was conducted using the VOSviewer software [11]. The analysis was based on terms extracted from the titles, abstracts, and keywords of the selected publications exported from Zotero. This network visualization, presented in Figure 1, reveals the thematic connections between key concepts, enabling the identification of dominant topics and their interrelationships in the context of aroma development in Camembert cheese.

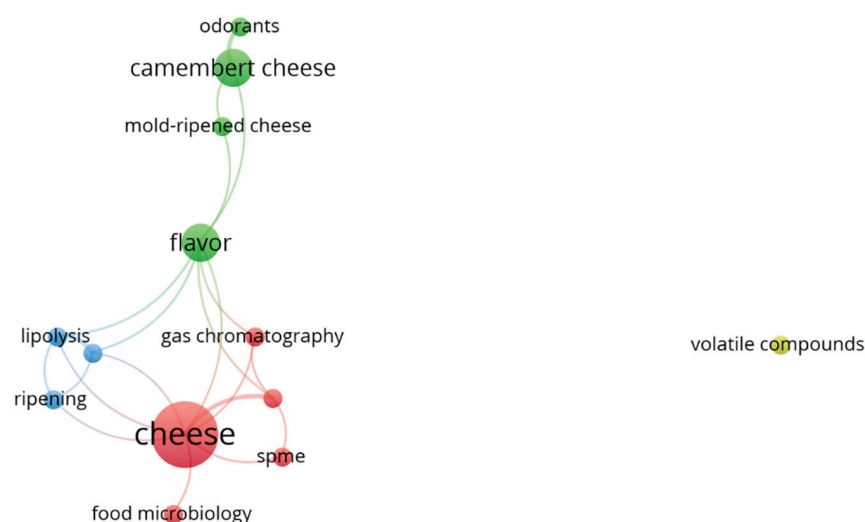


Figure 1. Co-occurrence network of terms from the selected literature corpus. Visualization generated using VOSviewer software [11].

As observed in the network, the literature is centered around interrelated themes such as biochemical pathways (e.g., lipolysis, proteolysis), sensory aspects (flavor), and analytical approaches (SPME, GC). These findings support the structure adopted in this review, which explores each of these domains in detail.

Advanced techniques, such as aroma extract dilution analysis (AEDA) and odor activity value (OAV) determination, although widely recognized in aroma studies of fermented foods, have been applied less systematically to white mold cheeses. Therefore, their relevance and potential future applications are discussed in detail throughout this review.

The collected data were organized into three main analytical categories: (i) classification of volatile compounds, grouping substances like alcohols, fatty acids, esters, and sulfur compounds; (ii) general biochemical pathways of volatile compound formation, with an emphasis on lipolysis, proteolysis, and carbohydrate metabolism; and (iii) microbial contributions, highlighting the role of microorganisms such as *Penicillium camemberti* and lactic acid bacteria in shaping Camembert's aroma profile.

3. Results

3.1. Odor-Active Volatile Profile of Camembert Cheese

Camembert cheese is typically produced from cow's milk, either raw or pasteurized, depending on the production region and legal framework. The process begins with milk standardization and acidification through the addition of mesophilic starter cultures, primarily *Lactococcus lactis*. Coagulation is induced using animal rennet, followed by gentle curd cutting and whey drainage. The curd is then ladled into molds and turned multiple times to promote uniform whey expulsion. After demolding, the cheese is salted—either by dry salting or brining—and surface-inoculated with *Penicillium camemberti* to initiate

rind development. The maturation process occurs over 2 to 4 weeks under controlled temperature (10–14 °C) and relative humidity (90–95%), allowing for the growth of surface microbiota and the development of the characteristic creamy texture and complex aroma profile [1,2].

Camembert cheese is characterized by a complex and diverse volatile profile, resulting from the interaction of biochemical, microbiological, and physical processes throughout ripening. This sensory richness stems from the occurrence of volatile compounds primarily formed through proteolysis, lipolysis, and microbial metabolism [3].

Microorganisms such as *Penicillium camemberti* and *Lactococcus lactis* play essential roles in generating these compounds, imparting fruity, buttery, pungent, and slightly ammoniacal notes to the cheese [5]. The aroma of Camembert arises from the presence and interaction of various classes of odor-active volatile compounds, predominantly lipophilic, formed through enzymatic reactions involving free fatty acids, peptides, amino acids, and carbohydrates [12]. During ripening, these compounds may undergo secondary transformations, including reduction, oxidation, and chemical interactions, forming more stable structures [5].

Nevertheless, the impact of odor-active volatile compounds on the aroma varies. Their influence depends on factors such as concentration, olfactory perception threshold, synergistic interactions within the cheese matrix, and environmental conditions such as temperature, humidity, and salinity, which modulate microbial and enzymatic activity [7,13]. Table 1 presents the main odor-active volatile compounds identified in Camembert and their associated sensory characteristics.

Table 1. Odor-active volatile compounds associated with Camembert.

Compound	Aroma Notes	References
Methanethiol	Cooked cabbage, sulfur	[9,14,15]
Ethanol	Alcoholic, mild	[7,9,14]
Acetone	Ethereal, fruity	[9,14]
Dimethyl sulfide	Boiled cabbage, sulfur	[9,14,15]
Acetic acid	Vinegar, pungent	[7,9,14,16]
Propionic acid	Vinegar, pungent	[14]
2-Pentanone	Fruity, sweet	[9,14]
Dimethyl disulfide	Cauliflower, garlic	[9,14,15]
Butanoic acid	Rancid, cheesy, sweaty	[7,9,14,16]
2-Heptanone	Blue cheese, spicy	[14]
Propanol	Alcoholic, sweet	[9]
2,3-Butanedione	Buttery	[9,15]
Ethyl acetate	Solvent, pineapple, fruity	[9,14]
Methyl thioacetate	Cooked cauliflower	[9]
3-Methylbutanol	Fruity, alcohol-like	[16]
3-Methylbutanal	Green, malty	[15,16]
2-Nonanone	Sweet, soapy, floral	[16]
Hexanoic acid	Rancid, sweaty, sour	[16]
Octanoic acid	Goaty, waxy, rancid	[16]
γ -Hexalactone	Caramel, nutty, woody	[16]
Benzeneacetaldehyde	Floral, sweet, fruity	[16]
3-Methylbutanoic acid	Rancid, sweaty, pungent	[16]

Although Table 1 presents a broad range of odor-active volatile compounds identified in Camembert cheese, not all of them contribute equally to its sensory perception. The aromatic relevance of each compound depends on factors such as its concentration, perception threshold, and interactions within the cheese matrix. Analytical–sensory techniques, including gas chromatography–olfactometry (GC–O) and solid-phase microextraction

combined with mass spectrometry (SPME–GC–MS), have been employed to characterize these compounds in surface-ripened cheeses, although their application to Camembert remains relatively limited and fragmented [9,12,14,17]. Among the identified compounds, methanethiol, dimethyl disulfide, 2,3-butanedione, ethyl acetate, and 3-methylbutanoic acid stand out due to their low detection thresholds and intense sensory attributes [9,12,17]. Addressing the current gap through more systematic use of omission tests or odor activity value (OAV) analysis could further refine our understanding of which compounds truly shape Camembert’s distinctive aroma. The main biochemical routes responsible for the generation of these compounds—such as lipolysis, proteolysis, and carbohydrate metabolism—are summarized in Figure 2, which illustrates their contribution to aroma formation throughout cheese ripening.

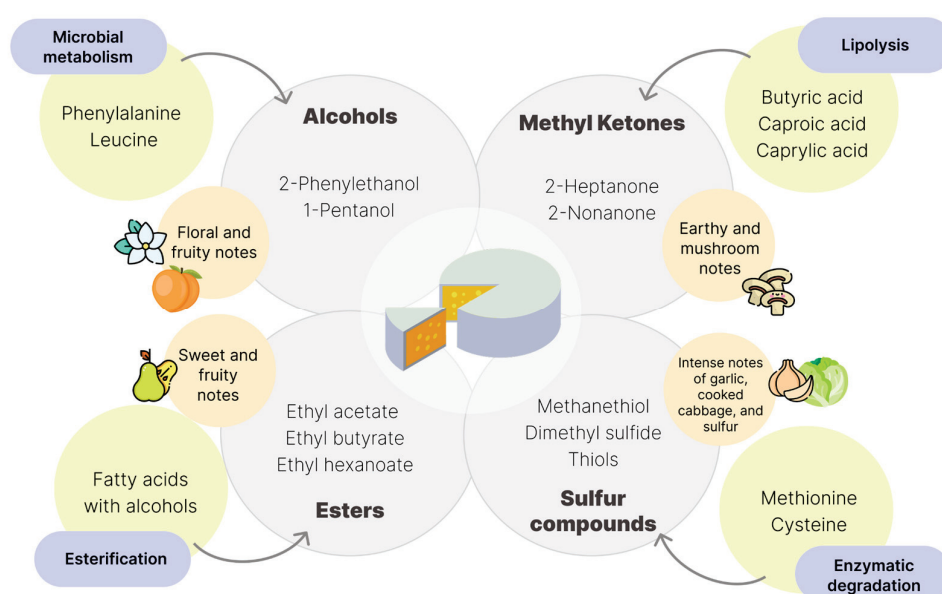


Figure 2. Key biochemical pathways contributing to the aroma development of Camembert cheese. Some icons used in this figure were sourced from Flaticon.com and are used in accordance with their free license with attribution.

3.2. Biochemical Origin of Odor-Active Volatile Compounds in Camembert Cheese

The sensory perception of ripened cheeses is directly related to their chemical composition, with volatile compounds being the primary contributors to their characteristic aroma. In the case of Camembert, these compounds are generated through a series of interconnected biochemical processes throughout production and ripening, including lipolysis, proteolysis, and carbohydrate metabolism [18].

As illustrated in Table 2, three main metabolic pathways influence the formation of volatile compounds responsible for the aroma of Camembert [19].

Table 2. Metabolic pathways for the formation of volatile compounds in Camembert cheese.

Process	Description	Formed Compounds	Sensory Contribution	References
Lactose Catabolism	Degradation of lactose, which generates organic acids, diacetyl, and volatile aroma precursor compounds..	Organic acids, diacetyl, volatile compounds	Acidic, buttery, mildly pungent notes	[2,12]

Table 2. Cont.

Process	Description	Formed Compounds	Sensory Contribution	References
Lipolysis	Breakdown of lipids into free fatty acids, which can be converted into ketones, esters, and lactones.	Ketones, esters, lactones	Fruity, buttery, pungent notes	[1,5]
Proteolysis	Hydrolysis of proteins into peptides and amino acids, serving as substrates for sulfur compounds, higher alcohols, and aldehydes.	Sulfur compounds, higher alcohols, aldehydes	Sulfurous, nutty notes	[2,9]

The quality of raw milk, along with fermentation and ripening conditions, plays a decisive role in shaping the aroma profile of Camembert. The composition of the present microorganisms directly influences the production of fatty acids, alcohols, esters, ketones, and lactones—compounds that define the cheese’s sensory nuances [20].

Ripening is the primary factor that determines the final flavor, as it involves a series of biochemical reactions that can be categorized into two major groups: primary and secondary metabolism [21]. These stages represent the sequential formation of aroma precursors—such as free fatty acids, amino acids, and organic acids—and their subsequent transformation into key volatile compounds [22,23]. A third dimension, the sensory profile, represents how these compounds contribute to the final aroma perceived in cheeses like Camembert [23]. Some metabolites, such as diacetyl and butanoic acid, play dual roles, appearing both as intermediates and as final odor-active compounds [24]. These processes are schematically illustrated in Figure 3.

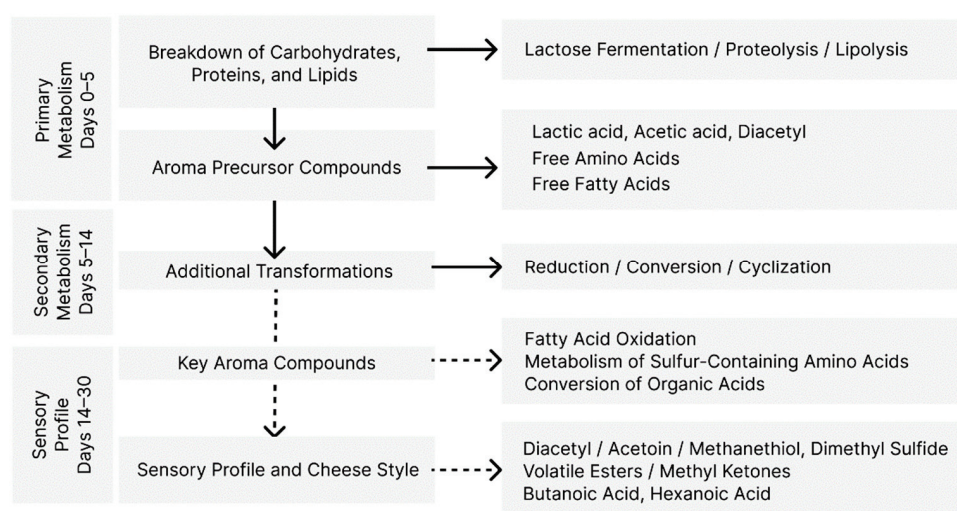


Figure 3. Conceptual overview of aroma compound formation during Camembert cheese ripening. Based on information from [5,7,10,18].

In addition to lactic acid bacteria, yeasts present during ripening play a crucial role by producing secondary metabolites such as carbonyl compounds, sulfur compounds, fatty acids, and higher alcohols, which enhance the aroma and flavor of Camembert [25]. Species such as *Geotrichum candidum* and *Debaryomyces hansenii* contribute significantly to the production of key aroma compounds. *G. candidum* initiates early lipolysis and

deaminates amino acids, leading to the formation of methyl ketones (e.g., 2-heptanone, 2-nonanone), alcohols (e.g., 2-phenylethanol), and esters (e.g., ethyl hexanoate). *D. hansenii* plays a detoxifying role while generating volatile sulfur compounds like methanethiol and dimethyl disulfide, as well as fruity esters that contribute to sweet and fermented notes [26,27].

Additionally, the fungus *Penicillium camemberti* plays a central role in the ripening of this cheese, promoting the development of its characteristic white rind and actively contributing to its sensory profile. This fungus produces proteolytic and lipolytic enzymes that degrade proteins and lipids, generating precursors of volatile compounds responsible for specific aroma notes [12,28].

Mushroom-like notes are associated with the presence of 1-octen-3-ol and methanethiol, while the buttery aroma is predominantly influenced by 2,3-butanedione (diacetyl) and acetoin. Meanwhile, ammoniacal nuances result from ammonia (NH₃) release and the metabolism of sulfur-containing amino acids, leading to the formation of compounds such as dimethyl sulfide, trimethylamine, and methanethiol, which play a fundamental role in defining Camembert's sensory profile [17]. The synergistic interaction between lactic acid bacteria, yeasts, and *Penicillium camemberti* results in a matrix rich in volatile compounds, whose complexity defines the unique aroma and flavor of Camembert [7,9,12,14–17,29]. The following sections will detail the general biochemical pathways involved in the formation of odor-active volatile compounds found in white mold-ripened cheeses.

3.2.1. Carbohydrate Metabolism and the Formation of Aroma Compounds

The degradation of carbohydrates plays a crucial role in the formation of volatile compounds in Camembert cheese. During the early stages of ripening, lactose is fermented by lactic acid bacteria (LAB), such as *Lactococcus lactis* and *Leuconostoc mesenteroides*, leading to the production of lactic acid, which contributes to the cheese's acidity and texture [20,22].

Following this initial metabolism, secondary transformations occur, involving the conversion of lactic acid into compounds such as acetic acid, propionic acid, ethanol, and diacetyl, which play an essential role in the aroma complexity of the cheese. These processes and their microbial interactions are detailed in Figure 4, which illustrates the formation of volatile compounds derived from carbohydrate metabolism throughout the ripening of Camembert.

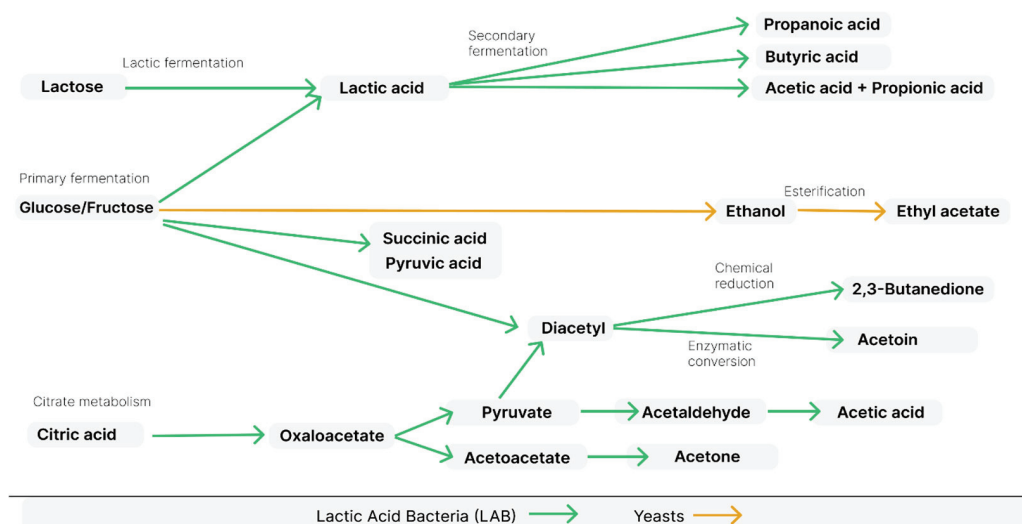


Figure 4. Pathways of aroma compound formation from carbohydrate metabolism (Based on information from [19]).

During ripening, lactic acid bacteria such as *Lactococcus lactis* and *Geotrichum candidum* metabolize milk lactose, generating glucose and other intermediates that can serve as substrates for secondary reactions. These processes lead to the formation of metabolites such as ethanol, which, in turn, acts as a precursor for volatile esters, including ethyl acetate and methyl butyrate, responsible for mild alcoholic and fruity notes [6].

Lactose is the primary carbohydrate in milk and can vary by up to 100 g/L among different mammalian species [20]. Its metabolism results in the production of compounds such as L-lactate and DL-lactate, which directly influence the flavor of ripened cheeses, including Camembert, Cheddar, and Dutch cheeses. L-lactate contributes to a slight acidity and freshness in flavor, while DL-lactate, depending on its concentration, can affect the perception of creaminess and aroma complexity. Consequently, these compounds serve as substrates for secondary reactions that lead to the formation of organic acids, such as acetic and propionic acid, responsible for mildly pungent and fruity notes in ripened cheeses [2].

Leuconostoc spp., a genus of lactic acid bacteria involved in Camembert ripening, plays an important role in the production of aroma compounds by metabolizing citrate, leading to the formation of diacetyl and acetate, which contribute buttery notes and complexity to the aroma. Additionally, its metabolic activity may contribute to ethanol and organic acid production, further enhancing the cheese's sensory diversity [30].

Citrate, present in milk at concentrations of up to 1.8 g/L, is often lost in the whey during processing but significantly impacts the sensory profile of certain cheeses. Mesophilic bacteria, such as *Lactococcus lactis* and *Leuconostoc* spp., metabolize citrate to produce compounds such as acetate, diacetyl, acetoin, and CO₂, which contribute to the aroma of cheeses like quark, cottage, and Dutch cheeses [31]. The main compounds resulting from citrate metabolism play fundamental roles in cheese sensory characteristics. Diacetyl is a key compound responsible for pronounced buttery notes, especially in intensely flavored cheeses. Carbon dioxide (CO₂) contributes to the formation of "eyes" in cheeses like Gouda, influencing their texture. Meanwhile, acetate and acetoin add slightly acidic characteristics, modulating flavor balance and enhancing aroma complexity. However, excessive citrate metabolism can affect cheese texture. In varieties such as Cheddar, its intense degradation can lead to structural softening, compromising the final product's integrity [32].

3.2.2. Odor-Active Volatiles Formed by Fatty Acid Metabolism

Lipolysis is one of the most important biochemical processes in the ripening of Camembert cheese, playing a central role in the development of its aroma, flavor, and texture. This process, which is best illustrated in Figure 5, involves the hydrolysis of triglycerides present in milk fat, releasing free fatty acids (FFA) that serve as precursors for a wide range of volatile compounds, including esters, lactones, alcohols, and ketones [31].

Lipid degradation in Camembert is primarily driven by the fungus *Penicillium camemberti*, which secretes lipases and esterases responsible for hydrolyzing the triacylglycerols present in the milk fat matrix throughout cheese ripening. These processes result in the release of free fatty acids (FFA) of various chain lengths, such as butyric, lauric, myristic, palmitic, oleic, and linoleic acids, each playing distinct roles in the cheese's sensory profile [5].

Each fatty acid released during lipid metabolism contributes specifically to the sensory profile of Camembert. Butyric acid, for example, is responsible for intense cheese and rancid notes, being one of the key compounds that impart pungency to ripened cheeses. Lauric and myristic acids, which have medium-chain structures, contribute slightly sweet and buttery nuances while also influencing the cheese's texture. Palmitic and oleic acids are associated with the perception of richness and body, enhancing the characteristic creaminess of Camembert. Linoleic acid, on the other hand, can act as a precursor for

secondary volatile compounds, such as aldehydes and ketones, which add complexity to the aroma with fruity, dairy, and nutty notes. The interaction between these fatty acids and the chemical reactions occurring during ripening determines the intensity and balance of the cheese's sensory attributes [12].

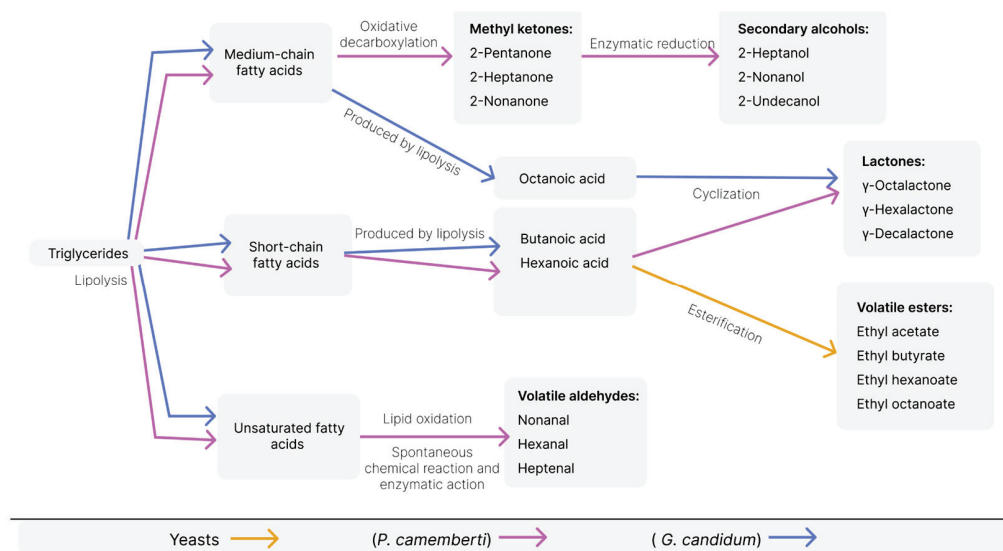


Figure 5. Fatty acid metabolism (Based on information from [31]).

Lipolysis in cheese is influenced by various enzymatic sources, including enzymes naturally present in milk, microbial cultures, and enzymes added during processing. Lipoprotein lipase, found in raw milk, plays a significant role in the release of free fatty acids, directly impacting the cheese's sensory profile [2]. In cheeses made from pasteurized milk, lower enzymatic activity can limit the formation of certain aroma compounds, making the selection of starter cultures and secondary microbiota essential to compensate for this difference [33]. Additionally, lipolytic enzymes present in rennet and exogenous enzymes added during production can modulate the intensity of lipolysis, contributing to the development of specific sensory characteristics [1].

The free fatty acids (FFA) resulting from lipolysis can follow different metabolic pathways, as illustrated in Figure 6, leading to the production of volatile compounds that define the sensory characteristics of Camembert.

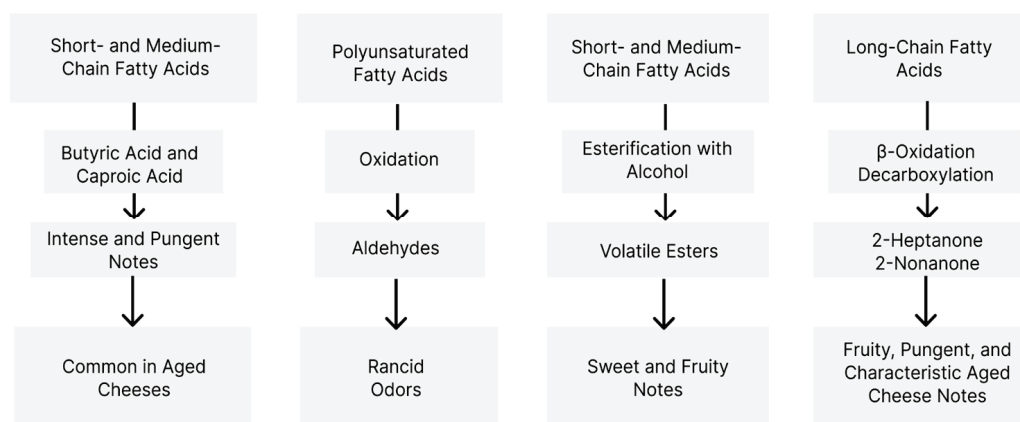


Figure 6. Pathways for the production of aroma compounds from FFA (Adapted from [5,31,34]).

Esters play a crucial role in the aroma profile of Camembert. Compounds such as ethyl acetate and methyl butyrate are responsible for fruity and sweet notes, balancing the intensity of free fatty acids. However, excessive concentrations of esters can result in overly sweet or artificial aromas, necessitating careful control of lipid metabolism and microbial activity [18].

Another important group is thioesters, such as methyl mercaptan, which contribute pungent and sulfurous notes, commonly found in washed-rind cheeses and cheeses with intense microbial activity during ripening. These compounds are formed from the metabolism of amino acids and fatty acids by specific microorganisms during maturation, including *Penicillium camemberti*, *Brevibacterium linens*, *Geotrichum candidum*, and certain species of *Lactococcus* and *Leuconostoc*, which play key roles in converting sulfur precursors into specific volatile compounds [28].

In addition to *P. camemberti*, the fungus *Geotrichum candidum* also actively participates in lipid metabolism in Camembert. This microorganism complements lipolysis by reducing bitterness, as it degrades bitter peptides generated by proteolysis. Furthermore, it enhances sulfurous notes by acting on the metabolism of amino acids and fatty acids. It also contributes to the cheese's creamy texture by modulating the lipid structure and influencing its smoothness. The combined impact of these microorganisms is essential for the sensory balance of Camembert, highlighting the importance of microbiota in shaping its characteristic aroma [13].

Technological factors, such as the type of salt used during ripening, also significantly influence lipid degradation and the formation of aroma compounds. Studies indicate that using unrefined salts can increase volatile fatty acid levels, such as octanoic acid, enhancing buttery and fruity notes. At the same time, it may reduce undesirable compounds, such as caproic acid, which is responsible for excessively pungent notes [13].

3.2.3. Odor-Active Volatiles Formed by Amino Acid Metabolism

Proteolysis represents a fundamental biochemical mechanism during the maturation of Camembert, playing a crucial role in the gradual degradation of dairy proteins, particularly caseins. This process is facilitated by an enzymatic consortium of diverse origins, including enzymes produced by *Penicillium camemberti*, *Geotrichum candidum*, and lactic acid microorganisms. The synchronized activity of these enzymes promotes the generation of peptides and free amino acids, which act as the chemical basis for flavor and aroma enhancement throughout maturation [2].

The free amino acids formed are essential not only as basic flavor molecules (contributing to sweet, umami, and bitter notes) but also as biochemical precursors of volatile compounds responsible for Camembert's characteristic aroma [35]. The subsequent metabolism of these components involves reactions such as transamination, decarboxylation, deamination, and oxidation, leading to the formation of alcohols, carboxylic acids, esters, and volatile sulfur compounds. These pathways are highly influenced by environmental conditions such as temperature, humidity, and pH, as well as the composition of the surface microbiota. The main transformations and their sensory contributions can be visualized in Figure 7, which illustrates the metabolic routes involved in the conversion of amino acids into aroma compounds.

Among the precursor amino acids, methionine and cysteine play a prominent role, as they give rise to volatile sulfur compounds, which are highly specific to white mold cheeses. Methionine metabolism leads to the formation of methanethiol, which is subsequently oxidized to dimethyl disulfide and dimethyl trisulfide—compounds with extremely low perception thresholds, responsible for the pungent, sulfurous, and ammoniacal notes of Camembert's rind [26]. In contrast, other amino acids, such as glutamate, do not contribute

to aroma directly but are involved in the formation of non-volatile flavor compounds, such as succinic acid and umami-related metabolites. These molecules play a key role in taste perception rather than olfactory characteristics, and therefore contribute to the overall flavor but not to the aroma of the cheese [2,35].

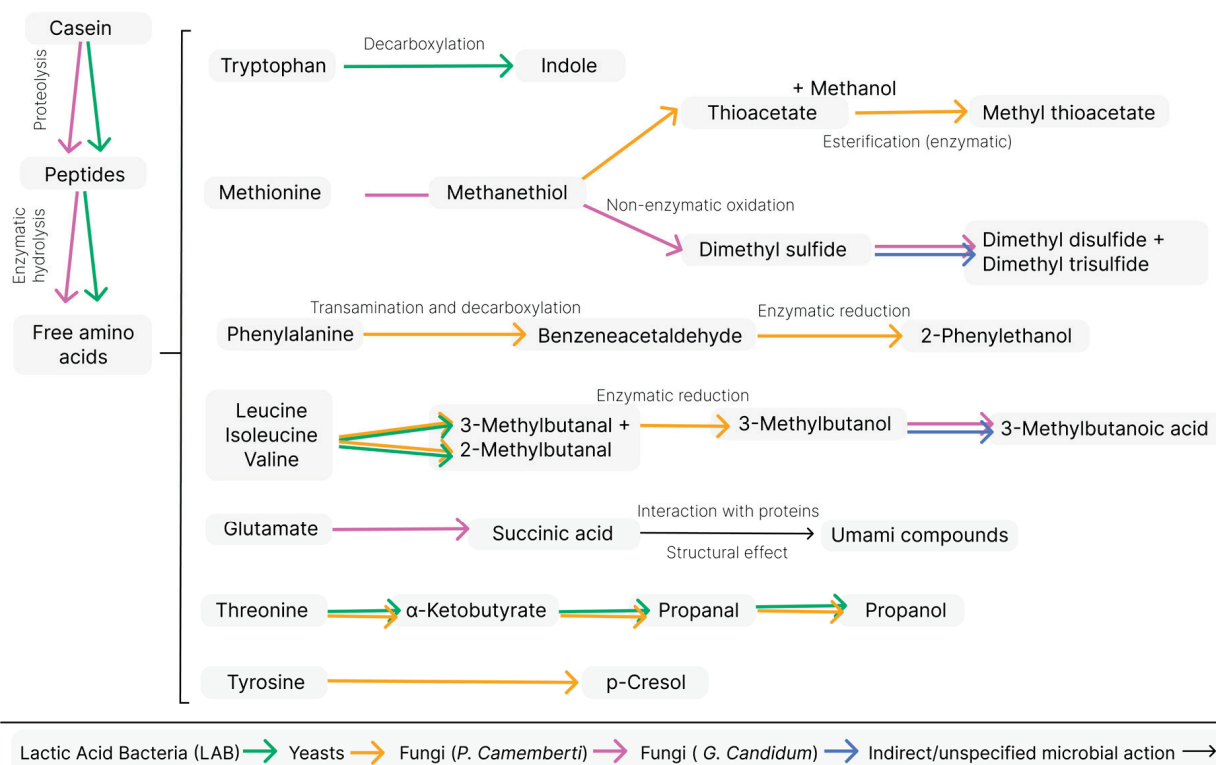


Figure 7. Pathways for the production of aroma and flavor compounds from amino acids. (Adapted from [2,26,36,37]).

Additionally, aroma and branched-chain amino acids contribute to the formation of alcohols and aldehydes responsible for floral, fruity, and malty notes, adding layers of complexity to the cheese's aroma. Table 3 summarizes the main metabolic pathways of amino acids, highlighting the compounds formed and their sensory relevance.

Table 3. Metabolic pathways of amino acids, formed compounds, and their sensory relevance.

Amino Acid Precursor	Conversion Step	Formed Compounds	Sensory Characteristics	Sensory Importance in Camembert	References
Methionine	Deamination and enzymatic conversion via transaminases	Methanethiol, Dimethyl disulfide, Dimethyl trisulfide	Sulfurous, pungent, and ammoniacal notes	Typical rind aroma, onion and sulfur notes	[10,28,33,38]
Cysteine	Similar reactions via sulfur metabolism	Volatile sulfur compounds	Similar to methionine	Complements the sulfurous profile of Camembert	[28,33,38]
Phenylalanine	Transamination and decarboxylation	Phenylethanol, Benzaldehyde	Floral and sweet notes	Contributes to floral/fruity notes	[1,10,33,39]

Table 3. Cont.

Amino Acid Precursor	Conversion Step	Formed Compounds	Sensory Characteristics	Sensory Importance in Camembert	References
Lysine	Decarboxylation by bacterial decarboxylase	Cadaverine	Metallic and pungent aroma at high concentrations	Related to sensory defects in excessive ripening	[1,10,39]
Arginine	Decarboxylation via the arginine deiminase system	Ornithine → Cadaverine	Similar to cadaverine	Can indicate uncontrolled fermentation	[1,10,39]
Glutamine and Asparagine	Enzymatic deamination	Ammonia (NH ₃)	Ammoniacal aroma; regulates pH	Essential for creamy texture and microbiological balance	[1,20,24]

This amino acid metabolism is strongly influenced by ripening conditions, including the temperature and humidity of the ripening chamber, which affect proteolytic activity and the rate of protein breakdown. Additionally, the pH of the cheese surface regulates the activity of key enzymes such as aminopeptidases and decarboxylases. Finally, the composition of the brine and the rind influences moisture retention and enzyme diffusion throughout the cheese matrix, directly impacting aroma and texture development.

Studies indicate that fine-tuning these parameters allows for the customization of the aroma profile, promoting the formation of desirable compounds while minimizing metabolites associated with sensory defects [39,40]. This ability to modulate the ripening process is particularly relevant for the industry, enabling adaptation to regional preferences and the creation of products with specific aroma profiles [29].

3.3. Role of Microbiota in the Ripening of Camembert Cheese

The ripening process of Camembert cheese is a complex phenomenon mediated by the interaction between filamentous fungi, LAB, and yeasts. These microorganisms carry out crucial biochemical transformations that shape the desired sensory characteristics, such as aroma, texture, and flavor. The coordinated action of the microbiota facilitates the breakdown of proteins, lipids, and carbohydrates, leading to the formation of essential volatile and non-volatile compounds that define the final sensory profile [5].

3.3.1. Role of *Penicillium camemberti*

During ripening, *P. camemberti* secretes proteolytic and lipolytic enzymes that play essential roles in the breakdown of macromolecules, releasing peptides and free amino acids. These amino acids are metabolized into secondary volatile compounds, which contribute to specific aroma notes that define Camembert's sensory profile [28].

One of the most notable processes is the metabolism of sulfur-containing amino acids, such as methionine, which leads to the formation of volatile sulfur compounds—methanethiol, dimethyl disulfide, and dimethyl trisulfide—responsible for the pungent, ammoniacal, and sulfurous notes characteristic of Camembert [18].

Additionally, the deamination of various free amino acids, such as glutamine and asparagine, results in the release of ammonia, which contributes to an increase in the pH of the cheese surface. This pH increase is essential for developing the creamy texture typical of Camembert's interior, while also influencing enzymatic activity and the microbial composition of the rind, as illustrated in Figure 8.

3.3.2. Role of Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB), such as *Lactococcus lactis*, *Lactococcus cremoris*, and *Leuconostoc mesenteroides*, play a fundamental role in the early stages of Camembert ripening. These bacteria ferment the lactose present in milk, converting it into lactic acid, which promotes the acidification of the cheese matrix. This acidification creates a hostile environment for undesirable microorganisms, favoring the growth of specific cultures, including ripening fungi such as *Penicillium camemberti* [1,34].

Additionally, LAB play a crucial role in generating aroma precursor compounds, such as diacetyl and acetoin, which originate from citrate metabolism. These compounds are subsequently metabolized by *Penicillium camemberti*, contributing to the characteristic aroma balance of Camembert [39]. LAB also participate in the breakdown of residual lipids, helping to reduce rancid notes by converting aldehydes and FFA into volatile esters and secondary alcohols, such as ethyl acetate and 2-butanol [1,16].

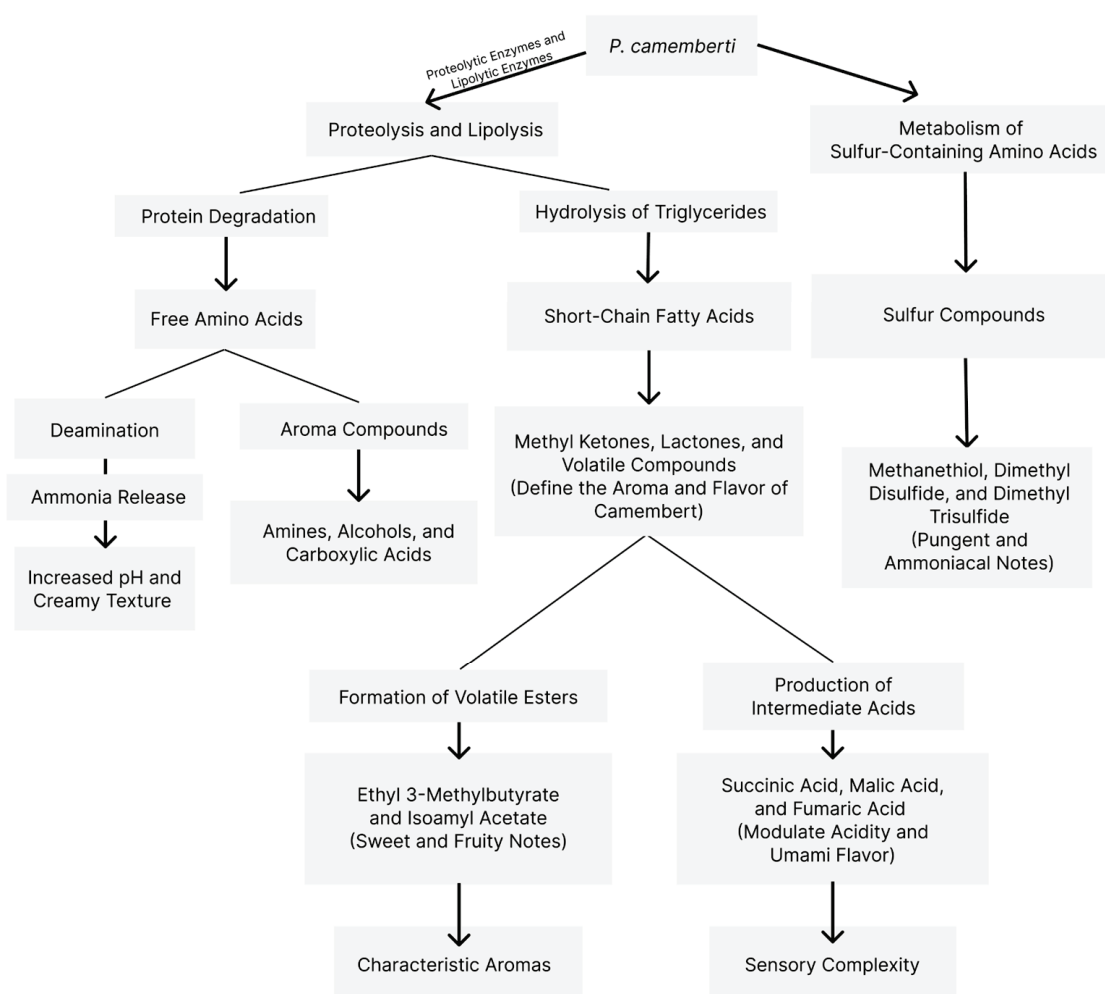


Figure 8. Metabolic pathways for the production of volatile (aroma) and non-volatile (flavor and texture) compounds by *Penicillium camemberti* (Adapted from [10,18,28,38,40–42]).

Another important function of LAB involves the metabolism of FFA, particularly through transamination and decarboxylation, leading to the formation of higher alcohols, aldehydes, and sulfur compounds that directly impact the aroma profile [43]. The main metabolic pathways involving LAB and their interaction with *Penicillium camemberti* can be observed in the Flowchart in Figure 9, which illustrates the synergistic contribution of these microorganisms to the sensory complexity of Camembert.

The symbiotic relationship between LAB and *P. camemberti* is crucial for the development of Camembert cheese. LAB produce lactic acid, creating an initially acidic environment. *P. camemberti* then consumes this acid, releasing ammonia and increasing the pH, which favors the growth of other microbial cultures and promotes uniform ripening [20].

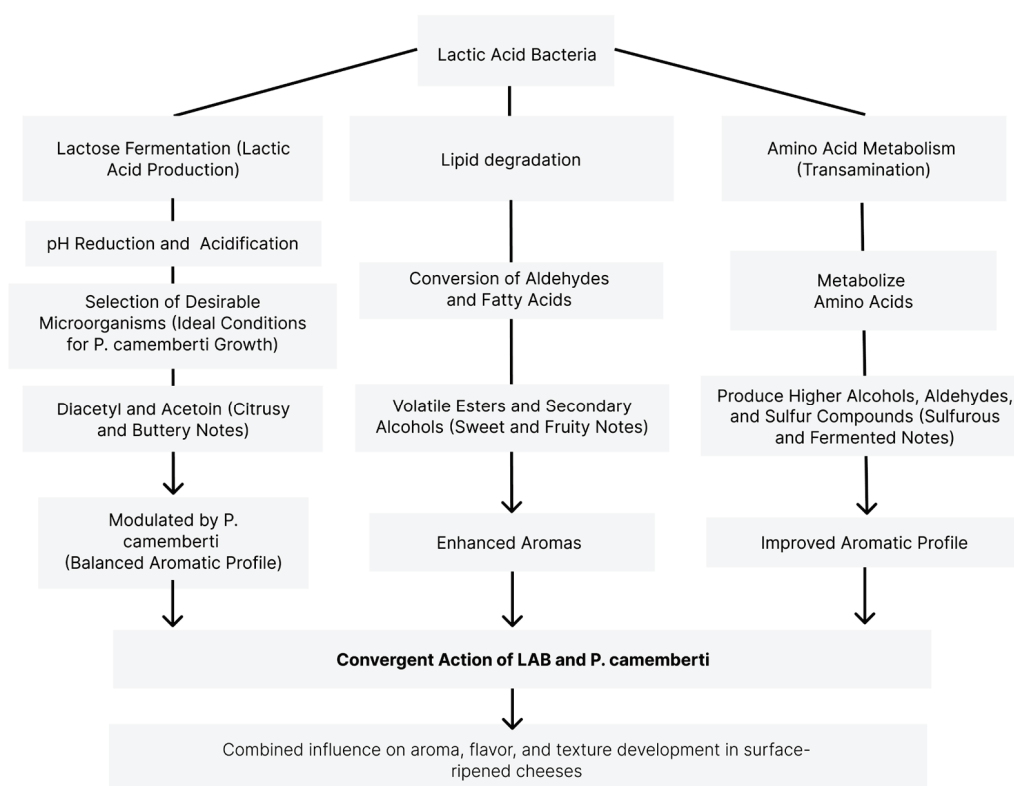


Figure 9. Synergistic pathways of aroma, flavor, and texture development in surface-ripened cheeses involving Lactic acid bacteria and *P. camemberti* (Adapted from [10,32,33,44]).

3.3.3. Role of Yeasts

Although often less emphasized, yeasts play essential roles in the sensory development of Camembert, primarily through the metabolism of residual sugars, lactate, and lipids, resulting in compounds that enhance the aroma and balance the acidity of this cheese [45]. Among the predominant species, *Debaryomyces hansenii* contributes to cheese deacidification and facilitates the growth of filamentous fungi such as *Penicillium camemberti* [33]. Meanwhile, *Yarrowia lipolytica* participates in lipid hydrolysis, producing volatile compounds such as esters and aldehydes, which add fruity and floral notes to the cheese [18].

The synergy between LAB, *Penicillium camemberti*, and yeasts is fundamental to Camembert's sensory profile. This interaction promotes the formation of aroma compounds derived from carbohydrates, such as ethyl lactate, which adds sweetness and complexity to the aroma. Additionally, *Geotrichum candidum* contributes to bitterness reduction and the formation of sulfur compounds, influencing both the aroma and texture of the cheese [46].

3.4. Methods for the Analysis of Volatile Compounds

The identification of volatile compounds in Camembert presents a challenge due to their structural complexity and the concentration variations of each component. The sensitivity of the human olfactory system surpasses that of many instrumental systems, requiring the application of pre-concentration and volatile extraction techniques before analysis [7]. The high water content in cheese can dilute these aroma compounds, making

their detection by CG-MS more difficult [47], while the presence of proteins, lipids, and carbohydrates can directly interfere with compound specificity and analysis [5].

In recent years, advancements in chromatographic and microextraction techniques, such as SPME, have enabled a more detailed characterization of the volatile profile of ripened cheeses [27]. However, analyzing these compounds remains challenging due to the synergistic and antagonistic interactions occurring within the cheese matrix, which complicate the isolated evaluation of each volatile compound's impact on the final perceived aroma [20].

On the other hand, identifying volatile compounds alone is not sufficient to fully understand the aroma of Camembert. Many instrumentally detected compounds have no direct sensory relevance, while others, present at extremely low concentrations, exert a strong olfactory impact [12]. To bridge this gap, techniques that combine instrumental analysis with sensory evaluation are indispensable, such as GC-O, which directly correlates each detected compound with its actual olfactory perception [14].

The sensory characterization of Camembert can also be enhanced using descriptive methodologies, such as quantitative descriptive analysis (QDA), which describes sensory attributes in standardized terms, and consumer preference mapping, which associates different aroma profiles with target consumer accessibility [8,29].

Thus, a comprehensive understanding of Camembert's aroma profile requires an integrated approach that combines analytical techniques for identify both chemically detectable and odor-active volatile compounds, along with sensory methods to validate human perception. Table 4 presents an overview of these approaches, highlighting their specific applications in Camembert analysis, along with their key advantages and limitations.

Table 4. Analytical and sensory techniques for characterizing volatile and odor-active compounds in Camembert cheese.

Technique	Description	Applications in Camembert	Advantages	Limitations	References
Gas Chromatography (GC)	Separation technique for identifying volatile compounds based on their interaction with the stationary and mobile phases.	Initial identification of alcohols, esters, short-chain fatty acids, and sulfur compounds.	High resolution and efficiency in compound separation.	Unable to directly identify odor-active compounds.	[5,7,48,49]
Gas Chromatography–Mass Spectrometry (GC–MS)	Combines GC with mass spectrometry for structural analysis and quantification of volatile compounds.	Detailed characterization of the volatile profile and initial correlation with sensory descriptors.	High sensitivity and precise identification.	Requires sophisticated equipment and longer analysis time.	[5,7,48]
Gas Chromatography–Olfactometry (GC–O)	Technique combining CG–O with sensory evaluation by a trained assessor at the olfactory detector.	Identification of odor-active compounds and their direct sensory contribution.	Allows direct correlation between volatile compounds and their olfactory perception.	Requires a trained panel and may be influenced by subjective perception.	[5,7,49]
Solid-Phase Microextraction (SPME)	Pre-concentration technique using sorbent fibers to capture volatiles before GC or GC–MS analysis.	Enhances the detection of compounds present at low concentrations in the cheese matrix.	Fast, simple, and non-destructive method.	Requires optimization for each matrix and analytical condition.	[7,48,49]
Quantitative Descriptive Analysis (QDA)	Formal sensory evaluation by a trained panel to describe and quantify aroma, flavor, and texture attributes.	Standardized description of Camembert's sensory profile, including its key aroma notes.	Enables mapping of key attributes and sample comparison.	Inherent subjectivity of human sensory analysis.	[8]

Table 4. Cont.

Technique	Description	Applications in Camembert	Advantages	Limitations	References
Consumer Preference Mapping	Consumer testing to assess preferences for different aroma profiles.	Identifies which volatile profiles are most accepted or desired by different consumer groups.	Links chemical and sensory profiles to product accessibility.	Lower technical precision and dependent on the selected consumer sample.	[8,50]

Recent technological advances have driven the development of innovative approaches for analyzing volatile compounds and evaluating aroma in cheeses like Camembert. These new methodologies combine advanced chemical detection techniques, high-precision sensing, and data modeling tools, enabling a more comprehensive and integrated understanding of the cheese's sensory profile [7,20].

Monitoring the volatile compounds of Camembert is essential to ensuring its sensory and commercial quality. Changes in the aroma profile may indicate microbiota imbalances, deviations in the ripening process, or variations in raw materials. Emerging technologies, such as electronic sensors (electronic noses), allow for rapid and non-destructive analysis of volatile compounds during production, enabling early detection of ripening deviations and supporting consistent product quality [18]. This continuous monitoring is also crucial for identifying sensory defects or microbial contaminants, ensuring not only sensory stability but also product safety and consumer satisfaction [5].

Additionally, metabolomics and metagenomics platforms provide a holistic view of the microbiota and its influence on the formation of volatile compounds during ripening [18]. Emerging techniques, such as ion mobility spectrometry (IMS), enable rapid, direct analysis of food matrices, while electronic noses simulate human olfactory perception, offering a direct technological interface for real-time quality control [51,52].

Despite their promising potential, the application of metabolomic and metagenomic tools in cheese aroma research still presents several limitations. One major challenge is the complexity of the cheese matrix, which can interfere with metabolite extraction and quantification, leading to variability in analytical results [27]. Additionally, metagenomic sequencing generates large datasets that require advanced bioinformatics tools and interpretation frameworks, which are not yet standardized for dairy systems [53]. Another limitation is the current difficulty in correlating specific microbial genes or pathways to the actual production of aroma-relevant compounds in situ, due to the influence of environmental and matrix-dependent factors [46]. Therefore, while these tools offer high-resolution insights, their integration into routine industrial or sensory-focused applications remains constrained by cost, data processing demands, and methodological validation requirements.

Table 5 summarizes the main innovative approaches used for characterizing aroma compounds in Camembert, highlighting their principles, applications, and benefits.

Table 5. Innovative approaches for the analysis of aroma compounds.

Innovative Technique	Description	Applications in Camembert	References
Nuclear Magnetic Resonance Spectroscopy (NMR)	Identifies and quantifies volatile and non-volatile metabolites, providing insights into metabolic processes.	Highlights the formation of aroma precursor compounds, such as amino acids and fatty acids.	[7,20]

Table 5. Cont.

Innovative Technique	Description	Applications in Camembert	References
Metabolomics and Metagenomics Platforms	Correlate the cheese microbiota with volatile compounds produced during ripening.	Identify critical metabolic pathways and potential technological adjustments to optimize the sensory profile.	[18,52]
Electronic Sensors (Electronic Nose)	Devices that simulate human olfactory perception, detecting and classifying volatile compounds in real time.	Monitor cheese quality during production and ripening, providing rapid and consistent data.	[7,51]

Although the analysis of volatile compounds in white mold cheeses such as Camembert already involves a wide range of chromatographic, spectrometric, and sensory techniques, certain relevant methodologies remain underexplored for this specific product. One such technique is AEDA, which combines GC–O with successive dilutions of aroma extracts, allowing for the objective ranking of odor-active compounds based on the persistence of their odors throughout the dilutions. This approach, widely used in studies of fermented products and complex foods such as dairy products, has proven highly effective in characterizing key compounds in cheeses of different origins, although its specific application to Camembert remains scarce [54].

Another valuable tool is the determination of the OAV, a parameter that relates the concentration of each volatile compound to its olfactory perception threshold, providing a quantitative assessment of the relative importance of each substance to the overall aroma. This technique has already been used in aroma studies of various dairy and fermented products, including soft cheeses, though its systematic application to Camembert is not yet well documented [55].

Additionally, complementary olfactometric evaluation techniques, such as Osme, which records the perceived odor intensity of each compound during chromatographic elution, and the GC–sniffing port, used as a descriptive sensory screening tool, are well-established methodologies for analyzing complex food matrices. These methods have already been applied in studies involving aged and fermented cheeses [56].

Another innovative technique with potential for future exploration is IMS, an analytical tool capable of performing rapid and sensitive real-time analyses of volatile compositions. Despite its increasing application in fermented foods, there are no well-documented records of its specific use in white mold cheeses, presenting a methodological opportunity for improving volatile monitoring throughout ripening and for developing real-time quality control tools [57].

However, its dedicated application in studies exclusively focused on Camembert and Brie remains limited. This lack of specific research highlights a methodological gap, suggesting that adopting these tools in future studies could significantly enhance our understanding of the relationship between chemical composition and sensory perception in traditional white mold cheeses such as Camembert.

3.5. Effect of Processing Conditions on the Formation of Volatile Compounds and the Aroma Profile of Camembert

Camembert cheese is highly valued for its unique aroma profile, which results from complex interactions between microbiota, biochemical processes, and ripening conditions.

In the industry, optimizing these factors is essential to ensure consistency in quality, meet consumer preferences, and explore new market opportunities [45].

Technological and processing factors, such as the type of salt used during ripening, directly affect the formation of volatile compounds. The use of non-organic salts, such as Himalayan pink salt, has been shown to enhance the levels of carboxylic acids and alcohols, improving sensory notes in aged cheeses [13]. Emerging technologies, such as X-ray irradiation, are also being investigated as a strategy to improve the volatile profile of aged cheeses, offering new perspectives for aroma preservation and enhancement [7].

The ripening of Camembert is a highly sensitive process, influenced by microbiota, environmental conditions, and milk formulation. Precise adjustments in parameters such as temperature, humidity, and oxygenation throughout ripening directly impact aroma development, promoting the proteolytic and lipolytic activity of microorganisms like *Penicillium camemberti*, resulting in a more complex aroma profile [10].

The use of selected cultures of *Penicillium camemberti* and genetically adapted LAB allows for the targeted production of desirable volatile compounds, such as methyl ketones and esters, while reducing undesirable compounds, leading to a more balanced and pleasant sensory profile [33]. Studies have shown that volatile profile modulation can be fine-tuned through microbial culture selection and ripening condition control. For example, species of *Monascus* and auxiliary cultures can increase levels of aroma compounds such as butanediol and 2-undecanone, contributing to distinct sensory profiles with floral and fruity notes [5].

Additionally, the introduction of digital and automated tools, such as automated fermenters and digitally controlled ripening chambers, enables precise process control. These systems can be integrated with electronic sensors and artificial intelligence algorithms, allowing for real-time monitoring and prediction of aroma profile development [20].

Innovations in the Ripening Process

Innovations in the ripening process of Camembert aim not only to optimize sensory quality and industrial reproducibility but also to create strategic opportunities for product innovation and diversification in the global market. The customization of the aroma profile can be used as a tool to develop Camembert varieties with specific sensory appeals, such as cheeses with intensified fruity, floral, or ammoniacal notes, tailored to regional consumer preferences [8].

In this context, the growing appreciation for artisanal and gourmet products strengthens Camembert's positioning as a premium product, especially when associated with limited editions or certification under a protected designation of origin (PDO), reinforcing the link between terroir and specific sensory characteristics [18].

Innovations also include the incorporation of functional or probiotic ingredients during production, addressing the increasing demand for foods that combine flavor with health benefits. Beyond promoting well-being, these products may also exhibit distinctive aroma profiles, thus expanding opportunities for market segmentation [33]. Commonly used probiotic strains in cheese production include *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Bifidobacterium animalis subsp. lactis*, which are associated with benefits such as improved gut health, immune modulation, and anti-inflammatory effects. Functional additions can also include prebiotics like inulin or resistant starch, as well as bioactive peptides derived from milk proteins. These ingredients can influence microbial dynamics during ripening, potentially altering the production of aroma compounds such as esters and short-chain fatty acids, while simultaneously enhancing the nutritional profile of the cheese [58,59].

At the same time, the use of electronic sensors and automated systems has gained traction as a tool for real-time monitoring of the ripening process, enabling proactive correction of deviations and directly contributing to final product consistency [18]. The combination of aroma profile customization and digital tools also facilitates the development of premium and differentiated products, driving innovation in the dairy sector [8].

3.6. Challenges and Opportunities for Research on Camembert Cheese Aroma

Despite advances in understanding the volatile compounds present in Camembert cheese, several scientific gaps and technological challenges still hinder a comprehensive characterization of its aromatic profile. This section outlines the main emerging research areas related to aroma biochemistry, the microbiota involved in ripening, technological modulation factors, and innovative analytical tools. The analysis of these aspects reveals promising avenues for both academic exploration and industrial applications aimed at sensory customization and standardization.

Unexplored Metabolic Pathways: Recent studies using gas chromatography–olfactometry (GC–O) have revealed sensorily relevant peaks that remain chemically uncharacterized or whose biochemical origins are unclear [46]. Volatile sulfur compounds, responsible for pungent and ammoniacal notes, are a classic example of metabolites whose formation may involve alternative pathways that remain underexplored, particularly under specific ripening conditions such as low oxygenation or osmotic stress [45]. Similarly, lactones and nitrogenous heterocyclic compounds, associated with fruity and toasted notes, are also detected, but their complete metabolic pathways are still under investigation [20].

Specific Microbial Interactions: The application of metagenomic and metabolomic tools has provided a new level of understanding regarding the interactions between microorganisms in Camembert and their direct influence on aroma compound formation [55]. For example, studies show that the initial lipolytic activity of *Geotrichum candidum* modifies the availability of substrates for *Penicillium camemberti*, altering the production of methyl ketones and directly impacting the aroma profile [45]. Additionally, lesser-studied species, such as *Debaryomyces hansenii*, play a significant role in detoxifying the ripening environment and producing alcohols and esters, contributing to characteristic fruity and fermented notes [20]. These microbial interactions remain underexplored under industrial conditions, representing a promising field for targeted optimization of the sensory profile.

Effects of Technological Adjustments: Factors such as temperature, humidity, and milk composition directly impact the formation of volatile compounds and, consequently, the sensory profile of Camembert [20]. Lower temperatures hinder lipolytic and proteolytic activity, resulting in a milder and less pungent aroma, while moderate temperatures accelerate the formation of methyl ketones and sulfur compounds, which are characteristic of Camembert's typical aroma [60]. The relative humidity of the ripening chamber regulates rind formation and the balance between yeasts and fungi, favoring milder profiles under high humidity or more intense, aged characteristics under low humidity conditions [20]. Meanwhile, milk composition, including fat and protein content, determines the availability of essential metabolic precursors, directly influencing the formation of key aroma compounds such as esters, lactones, and sulfur derivatives [60].

Innovative Approaches for Chemical-Sensory Correlation: Although Camembert has been extensively analyzed through chromatographic and spectrometric methods, the use of approaches that directly correlate chemical composition with sensory perception remains limited for this specific type of cheese. Techniques such as AEDA, which combines serial dilutions of aroma extracts with sensory evaluation via GC–O, and OAV determination, which prioritizes key compounds based on their relative concentration and perception

thresholds, are widely applied in other fermented products but remain underexplored in white mold cheeses, including Camembert [54].

Additionally, methods such as Osme, which records the perceived intensity of each compound during chromatographic elution, and the systematic application of GC–sniffing port as a sensory screening tool, could provide groundbreaking insights into compounds responsible for Camembert’s distinctive sensory characteristics [56].

4. Conclusions

A critical analysis of the techniques used to characterize volatile compounds and their relationship with sensory perception in Camembert cheese reveals significant challenges for both industry and scientific research. The complexity of the cheese matrix, combined with the diversity and variability of volatile compounds, complicates precise and reproducible aroma characterization.

Integrating traditional chromatographic techniques with sensory evaluation, targeted metabolomics, and artificial intelligence offers promising avenues to address these issues. However, effective application in industrial settings still requires method validation and standardization.

Another major challenge lies in customizing the aroma profile through microbial and process control. Experimental studies have shown that adjusting ripening temperatures (e.g., 10–14 °C), humidity (85–95%), and inoculation rates of fungi such as *Penicillium camemberti* (~10⁶ CFU/g) can significantly influence the formation of key compounds like 2-undecanone and methanethiol. Incorporating such strategies can support the development of customized products aligned with consumer preferences.

Camembert thus emerges as a model system for exploring microbial interactions and process optimization in surface-ripened cheeses. Future research focused on filling current gaps—such as the role of lesser-known microorganisms and non-canonical metabolic routes—may further advance innovation, quality control, and traceability in the dairy sector.

Author Contributions: Conceptualization, B.F.D.R. and M.R.M.J.; methodology, B.F.D.R.; investigation, B.F.D.R.; writing—original draft preparation, B.F.D.R.; writing—review and editing, J.L.B., B.F.D.R. and G.A.M.; supervision, J.L.B. and M.R.M.J. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001; Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (grants 307314/2023-5, 403976/2021-9, 301496/2019-6, and 303033/2021-5); and Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP (grants 2022/09493-9 and 2023/17076-1).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: The authors thank the institutions and funding agencies that supported this work, including CAPES, CNPq, and FAPESP, as well as the laboratories and departments that provided infrastructure and access to scientific resources.

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

Abbreviations

The following abbreviations are used in this manuscript:

GC–MS	Gas Chromatography–Mass Spectrometry
GC–O	Gas Chromatography–Olfactometry
SPME	Solid-Phase Microextraction
GC	Gas Chromatography
AEDA	Aroma Extract Dilution Analysis
OAV	Odor Activity Value
LAB	Lactic Acid Bacteria
FFA	Free Fatty Acids
QDA	Quantitative Descriptive Analysis
IMS	Ion Mobility Spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy

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ISBN 978-3-7258-8848-1