

Special Issue Reprint

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# Anticancer Activities of Dietary Phytochemicals

2nd Edition

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Edited by  
Ching-Hsein Chen and Yi-Wen Liu

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# **Anticancer Activities of Dietary Phytochemicals: 2nd Edition**



# Anticancer Activities of Dietary Phytochemicals: 2nd Edition

Guest Editors

**Ching-Hsein Chen**

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# Contents

|                                                                                                                                                                          |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| About the Editors . . . . .                                                                                                                                              | vii |
| Preface . . . . .                                                                                                                                                        | ix  |
| <b>Ching-Hsein Chen and Yi-Wen Liu</b>                                                                                                                                   |     |
| Dietary Phytochemicals in Cancer Therapy—From Mechanistic Insights to Combination Strategies                                                                             |     |
| Reprinted from: <i>Nutrients</i> <b>2026</b> , <i>18</i> , 1524, <a href="https://doi.org/10.3390/nu18101524">https://doi.org/10.3390/nu18101524</a> . . . . .           | 1   |
| <b>Yu-Hsiang Liao, Kai-Chien Yang, Heng-Jing Chen, Ching-Wei Shih, Yi-Cheng Yeh, Jyun-Yu Peng, et al.</b>                                                                |     |
| Combined Curcumin and Doxorubicin Induce Apoptosis via JNK-Dependent MAPK Signaling Independent of TXNDC5 in Human Osteosarcoma Cells                                    |     |
| Reprinted from: <i>Nutrients</i> <b>2026</b> , <i>18</i> , 1235, <a href="https://doi.org/10.3390/nu18081235">https://doi.org/10.3390/nu18081235</a> . . . . .           | 6   |
| <b>Irma Airoidi, Lucrezia Canè, Chiara Brignole, Eleonora Ciampi, Daniela Montagna and Fabio Morandi</b>                                                                 |     |
| Olive Leaf Extract (OLE) Anti-Tumor Activities Against Hematologic Tumors: Potential Therapeutic Implications for Pediatric Patients with B-Acute Lymphoblastic Leukemia |     |
| Reprinted from: <i>Nutrients</i> <b>2026</b> , <i>18</i> , 15, <a href="https://doi.org/10.3390/nu18010015">https://doi.org/10.3390/nu18010015</a> . . . . .             | 27  |
| <b>Aly A. M. Shaalan, Ekramy M. Elmorsy, Eman M. Embaby, M. Alfawaz, Nagwa M. Aly, Ahmed S. Shams, et al.</b>                                                            |     |
| Antitumor, Antioxidant, and Hepatoprotective Effects of Grape Seed Oil Nanoemulsion as a Dietary Phytochemical Intervention in Ehrlich Solid Tumors                      |     |
| Reprinted from: <i>Nutrients</i> <b>2025</b> , <i>17</i> , 3450, <a href="https://doi.org/10.3390/nu17213450">https://doi.org/10.3390/nu17213450</a> . . . . .           | 48  |
| <b>Dongge Yin, Hongyue Chen, Xiaohong Jing, Shuting Lin, Yufei Sun, Rongrong Chang, et al.</b>                                                                           |     |
| Cucurbitacin B from Cucurbitaceae Plants: Treating Pancreatic Cancer via Inducing Mitophagy, Inhibiting Glycolysis, and Enhancing Immune Function                        |     |
| Reprinted from: <i>Nutrients</i> <b>2025</b> , <i>17</i> , 2809, <a href="https://doi.org/10.3390/nu17172809">https://doi.org/10.3390/nu17172809</a> . . . . .           | 76  |
| <b>Sascha D. Markowitsch, Thao Pham, Jochen Rutz, Felix K.-H. Chun, Axel Haferkamp, Igor Tsaur, et al.</b>                                                               |     |
| Growth of Renal Cancer Cell Lines Is Strongly Inhibited by Synergistic Activity of Low-Dosed Amygdalin and Sulforaphane                                                  |     |
| Reprinted from: <i>Nutrients</i> <b>2024</b> , <i>16</i> , 3750, <a href="https://doi.org/10.3390/nu16213750">https://doi.org/10.3390/nu16213750</a> . . . . .           | 99  |
| <b>Chun-Yik Lew, Yi-Teng Tang, Amanda Yee-Jing Lee, Zhi-Jian Chin, Wan-Ling Chang, Ching-Hsein Chen and Soi-Moi Chye</b>                                                 |     |
| Shikonin as a Dietary Phytochemical with Multi-Target Anti-Cancer Activities: From Molecular Mechanisms to Translational Applications                                    |     |
| Reprinted from: <i>Nutrients</i> <b>2025</b> , <i>17</i> , 3085, <a href="https://doi.org/10.3390/nu17193085">https://doi.org/10.3390/nu17193085</a> . . . . .           | 115 |
| <b>See-Hyoung Park</b>                                                                                                                                                   |     |
| Recent Research on the Role of Phytochemicals from Ginseng in Management of Osteosarcoma, Osteoporosis, and Osteoarthritis                                               |     |
| Reprinted from: <i>Nutrients</i> <b>2025</b> , <i>17</i> , 1910, <a href="https://doi.org/10.3390/nu17111910">https://doi.org/10.3390/nu17111910</a> . . . . .           | 152 |
| <b>Gisella Campanelli and Anait S. Levenson</b>                                                                                                                          |     |
| Gnetin C in Cancer and Other Diseases: What Do We Know So Far?                                                                                                           |     |
| Reprinted from: <i>Nutrients</i> <b>2025</b> , <i>17</i> , 863, <a href="https://doi.org/10.3390/nu17050863">https://doi.org/10.3390/nu17050863</a> . . . . .            | 181 |



# About the Editors

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Ching-Hsein Chen is a professor in the Department of Microbiology, Immunology and Biopharmaceuticals at National Chiayi University, Taiwan. His research interests focus on cancer biology, molecular signaling pathways, oxidative stress, apoptosis, autophagy, and the anticancer activities of natural compounds and combination therapies. His recent studies have particularly emphasized the roles of MAPK signaling, PI3K/Akt pathways, NF- $\kappa$ B, and reactive oxygen species in colorectal cancer and osteosarcoma models. He has published numerous peer-reviewed articles related to dietary phytochemicals and anticancer mechanisms and currently serves as a Guest Editor and reviewer for several international scientific journals.

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# Preface

Cancer remains one of the leading causes of morbidity and mortality worldwide, and the development of effective and safer therapeutic strategies continues to be an important challenge in biomedical research. In recent years, dietary phytochemicals have attracted increasing attention because of their diverse biological activities, including antioxidant, anti-inflammatory, antiproliferative, and anticancer effects. Furthermore, many natural compounds have shown promising potential in combination with conventional chemotherapeutic agents, thereby improving therapeutic efficacy and reducing adverse effects. This Reprint, entitled “Anticancer Activities of Dietary Phytochemicals: 2nd Edition”, requests recent studies and review articles focusing on the molecular mechanisms and therapeutic applications of dietary phytochemicals in cancer prevention and treatment. The topics covered include apoptosis, oxidative stress, signaling pathways, metastasis, and combination therapies in various cancer models. We hope that this Reprint will provide readers with updated knowledge and valuable insights into the rapidly evolving field of phytochemical-based anticancer research and stimulate further investigations in this area.

**Ching-Hsein Chen and Yi-Wen Liu**

*Guest Editors*



*Editorial*

# Dietary Phytochemicals in Cancer Therapy—From Mechanistic Insights to Combination Strategies

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## 1. Introduction

Dietary phytochemicals have attracted increasing attention as promising anticancer agents due to their multi-target biological activities and relatively low toxicity [1,2]. Unlike conventional therapies that often focus on single molecular targets, phytochemicals exert pleiotropic effects by modulating interconnected signaling pathways involved in cancer cell survival, proliferation, metabolism, and the tumor microenvironment [3].

This Special Issue, *Anticancer Activities of Dietary Phytochemicals: 2nd Edition*, gathers eight contributions, including five original research articles and three review articles, that collectively provide new insights into the mechanisms and therapeutic potential of dietary phytochemicals. A central theme emerging from these studies is the critical role of reactive oxygen species (ROS)-mediated stress responses and mitogen-activated protein kinase (MAPK) signaling pathways in mediating the anticancer effects of dietary phytochemicals. This Editorial provides an integrative overview of the key findings and emerging mechanistic themes highlighted in this Special Issue.

## 2. Overview of This Special Issue

The contributions included in this Special Issue highlight the diverse anticancer activities of phytochemicals across multiple cancer types and experimental models. Several studies emphasize the importance of combination strategies in enhancing the therapeutic efficacy of dietary phytochemicals. For instance, the combination of amygdalin and sulforaphane strongly inhibits renal cancer cell proliferation through coordinated modulation of signaling pathways (contribution 1). Similarly, curcumin significantly increases doxorubicin-induced apoptosis compared with that of doxorubicin alone via JNK-dependent MAPK signaling in osteosarcoma cells (contribution 2). In hematologic malignancies, olive leaf extract potentiates the anticancer effects of cytarabine, suggesting its role as a chemosensitizer (contribution 4).

Cucurbitacin B provides insight into the multi-layered mechanisms involving metabolic reprogramming and immune modulation (contribution 3), and the nanoemulsion-based delivery of grape seed oil highlights the importance of increasing bioavailability and therapeutic efficacy (contribution 5). Review articles focusing on shikonin, Gnetin C, and ginseng-derived compounds further expand the mechanistic and translational perspectives on the use of phytochemicals in cancer therapy (contributions 6–8).

## 3. ROS and MAPK Signaling as Central Mechanistic Themes

The studies included in this Special Issue collectively highlight the central role of ROS-mediated stress responses in phytochemical-induced anticancer activity, which are



key mechanisms in cancer therapy [4]. Several contributions demonstrate that increased ROS levels act as an upstream trigger for activating stress-responsive signaling pathways, particularly MAPK cascades, thereby promoting apoptotic responses (contributions 2, 3, and 6).

In this context, MAPK signaling emerges as a key convergence node linking oxidative stress to downstream cellular responses. The activation of the JNK and p38 pathways has been consistently associated with apoptosis induction. The modulation of ERK signaling exhibits context-dependent roles in regulating cell survival and death (contributions 1 and 2). These findings underscore a shared mechanistic framework across phytochemicals and cancer models.

The cross-talk between MAPK pathways and survival signaling networks further refines our understanding of the cellular responses to cancer treatment. Inhibiting pro-survival pathways, such as the PI3K/Akt pathway, enhances the pro-apoptotic effects of MAPK activation, thereby contributing to the overall therapeutic outcome (contribution 1).

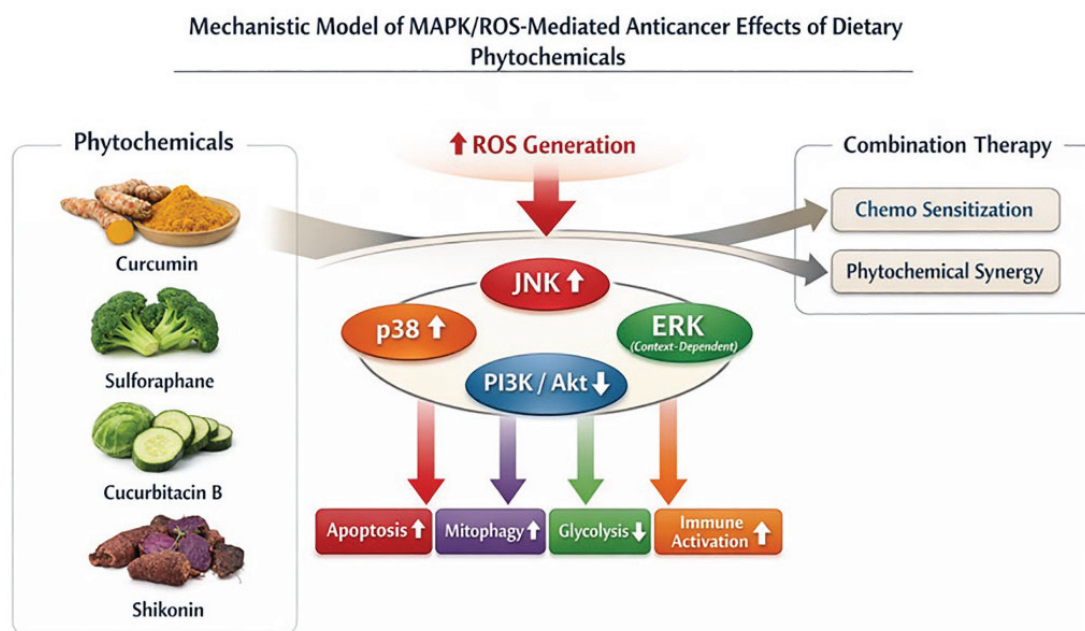
#### 4. Combination Strategies as a Core Therapeutic Concept

A key insight from this Special Issue is that phytochemicals exert enhanced anticancer effects when applied in combination strategies than when applied alone, an approach that is a promising direction in cancer therapy [5]. These combinations may involve phytochemical–chemotherapy interactions or phytochemical–phytochemical synergy, both of which contribute to increased therapeutic efficacy.

Phytochemical–chemotherapy combinations improve treatment outcomes by amplifying oxidative stress and modulating stress-responsive signaling pathways. For example, curcumin enhances doxorubicin-induced apoptosis through JNK activation, and olive leaf extract increases the efficacy of cytarabine (contributions 2 and 4). These findings suggest that phytochemicals function as sensitizers that lower the apoptotic threshold and enhance drug responsiveness.

Phytochemical–phytochemical combinations similarly enable the coordinated targeting of complementary signaling pathways. The combination of amygdalin and sulforaphane illustrates how the modulation of two molecular pathways can result in synergistic anticancer effects (contribution 1). Such strategies reduce the likelihood of compensatory signaling and provide broader-spectrum activity against tumor cells.

Taken together, these findings support a unified mechanistic model in which combination therapy converges on the ROS-mediated activation of MAPK pathways and the simultaneous suppression of survival signaling, as illustrated in Figure 1, which summarizes the integrated signaling framework underlying these mechanisms. This dual regulation promotes a coordinated shift toward cancer cell death and enhances therapeutic efficacy.



**Figure 1.** Proposed mechanistic model illustrating anticancer effects of dietary phytochemicals through ROS-mediated MAPK signaling pathways. Phytochemicals induce ROS generation, activating MAPK pathways (JNK, p38, ERK) and suppressing PI3K/Akt signaling. These effects lead to apoptosis, mitophagy, glycolysis, and immune activation, which are further enhanced under combination therapy conditions. This model highlights the integrated and multi-target nature of phytochemical-mediated anticancer mechanisms.

## 5. Tumor Metabolism and Microenvironment

In addition to their direct cytotoxic effects, several contributions highlight the role of phytochemicals in regulating tumor metabolism and the tumor microenvironment. Metabolic reprogramming, particularly enhanced glycolysis, is a hallmark of cancer that supports rapid cell proliferation and survival. The evidence is increasingly indicating that phytochemicals regulate metabolic processes [6].

Cucurbitacin B provides a representative example of how phytochemicals can disrupt metabolic homeostasis by inhibiting glycolysis and inducing mitophagy, thereby impairing tumor energy metabolism (contribution 3). In addition, the modulation of metabolic pathways by cucurbitacin B is closely linked to immune regulation, as alterations in lactate production and redox balance can influence immune cell function and tumor progression.

These findings suggest that phytochemicals act as not only direct anticancer agents but also regulators of the tumor microenvironment, contributing to a more comprehensive therapeutic effect.

## 6. Translational Perspectives

Despite the promising anticancer activities of phytochemicals, the clinical application of phytochemicals remains limited by challenges related to bioavailability and stability. Several contributions highlight the importance of formulation strategies in overcoming these limitations.

Nanoformulation approaches, such as nanoemulsion-based delivery systems, can enhance the bioavailability and therapeutic efficacy of phytochemicals (contribution 5). In addition, the structural optimization of compounds, such as Gnetin C and shikonin, further supports the translational potential of phytochemicals (contributions 6 and 7).

These advances underscore the importance of integrating mechanistic insights with delivery technologies to facilitate the clinical translation of phytochemicals. Despite these

advances, challenges related to bioavailability and pharmacokinetics remain major barriers to the clinical application of phytochemicals [7].

## 7. Future Perspectives

The findings presented in this Special Issue highlight several important directions for future research. First, further studies are needed to validate the anticancer efficacy of phytochemicals in clinical settings. Second, a deeper understanding of the molecular mechanisms underlying phytochemical activity will be essential for optimizing their therapeutic application. In particular, the integration of phytochemicals with emerging therapeutic strategies, such as immunotherapy and targeted therapy, represents a promising avenue for enhancing treatment outcomes. Advances in delivery systems, combined with mechanism-based approaches, may further enable the clinical applicability of these compounds. These advances will be critical for translating phytochemical-based strategies into clinically applicable cancer therapies.

## 8. Conclusions

The studies included in this Special Issue collectively demonstrate that dietary phytochemicals are highly promising multi-target anticancer agents capable of modulating complex signaling networks. A central theme emerging from these contributions is the critical role of ROS-mediated stress responses and MAPK signaling pathways in regulating cancer cell fate.

Phytochemicals exert their strongest therapeutic effects when applied in combination strategies that simultaneously enhance pro-apoptotic signaling and suppress survival pathways. In addition, the ability of phytochemicals to modulate tumor metabolism and the tumor microenvironment further expands their functional role in cancer therapy.

These findings together support a conceptual framework in which dietary phytochemicals act as integrative modulators of cancer-related pathways. This integrated approach may ultimately bridge the gap between experimental findings and clinical application, thereby accelerating the translation of dietary phytochemicals into evidence-based cancer therapies. The major findings and mechanisms of the studies included in this Special Issue are summarized in Table 1.

**Table 1.** Summary of studies included in this Special Issue.

| Contribution | Compound/Strategy      | Cancer Type       | Mechanisms                            | Key Feature           |
|--------------|------------------------|-------------------|---------------------------------------|-----------------------|
| 1            | Amygdalin + SFN        | Renal cancer      | Akt downregulation; cell cycle arrest | Synergy               |
| 2            | Curcumin + Doxorubicin | Osteosarcoma      | JNK activation; apoptosis             | Chemosensitization    |
| 3            | Cucurbitacin B         | Pancreatic cancer | Mitophagy; glycolysis inhibition      | Multiple targets      |
| 4            | Olive leaf extract     | Leukemia          | Apoptosis; signaling                  | Drug synergy          |
| 5            | Grape seed oil (nano)  | Solid tumor       | ROS modulation                        | Delivery enhancement  |
| 6            | Shikonin               | Multiple          | ROS; cell death pathways              | Multiple pathways     |
| 7            | Gnetin C               | Prostate          | Anti-inflammatory                     | Bioavailability issue |
| 8            | Ginseng compounds      | Bone cancers      | Multiple targets                      | Broad effects         |

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

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1. Markowitsch, S.D.; Pham, T.; Rutz, J.; Chun, F.K.-H.; Haferkamp, A.; Tsaur, I.; Juengel, E.; Ries, N.; Thomas, A.; Blaheta, R.A. Growth of renal cancer cell lines is strongly inhibited by synergistic activity of low-dosed amygdalin and sulforaphane. *Nutrients* **2024**, *16*, 3750. <https://doi.org/10.3390/nu16213750>.
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## Article

# Combined Curcumin and Doxorubicin Induce Apoptosis via JNK-Dependent MAPK Signaling Independent of TXNDC5 in Human Osteosarcoma Cells

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## Abstract

**Background:** Curcumin, a dietary polyphenol with anticancer potential, has been reported to enhance the efficacy of chemotherapeutic agents. **Methods:** The effects of combined curcumin and doxorubicin treatment on apoptosis and associated signaling pathways were investigated in human osteosarcoma HOS cells. **Results:** Combined treatment significantly reduced cell viability and induced apoptotic morphological changes, which were confirmed by increased sub-G1 population, enhanced DNA fragmentation, and elevated cleaved poly(ADP-ribose) polymerase (PARP) levels. Mechanistically, combined treatment markedly increased c-Jun N-terminal kinase (JNK) phosphorylation, whereas extracellular signal-regulated kinase (ERK) phosphorylation showed no appreciable change. Pharmacological inhibition revealed that JNK suppression attenuated PARP cleavage, while ERK inhibition also reduced apoptotic responses, suggesting a permissive role of basal ERK activity. In addition, combined treatment was associated with increased expression of the endoplasmic reticulum stress marker GRP78 and modulation of autophagy-associated markers. Although thioredoxin domain-containing protein 5 (TXNDC5) expression was reduced, TXNDC5 overexpression failed to attenuate apoptosis, indicating that apoptosis induction occurs independently of TXNDC5. **Conclusions:** These findings indicate that combined curcumin and doxorubicin induce apoptosis primarily through JNK-dependent MAPK signaling, accompanied by stress-associated cellular responses.

**Keywords:** curcumin; doxorubicin; MAPK; TXNDC5; apoptosis; osteosarcoma



## 1. Introduction

Osteosarcoma is an aggressive primary bone malignancy for which doxorubicin remains a cornerstone chemotherapeutic agent in both neoadjuvant and adjuvant treatment protocols. Despite its clinical utility, the effectiveness of doxorubicin is frequently compromised by intrinsic or acquired resistance mechanisms in osteosarcoma cells [1–4]. One well-recognized mechanism involves the overexpression of multidrug transporters such as P-glycoprotein, which actively export chemotherapeutic agents and reduce intracellular drug accumulation [5]. Resistance to platinum-based agents has also been associated with impaired drug uptake, enhanced DNA repair mechanisms, and elevated intracellular antioxidant defenses, including glutathione-mediated detoxification of cytotoxic stress [6,7]. In addition to therapeutic resistance, doxorubicin-induced cardiotoxicity represents a major dose-limiting adverse effect and may lead to progressive cardiomyopathy and heart failure [8]. Over the past three decades, standard combination regimens incorporating methotrexate, doxorubicin, and cisplatin have produced limited improvement in long-term survival, particularly in patients with metastatic disease or poor chemotherapeutic response [9]. These limitations highlight the urgent need for novel strategies that enhance therapeutic efficacy while minimizing systemic toxicity.

Growing interest has focused on dietary phytochemicals and natural compounds as adjuvant agents capable of potentiating chemotherapy while reducing adverse effects [10]. Curcumin, a polyphenolic compound derived from *Curcuma longa*, exhibits diverse biological activities including anti-inflammatory, antioxidant, immunomodulatory, and anticancer properties [11]. Extensive evidence indicates that curcumin exerts antitumor activity through modulation of multiple molecular targets involved in tumor initiation and progression [12,13]. Moreover, curcumin suppresses oncogenic signaling networks while enhancing pro-apoptotic pathways, thereby promoting programmed cell death in cancer cells [14,15]. Owing to its pleiotropic actions and favorable safety profile, curcumin has emerged as a promising candidate for combination therapy aimed at overcoming chemoresistance and improving therapeutic outcomes.

Mitogen-activated protein kinase (MAPK) signaling pathways regulate fundamental cellular processes including proliferation, differentiation, stress responses, and apoptosis [16]. The MAPK family consists primarily of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), each responding to distinct extracellular and intracellular stimuli [17]. Activation of ERK, p38, and JNK has been implicated in apoptosis induction under specific conditions, particularly in response to oxidative stress and chemotherapeutic exposure [18]. Because MAPK pathways integrate stress signals and influence cell fate decisions, they represent attractive therapeutic targets in cancer treatment. Modulation of MAPK signaling has therefore been proposed as a strategy to enhance anticancer efficacy and overcome therapeutic resistance [19,20]. Nevertheless, the relative contribution of individual MAPK branches to chemotherapy-induced apoptosis remains context-dependent and requires further clarification in osteosarcoma.

Thioredoxin domain-containing protein 5 (TXNDC5) has recently attracted attention as an oncogenic factor due to its elevated expression in multiple malignancies and its involvement in tumor progression [21]. TXNDC5 contributes to cancer cell survival by regulating redox homeostasis and cellular stress adaptation. Under hypoxic conditions, TXNDC5 expression is upregulated via hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), reducing reactive oxygen species-induced endoplasmic reticulum (ER) stress and supporting cancer cell survival [22,23]. Beyond stress adaptation, TXNDC5 has been implicated in cell cycle regulation; its overexpression increases the proportion of cells in the G2/M phase, whereas its suppression results in G0/G1 arrest [21,24]. Functionally, TXNDC5 acts as a protein disulfide isomerase that facilitates protein folding within the ER and protects cells from

ER stress-induced apoptosis [22,25]. Elevated TXNDC5 expression has been associated with poor prognosis in several cancers and may serve as a prognostic biomarker [21,22,26]. Inhibition of TXNDC5 has been shown to suppress malignant phenotypes and enhance susceptibility to apoptosis, supporting its potential as a therapeutic target [21–23,26]. Our previous work further demonstrated that TXNDC5 suppression enhances chemosensitivity in cancer cells [23].

Given the limitations of doxorubicin therapy and the multifaceted anticancer properties of curcumin, combining these agents represents a rational strategy for osteosarcoma treatment. Curcumin has been shown to sensitize cancer cells to chemotherapeutic agents by modulating pathways associated with survival, oxidative stress, apoptosis, and drug resistance, thereby enhancing anticancer efficacy and potentially allowing dose reduction of cytotoxic drugs [12,27–29]. Importantly, curcumin has also been reported to mitigate chemotherapy-induced toxicity in normal tissues, improving the therapeutic index of conventional anticancer therapies [29–32]. However, the molecular mechanisms underlying the combined effects of curcumin and doxorubicin in osteosarcoma cells remain incompletely defined. Therefore, the present study investigated the effects of combined curcumin and doxorubicin treatment on apoptosis in human osteosarcoma cells and examined the associated signaling pathways, with particular emphasis on MAPK-dependent mechanisms.

## 2. Materials and Methods

### 2.1. Reagents and Chemicals

Minimum Essential Medium (MEM) was obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS), penicillin–streptomycin, sodium pyruvate, and MEM non-essential amino acids (NEAA) were also purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Curcumin (from *Curcuma longa* (turmeric), powder form; CAS No. 458-37-7; Sigma-Aldrich, St. Louis, MO, USA) was used in this study. Doxorubicin hydrochloride was acquired from Sigma-Aldrich (St. Louis, MO, USA). Propidium iodide (PI), RNase A, paraformaldehyde (PFA), trypan blue, and routine laboratory reagents were obtained from Sigma-Aldrich. The Cell Counting Kit-8 (CCK-8) assay kit was purchased from Dojindo Laboratories (Kumamoto, Japan), and the Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). X-tremeGENE™ HP DNA transfection reagent was purchased from Roche (Raleigh, NC, USA). MAPK inhibitors PD98059, SB203580, and SP600125 were obtained from Sigma-Aldrich. The TXNDC5 overexpression plasmid and corresponding control vector were obtained from Addgene (Watertown, MA, USA). All other chemicals were of analytical grade. Primary antibodies against cleaved PARP, phosphorylated and total ERK, p38 MAPK, and JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TXNDC5 antibody was purchased from Abcam (Waltham, MA, USA). All primary antibodies were used at dilutions of 1:1000 unless otherwise specified. Horseradish peroxidase–conjugated secondary antibodies were used at a dilution of 1:10,000.

### 2.2. Cell Culture

Human osteosarcoma HOS and MG-63 cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin G, 100 µg/mL streptomycin, 250 ng/mL amphotericin B, 1 mM sodium pyruvate, and MEM non-essential amino acids (NEAA). Cells were cultured at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. Upon reaching approximately 80% confluence, cells were rinsed with phosphate-buffered saline (PBS) and detached using trypsin–EDTA. Enzymatic activity was neutralized with complete medium, and cells were collected by centrifugation at 1000 rpm for 3 min. Cell pellets were resuspended in fresh

medium, and viable cells were quantified using trypan blue exclusion and a hemocytometer before seeding at the desired density.

### 2.3. Cell Viability Assay

Cell viability was determined using the CCK-8 assay according to the manufacturer's protocol. HOS and MG-63 cells were seeded at  $4 \times 10^4$  cells per well in 24-well plates and allowed to attach for at least 16 h prior to treatment. Cells were treated with curcumin, doxorubicin, or their combination. For curcumin-only treatment, concentrations ranged from 5–20  $\mu\text{M}$ ; for doxorubicin alone, concentrations ranged from 25–500 nM. For combination treatment, curcumin (7.5–9  $\mu\text{M}$ ) was combined with doxorubicin (0.2  $\mu\text{M}$ ). The selected concentration range of curcumin was based on preliminary dose–response experiments to identify sub-cytotoxic conditions (20–40% reduction in cell viability) suitable for evaluating combination effects. Doxorubicin was used as an internal reference control due to its well-established cytotoxic effects in osteosarcoma cells. After 48 h, 20  $\mu\text{L}$  CCK-8 reagent was added to each well and incubated for 1.5 h at 37 °C. Aliquots (200  $\mu\text{L}$ ) were transferred to a 96-well plate, and absorbance was measured at 450 nm. Cell viability was expressed relative to untreated controls.

### 2.4. Flow Cytometry Analysis of Sub-G1 Population

Flow cytometric analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). Data acquisition was conducted under standard hydrodynamic focusing conditions, and at least 10,000 events were collected per sample. Cell cycle distribution and the sub-G1 population were analyzed by flow cytometry following propidium iodide (PI) staining, which was used as a single-color parameter to determine DNA content. After drug treatment, cells were washed with phosphate-buffered saline (PBS), harvested, and centrifuged at 1300 rpm for 5 min. The supernatant was removed, and the cell pellet was resuspended in 1 mL of PBS. Cells were then transferred to 1.5 mL microcentrifuge tubes. RNase A (4  $\mu\text{L}$ ) and propidium iodide (PI; 20  $\mu\text{L}$ ) were added to each sample, and the mixtures were incubated for 30 min at room temperature in the dark. All samples were analyzed within 3 h using a flow cytometer to determine cell cycle distribution. The sub-G1 population, defined as a population with DNA content lower than that of the G1 peak, appears to the left of the G1 phase in the DNA content histogram. This population arises from apoptotic cells undergoing DNA fragmentation, resulting in reduced PI incorporation and lower fluorescence intensity. Therefore, the percentage of cells in the sub-G1 fraction was quantified as an indicator of apoptotic cell death.

### 2.5. TUNEL Assay

For detection of apoptotic DNA fragmentation, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the APO-BrdU<sup>TM</sup> TUNEL assay kit with Alexa Fluor<sup>TM</sup> 488 anti-BrdU (Thermo Fisher Scientific; Cat. No. A23210) according to the manufacturer's instructions. Following labeling, cells were subjected to dual-color staining with Alexa Fluor<sup>TM</sup> 488–conjugated anti-BrdU and propidium iodide (PI). Alexa Fluor<sup>TM</sup> 488 fluorescence was used to detect TUNEL-positive cells, while PI staining was used to determine total DNA content. Dual-color flow cytometric analysis was performed to distinguish apoptotic (TUNEL-positive) cells from non-apoptotic populations. Data acquisition and analysis were conducted using appropriate flow cytometry software. A minimum of 10,000 events per sample were collected.

After drug treatment, cells were collected and washed twice with phosphate-buffered saline (PBS) and transferred to 15 mL centrifuge tubes. Cells were then detached by incubation with trypsin at 37 °C in a humidified incubator containing 5% CO<sub>2</sub> for approximately 3 min. Detached cells were collected, washed with PBS, combined into the same tube,



and centrifuged at 1300 rpm for 5 min. The supernatant was carefully removed. Cells were fixed with 5 mL of 1% paraformaldehyde (PFA) at room temperature for 15 min, followed by two washes with PBS to remove residual fixative. Cells were then resuspended in 5 mL of ice-cold 70% ethanol and stored at  $-20^{\circ}\text{C}$  for at least 16–18 h prior to analysis. After fixation and permeabilization, cells were centrifuged at 1300 rpm for 5 min, and the supernatant was removed. Cells were resuspended in 1 mL of wash buffer and centrifuged again at 1300 rpm for 5 min. This washing step was repeated twice to ensure complete removal of residual medium and debris. Subsequently, 50  $\mu\text{L}$  of DNA labeling solution was added to each sample, and cells were incubated at  $37^{\circ}\text{C}$  for 60 min in a hybridization incubator. During incubation, samples were gently mixed every 15 min to ensure uniform reagent distribution. Following labeling, cells were washed twice with 1 mL of rinse buffer (1300 rpm, 5 min), with complete removal of the supernatant after each wash. Cells were then resuspended in 0.1 mL of antibody solution and incubated at room temperature for 30 min in the dark. Subsequently, 0.9 mL of PI/RNase A solution was added, and samples were further incubated at room temperature for 30 min in the dark. All samples were analyzed by flow cytometry within 3 h of completion of staining. The percentage of TUNEL-positive cells was quantified as an indicator of apoptotic cell death.

## 2.6. Western Blot Analysis

Protein expression levels of cleaved poly(ADP-ribose) polymerase (PARP), mitogen-activated protein kinase (MAPK) signaling proteins, and thioredoxin domain-containing protein 5 (TXNDC5) were analyzed by Western blotting. Protein samples were prepared from whole-cell lysates. After treatment, cells were washed with phosphate-buffered saline (PBS) and lysed in protein extraction buffer on ice for 10 min. The lysates were then centrifuged at  $12,000\times g$  for 10 min at  $4^{\circ}\text{C}$ , and the supernatants were collected as total cellular proteins. Protein concentrations were determined using a protein assay reagent (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. Protein samples were retrieved from  $-80^{\circ}\text{C}$  storage and kept on ice prior to analysis. For experiments performed in HOS cells, four treatment groups were included: untreated control, curcumin-treated (CUR, 9  $\mu\text{M}$ ), doxorubicin-treated (DOX, 0.2  $\mu\text{M}$ ), and combined curcumin (9  $\mu\text{M}$ ) and doxorubicin (0.2  $\mu\text{M}$ ) treatment, as indicated in the corresponding figures. For validation experiments performed in MG-63 cells (Supplementary Figure S2), cells were treated with curcumin (15  $\mu\text{M}$ ), doxorubicin (0.3  $\mu\text{M}$ ), or the indicated combinations for 48 h. Higher concentrations of doxorubicin were selected in MG-63 cells to facilitate clearer detection of apoptotic signaling, as assessed by cleaved PARP expression.

Protein concentrations were determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions. Equal amounts of protein (20–30  $\mu\text{g}$  per lane) from each sample were mixed with distilled water and sample buffer to a final volume of 20  $\mu\text{L}$ . Samples were heated for 5 min, immediately placed on ice for 5 min, and briefly centrifuged prior to electrophoresis. Proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 0.05% bovine serum albumin (BSA) for 90–120 min at room temperature, followed by washing with phosphate-buffered saline containing 0.1% Tween-20 (PBST) three times for 10 min each. Membranes were then incubated with primary antibodies at  $4^{\circ}\text{C}$  for 16–18 h. After primary antibody incubation, membranes were washed three times with PBST for 10 min each and incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies at room temperature for approximately 1 h. Membranes were subsequently washed three times with PBST for 10 min each. Protein bands were detected using enhanced chemiluminescence (ECL) reagents, and immunoreactive signals were visualized

and quantified using a chemiluminescence imaging system. Membranes were trimmed according to molecular weight markers prior to antibody incubation when appropriate. Each target protein corresponds to the same sample as the loading control, but was obtained from a trimmed membrane processed separately. Band intensities were quantified using image analysis software, normalized to the corresponding loading control (GAPDH or tubulin), and expressed relative to control conditions. Phosphorylation levels were evaluated as relative changes and were not normalized to corresponding total protein levels. All quantitative analyses were performed using consistent regions of interest and standardized analysis parameters across all treatment groups to ensure comparability.

### 2.7. MAPK Inhibitor Pretreatment Experiments

To investigate the involvement of mitogen-activated protein kinase (MAPK) signaling pathways in apoptosis induced by combined curcumin and doxorubicin treatment, specific pharmacological inhibitors were used. HOS cells were pretreated with the ERK inhibitor PD98059 (25  $\mu$ M), the p38 MAPK inhibitor SB203580 (25  $\mu$ M), or the JNK inhibitor SP600125 (25  $\mu$ M) for 1 h prior to drug treatment. Following inhibitor pretreatment, cells were treated with curcumin (9  $\mu$ M) and doxorubicin (0.2  $\mu$ M), either alone or in combination, as indicated. Cells were then incubated under standard culture conditions for an additional 48 h. After treatment, cells were harvested, and total cellular proteins were extracted for subsequent Western blot analysis. The effects of MAPK inhibition on apoptosis were evaluated by assessing the expression level of cleaved poly(ADP-ribose) polymerase (PARP) using Western blotting, as described above. These experiments were performed to determine the functional contribution of individual MAPK pathways to apoptosis induced by combined curcumin and doxorubicin treatment.

### 2.8. TXNDC5 Overexpression and Cell Transfection

TXNDC5 overexpression was achieved by plasmid transfection in HOS cells. One day prior to transfection, cells were seeded in culture dishes to reach approximately 80% confluence at the time of transfection. A TXNDC5 overexpression plasmid was used, with an empty vector serving as the control. Transfections were performed using X-tremeGENE™ HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, serum-free culture medium was added to 1.5 mL microcentrifuge tubes, followed by the addition of X-tremeGENE™ HP reagent at a ratio of 3  $\mu$ L per 1  $\mu$ g of plasmid DNA. Plasmid DNA was then added at a ratio of 1  $\mu$ g per transfection reaction, and the mixture was gently mixed and incubated at room temperature for 15 min to allow formation of DNA–reagent complexes. The complexes were subsequently added dropwise to cells cultured in complete medium. After transfection, cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were harvested 24 h after transfection for protein extraction and Western blot analysis to confirm TXNDC5 overexpression. Successfully transfected cells were then used for subsequent functional assays, including apoptosis analysis following combined curcumin and doxorubicin treatment.

### 2.9. Statistical Analysis

All quantitative data are presented as the mean  $\pm$  standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using GraphPad Prism software (version 9.0; GraphPad Software, San Diego, CA, USA). Comparisons between two groups were conducted using Student's *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied. The assumptions of normality and homogeneity of variance were considered to be satisfied

based on the experimental design and comparable variance observed among groups. A *p*-value of less than 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Effects of Curcumin and Doxorubicin, Alone and in Combination, on Cell Viability

Curcumin and doxorubicin individually reduced cell viability in a dose-dependent manner in HOS cells. As shown in Figure 1A, treatment with curcumin at concentrations ranging from 5 to 20  $\mu$ M significantly decreased cell viability from approximately 100% in the control group to about 80%, 75%, 70%, 65%, and 45%, respectively. Similarly, doxorubicin hydrochloride treatment (25–500 nM) resulted in a progressive reduction in cell viability, decreasing from approximately 100% to about 80%, 75%, 70%, 60%, 50%, 45%, and 45% across the indicated concentrations (Figure 1B). Based on the dose–response profiles, sub-cytotoxic concentrations of curcumin (7.5, 8, and 9  $\mu$ M) and doxorubicin (0.2  $\mu$ M) were selected for combination treatment. The sub-cytotoxic concentrations refer to doses that result in less than approximately 20–40% reduction in cell viability. As shown in Figure 1C, treatment with curcumin alone reduced cell viability to approximately 78%, 72%, and 68% at 7.5, 8, and 9  $\mu$ M, respectively, whereas doxorubicin (0.2  $\mu$ M) alone resulted in a viability of approximately 75%. Notably, combined treatment further decreased cell viability to approximately 60%, 55%, and 30% at the corresponding curcumin concentrations, respectively, indicating a markedly enhanced inhibitory effect compared with either single treatment. The inhibitory effect of the combination treatment was more pronounced at higher curcumin concentrations, suggesting a concentration-dependent enhancement of growth suppression under combined treatment conditions. These results indicate that the combination treatment leads to a greater reduction in cell viability than either agent alone under comparable conditions.

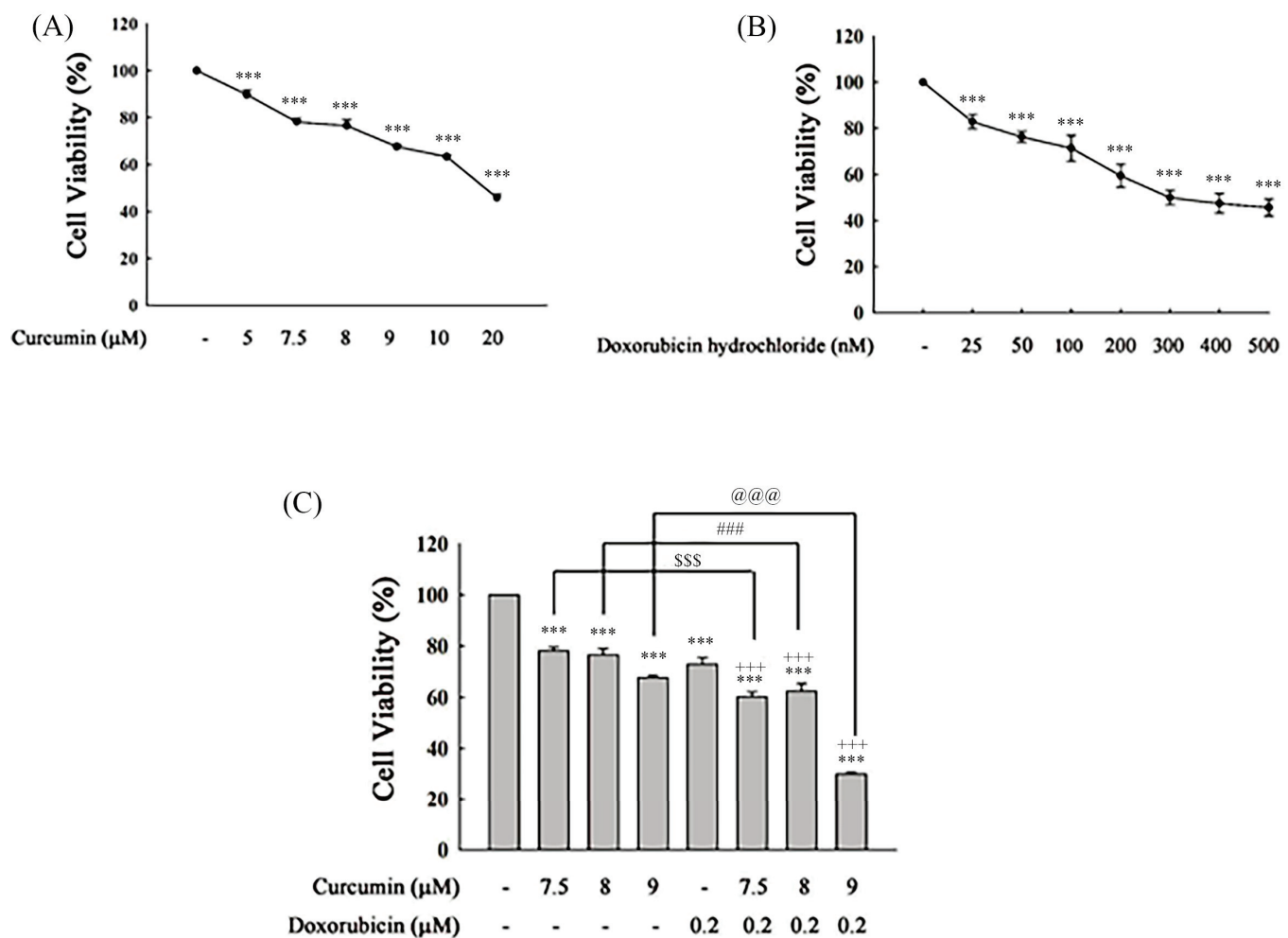
#### 3.2. Morphological Changes in HOS Cells Following Treatment with Curcumin and Doxorubicin

To examine the morphological effects of curcumin and doxorubicin on HOS cells, phase-contrast microscopy was used to observe cellular changes following different treatments. As shown in Figure 2, untreated cells exhibited a typical spindle-shaped morphology with intact cell–cell adhesion. Treatment with curcumin (9  $\mu$ M) or doxorubicin (0.2  $\mu$ M) alone resulted in moderate morphological alterations, including cell rounding and the appearance of cytoplasmic vacuoles. In contrast, combined treatment with curcumin and doxorubicin induced more pronounced morphological changes, characterized by increased cell shrinkage, extensive vacuolization, and the presence of apoptotic-like features, as indicated by red arrows. These observations suggest that the combination treatment caused greater morphological disruption compared with either agent alone.

#### 3.3. Curcumin Enhances Doxorubicin-Induced Apoptosis in HOS Cells

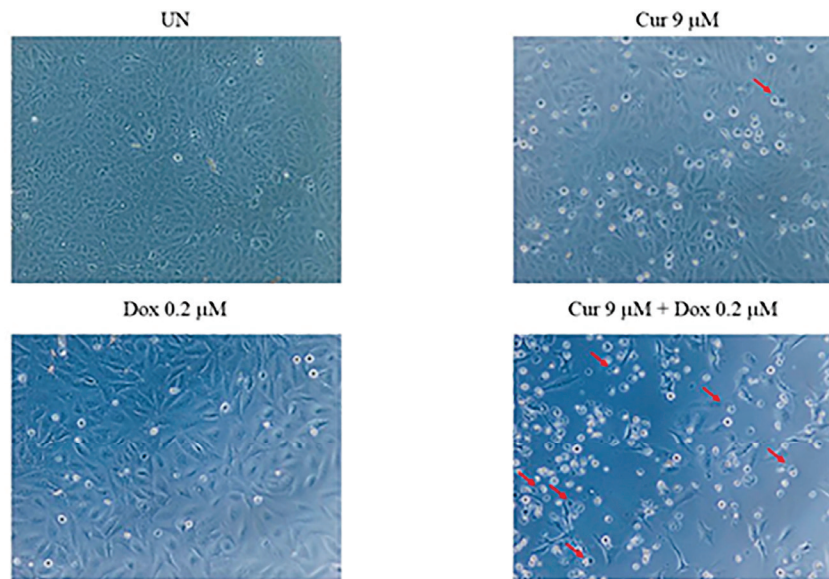
Based on the observed reduction in cell viability following combined curcumin and doxorubicin treatment (Figure 1), together with apoptotic-like morphological changes revealed by phase-contrast microscopy (Figure 2), further analyses were performed to determine whether the enhanced growth inhibition was associated with increased and quantifiable apoptotic cell death. As shown in Figure 3A, flow cytometric analysis of propidium iodide (PI)-stained cells revealed a low sub-G1 population in untreated cells (0.27%). Treatment with curcumin (9  $\mu$ M) or doxorubicin (0.2  $\mu$ M) alone moderately increased the sub-G1 fraction to 4.98% and 3.01%, respectively. Notably, combined treatment with curcumin and doxorubicin resulted in a marked accumulation of sub-G1 cells, reaching 20.49%, indicating enhanced apoptotic cell death. Consistent with these findings, TUNEL assay demonstrated minimal DNA fragmentation in untreated cells (0.05%), whereas treatment

with curcumin or doxorubicin alone increased the percentage of TUNEL-positive cells to 5.51% and 26.02%, respectively (Figure 3B). In contrast, combination treatment dramatically elevated the proportion of TUNEL-positive cells to 93.87%, indicating extensive DNA fragmentation under combined treatment conditions. Furthermore, Western blot analysis showed increased levels of cleaved PARP in cells treated with the combination of curcumin and doxorubicin compared with either agent alone (Figure 3C), further supporting the induction of apoptosis. Together, these results indicate that curcumin markedly potentiates doxorubicin-induced apoptotic responses in HOS cells.

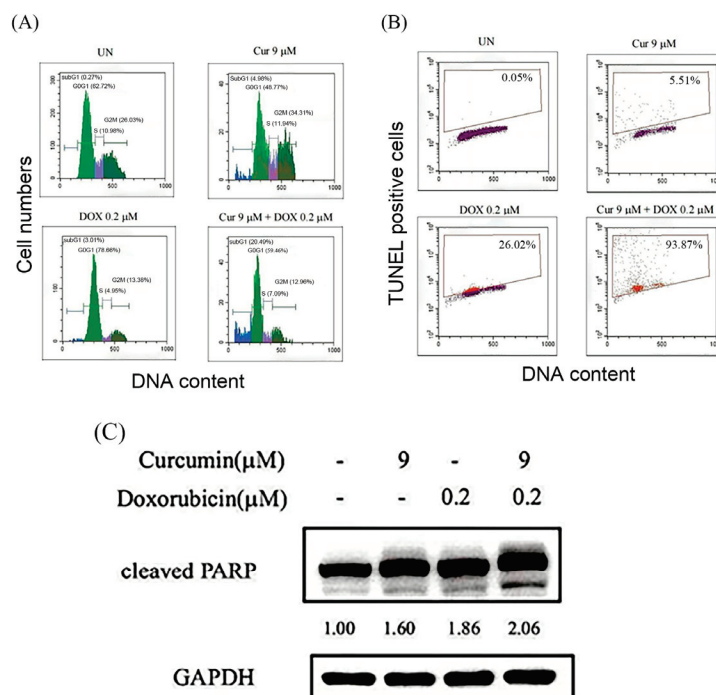


**Figure 1.** Effects of curcumin and doxorubicin on cell viability in HOS cells. (A,B) HOS cells were treated with increasing concentrations of curcumin (5–20 μM) or doxorubicin hydrochloride (25–500 nM) for 48 h, and cell viability was assessed using the CCK-8 assay. (C) Cells were treated with curcumin (7.5, 8, and 9 μM) alone or in combination with doxorubicin (0.2 μM) for 48 h. Cell viability was expressed as a percentage relative to the untreated control. Data are presented as mean ± SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. \*\*\*  $p < 0.001$  vs. untreated control; +++  $p < 0.001$  vs. doxorubicin alone; \$\$\$, ###, and @@@  $p < 0.001$  indicate comparisons between curcumin-alone and combination treatment groups, as indicated.





**Figure 2.** Morphological changes of HOS cells following treatment with curcumin and doxorubicin. Representative phase-contrast microscopic images of HOS cells under different treatment conditions. Cells were untreated (UN), treated with curcumin (9  $\mu$ M), treated with doxorubicin (0.2  $\mu$ M), or treated with a combination of curcumin (9  $\mu$ M) and doxorubicin (0.2  $\mu$ M) for 48 h. Red arrows indicate cytoplasmic vacuolization and morphological features associated with apoptotic cell death. Images were captured using an optical microscope.



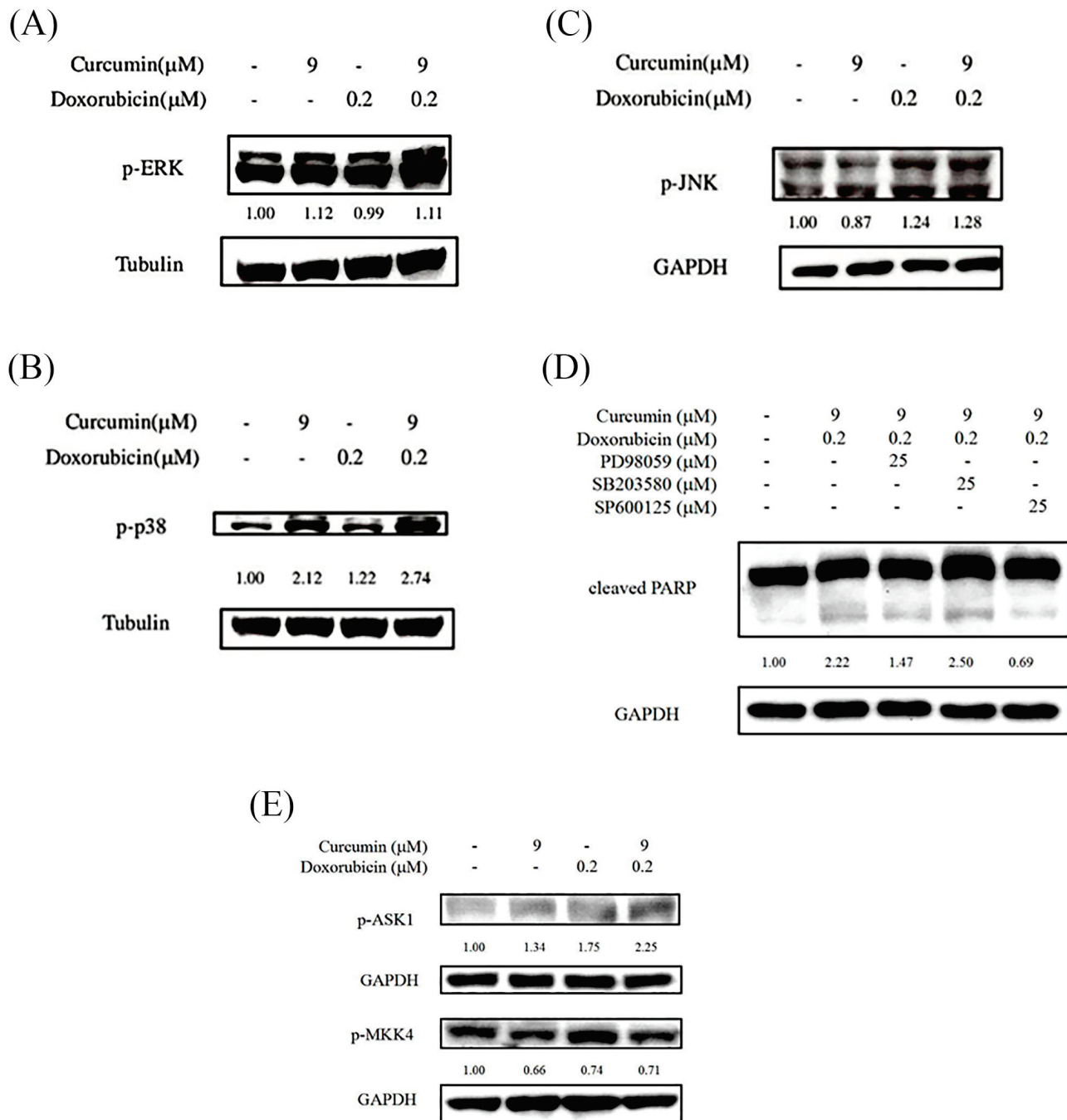
**Figure 3.** Effects of curcumin and doxorubicin on apoptosis induction in HOS cells. (A) Cell cycle distribution and sub-G1 population were analyzed by flow cytometry following propidium iodide (PI) staining after 48 h of treatment. HOS cells were left untreated (UN) or treated with curcumin (9  $\mu$ M), doxorubicin (0.2  $\mu$ M), or their combination. The sub-G1 fraction was used as an indicator of apoptotic cell death. (B) DNA fragmentation was assessed by TUNEL assay using an APO-BrdU<sup>TM</sup> TUNEL assay kit. Representative flow cytometric dot plots show TUNEL-positive cells, and percentages indicate the proportion of apoptotic cells. (C) Expression of apoptosis-associated protein cleaved PARP was analyzed by Western blotting following 48 h of treatment. GAPDH was used as a loading control. Relative protein expression levels were quantified by densitometric analysis.

### 3.4. Activation of p38 and JNK, but Not ERK, Contributes to Curcumin- and Doxorubicin-Induced Apoptosis in HOS Cells

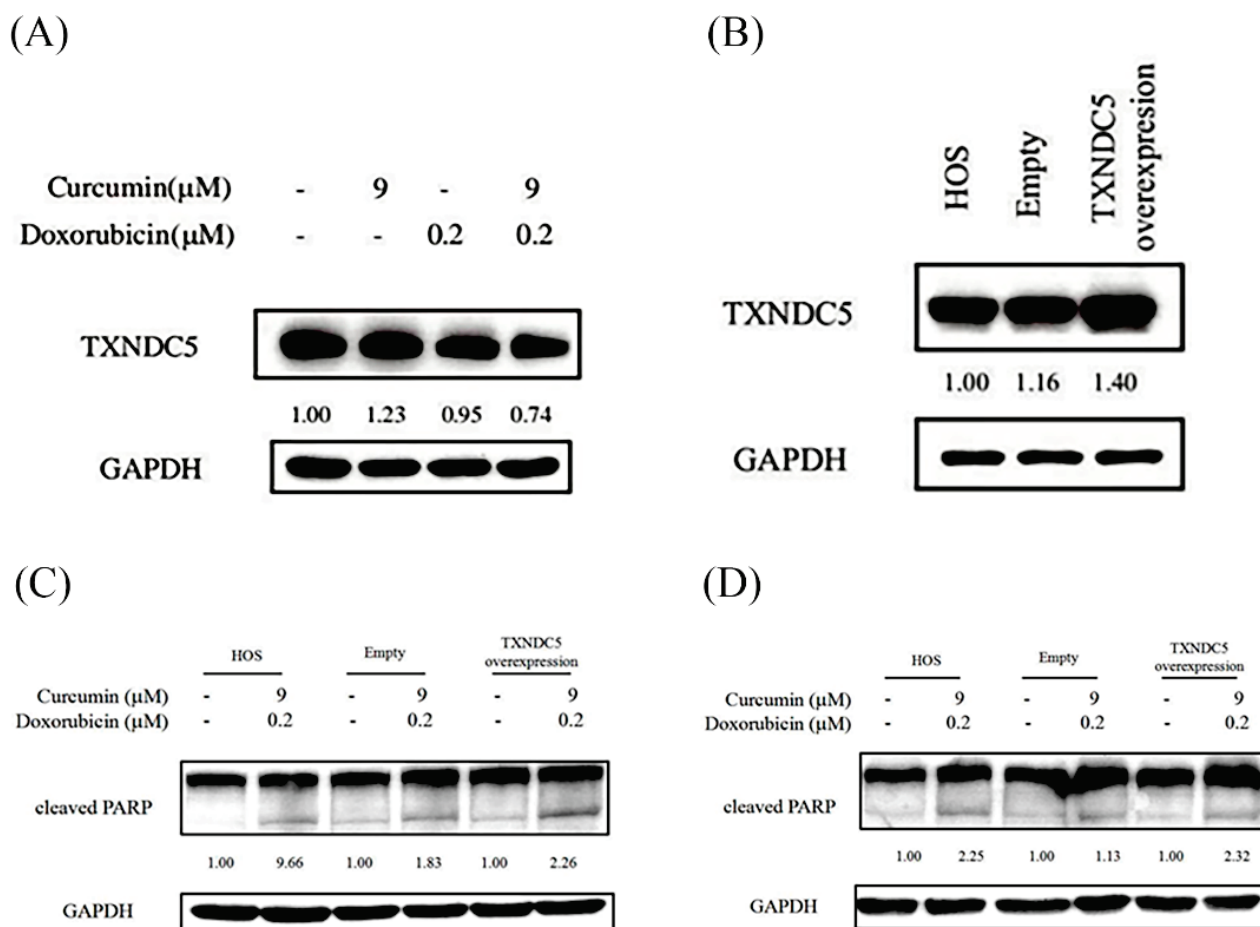
To further elucidate the signaling pathways involved in apoptosis induced by combined curcumin and doxorubicin treatment, the activation status of major MAPK pathways was examined. As shown in Figure 4A–C, combined treatment markedly increased the phosphorylation levels of p38 MAPK and c-Jun N-terminal kinase (JNK) compared with untreated or single-agent-treated cells. In contrast, no apparent increase in extracellular signal-regulated kinase (ERK) phosphorylation was observed following the combination treatment. To assess the functional relevance of MAPK signaling in apoptosis induction, specific MAPK inhibitors were applied prior to combined drug treatment. As shown in Figure 4D, inhibition of ERK phosphorylation by PD98059 or inhibition of JNK phosphorylation by SP600125 significantly reduced the level of cleaved PARP induced by the combination of curcumin and doxorubicin. To further examine whether early JNK activation is a conserved response across osteosarcoma cell lines, JNK phosphorylation was also assessed in MG-63 cells. As shown in Supplementary Figure S3, combined curcumin and doxorubicin treatment induced a rapid increase in JNK phosphorylation within 1 h, supporting early activation of JNK signaling in response to combination treatment. In contrast, inhibition of p38 signaling by SB203580 did not attenuate cleaved PARP expression. These results suggest that JNK activation and ERK signaling, but not p38 activation, are involved in mediating apoptosis induced by combined curcumin and doxorubicin treatment in HOS cells. Given the prominent involvement of JNK signaling in apoptosis induction, upstream signaling events regulating JNK activation were further investigated. As shown in Figure 4E, phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) was increased following combined curcumin and doxorubicin treatment at an early time point (1 h). In contrast, phosphorylation of mitogen-activated protein kinase kinase 4 (MKK4), a known upstream kinase of JNK, was not markedly altered under the same conditions. This apparent discrepancy suggests that phosphorylation status alone may not directly reflect functional involvement in apoptotic execution, and that distinct MAPK branches may differentially contribute to stress signaling and apoptosis under these conditions. These results reflect relative changes in phosphorylation levels under the indicated conditions rather than absolute activation of MAPK pathways.

### 3.5. TXNDC5 Downregulation Is Not Sufficient to Prevent Apoptosis Induced by Combined Curcumin and Doxorubicin Treatment

To investigate the role of TXNDC5 in apoptosis induced by combined curcumin and doxorubicin treatment, TXNDC5 protein expression was first examined. As shown in Figure 5A, treatment with the combination of curcumin and doxorubicin resulted in a reduction of TXNDC5 protein levels compared with untreated or single-agent-treated cells. Overexpression of TXNDC5 was confirmed by Western blot analysis in TXNDC5-overexpressing cells relative to parental HOS cells and empty vector-transfected controls (Figure 5B). Next, the effect of TXNDC5 overexpression on apoptosis induced by combined drug treatment was evaluated. As shown in Figure 5C,D, combined curcumin and doxorubicin treatment markedly increased cleaved PARP levels at both 24 h and 48 h. However, enforced overexpression of TXNDC5 did not attenuate cleaved PARP induction induced by the combination treatment at either time point. These results indicate that although TXNDC5 expression is downregulated following combined treatment, restoration of TXNDC5 expression is insufficient to suppress apoptosis induced by curcumin and doxorubicin in HOS cells.



**Figure 4.** Involvement of MAPK signaling pathways in curcumin- and doxorubicin-induced apoptosis in HOS cells. HOS cells were treated with curcumin (9  $\mu$ M), doxorubicin (0.2  $\mu$ M), or their combination, as indicated. (A–C) The phosphorylation levels of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (p-ERK), p38 MAPK (p-p38), and c-Jun N-terminal kinase (p-JNK), were analyzed by Western blotting following 48 h of treatment and normalized to GAPDH. (D) The involvement of MAPK signaling pathways in apoptosis was further evaluated using specific MAPK inhibitors. Cells were pretreated with PD98059 (ERK inhibitor, 25  $\mu$ M), SB203580 (p38 inhibitor, 25  $\mu$ M), or SP600125 (JNK inhibitor, 25  $\mu$ M) for 1 h prior to combined curcumin and doxorubicin treatment, and cleaved PARP expression was analyzed after 48 h to evaluate the functional involvement of MAPK signaling in apoptosis. (E) To investigate upstream signaling events involved in JNK activation, phosphorylation of apoptosis signal-regulating kinase 1 (p-ASK1) and mitogen-activated protein kinase kinase 4 (p-MKK4) was examined by Western blot analysis following 1 h of treatment.

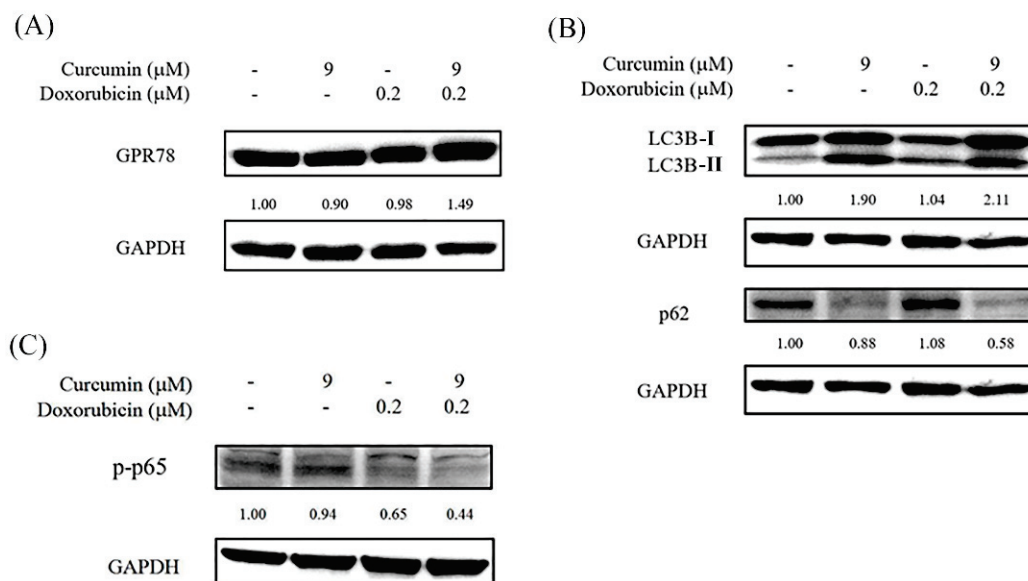


**Figure 5.** Role of TXNDC5 in modulating apoptosis induced by combined curcumin and doxorubicin treatment in HOS cells. (A) The protein expression level of thioredoxin domain-containing protein 5 (TXNDC5) was analyzed by Western blotting in HOS cells following treatment with curcumin (9  $\mu$ M), doxorubicin (0.2  $\mu$ M), or their combination, as indicated. (B) TXNDC5 expression was confirmed in parental, vector control, and TXNDC5-overexpressing HOS cells. (C,D) Parental HOS cells, empty vector-transfected cells, and TXNDC5-overexpressing cells were treated with curcumin (9  $\mu$ M) and doxorubicin (0.2  $\mu$ M) for 24 h or 48 h, followed by analysis of cleaved PARP expression by Western blotting. GAPDH was used as a loading control. Relative protein expression levels were quantified by densitometric analysis and are shown below the corresponding blots.

### 3.6. Combined Curcumin and Doxorubicin Treatment Modulates ER Stress, Autophagy, and NF- $\kappa$ B-Associated Signaling

In addition to MAPK-dependent apoptotic signaling, the effects of combined curcumin and doxorubicin treatment on other stress-associated pathways were further examined. As shown in Figure 6A, expression of glucose-regulated protein 78 (GRP78), a key marker of endoplasmic reticulum (ER) stress, was increased following combined treatment compared with untreated or single-agent-treated cells. Autophagy-associated markers were also evaluated. As shown in Figure 6B, combined treatment resulted in increased levels of LC3B-II accompanied by a reduction in p62 expression, consistent with alterations in autophagy-associated protein markers. In parallel, the effect of combined treatment on nuclear factor kappa B (NF- $\kappa$ B) signaling was assessed. As shown in Figure 6C, phosphorylation of NF- $\kappa$ B p65 was reduced following combined curcumin and doxorubicin treatment. Together, these findings suggest that combined treatment is associated with concurrent modulation of ER stress, autophagy-related responses, and NF- $\kappa$ B signaling in HOS cells.





**Figure 6.** Combined curcumin and doxorubicin treatment modulates ER stress-, autophagy-, and NF-κB-associated signaling pathways in HOS cells. HOS cells were treated with curcumin (9 μM), doxorubicin (0.2 μM), or their combination, as indicated, for 48 h. **(A)** GRP78 expression was analyzed as a marker of ER stress. **(B)** Autophagy-associated markers LC3B and p62 were analyzed by Western blotting. **(C)** The phosphorylation of NF-κB p65 was analyzed to assess NF-κB signaling activity. GAPDH was used as a loading control. Relative protein expression levels were quantified by densitometric analysis.

### 3.7. Validation of the Inhibitory Effect of Combined Curcumin and Doxorubicin in MG-63 Osteosarcoma Cells

To evaluate whether the growth-inhibitory effect of combined curcumin and doxorubicin treatment was reproducible in another osteosarcoma cell model, MG-63 cells were subjected to cell viability analysis. As shown in Supplementary Figure S1, treatment with curcumin or doxorubicin alone moderately reduced MG-63 cell viability after 48 h. Notably, combined treatment with curcumin (15 μM) plus doxorubicin (0.2 μM), as well as curcumin (15 μM) plus doxorubicin (0.3 μM), resulted in a more pronounced reduction in cell viability compared with either agent alone. These findings indicate that the enhanced inhibitory effect of combined curcumin and doxorubicin treatment is not restricted to HOS cells but can also be observed in MG-63 osteosarcoma cells, supporting the generalizability of the combination effect across osteosarcoma cell lines. To further determine whether the enhanced growth inhibition observed in MG-63 cells was associated with apoptotic cell death, the expression of cleaved poly(ADP-ribose) polymerase (PARP) was examined by Western blot analysis. To allow clearer detection of apoptotic signaling, MG-63 cells were treated with curcumin (15 μM), doxorubicin (0.3 μM), or the indicated combinations for 48 h. As shown in Supplementary Figure S2, treatment with either curcumin or doxorubicin alone resulted in modest induction of cleaved PARP. In contrast, combined treatment with curcumin (15 μM) plus doxorubicin (0.3 μM) led to a more pronounced increase in cleaved PARP levels. Consistent with the viability data, combined curcumin and doxorubicin treatment also enhanced apoptotic signaling in MG-63 cells, as evidenced by increased cleaved PARP expression under combination conditions (Supplementary Figure S2).

## 4. Discussion

Consistent with the concept that curcumin can function as a chemosensitizing phytochemical, studies published since 2021 have demonstrated that curcumin enhances the anticancer response to doxorubicin through modulation of stress-adaptive and survival-

related pathways, although the dominant molecular nodes appear to vary across tumor contexts. For instance, in doxorubicin-insensitive breast cancer models, curcumin has been reported to restore doxorubicin responsiveness primarily by targeting Aurora A-centered signaling networks and drug-resistance programs [33], rather than directly implicating MAPK signaling as the central mediator of the synergistic effect. In contrast, accumulating mechanistic evidence has highlighted the c-Jun N-terminal kinase (JNK) cascade as a key stress-activated pathway through which curcumin promotes tumor cell death under specific cellular conditions, including oxidative stress-driven apoptosis or regulated cell death [28,34–36].

Building on this framework, our data suggest that JNK activation represents a principal execution-associated MAPK signal in human osteosarcoma HOS cells subjected to combined curcumin and doxorubicin treatment. This conclusion is supported by the marked increase in JNK phosphorylation and by the ability of the JNK inhibitor SP600125 to significantly attenuate PARP cleavage (Figure 4), indicating a functional requirement for JNK signaling in apoptotic execution.

Interestingly, although combined curcumin and doxorubicin treatment elicited a pronounced increase in p38 MAPK phosphorylation (Figure 4B), pharmacological inhibition of p38 signaling using SB203580 failed to attenuate PARP cleavage induced by the combination treatment (Figure 4D). This dissociation between p38 activation and apoptotic execution suggests that p38 MAPK activation in this context is not functionally required for apoptosis induction. Instead, p38 activation may reflect a stress-responsive or adaptive signaling event triggered by chemotherapeutic and phytochemical stress, rather than serving as a dominant execution pathway. Indeed, p38 MAPK has been reported to exert context-dependent functions in cancer cells, acting either as a pro-apoptotic mediator or as a regulator of stress adaptation and survival depending on cellular context and stimulus intensity [37]. In several models, p38 activation accompanies cellular stress without being essential for downstream apoptotic execution once stronger death-promoting pathways are engaged [38–40].

In the present study, our data support a model in which p38 MAPK activation represents an upstream stress-sensing response to combined curcumin and doxorubicin treatment, whereas JNK signaling functions as the principal execution-associated MAPK pathway driving apoptotic cell death. Notably, although ERK phosphorylation was not elevated by the combination treatment, pharmacological inhibition of ERK signaling nevertheless reduced cleaved PARP expression (Figure 4). This finding suggests that basal ERK activity may function in a permissive or cooperative manner to support JNK-dependent apoptotic signaling, rather than acting as an inducible driver of apoptosis under these experimental conditions.

It should be noted that phosphorylation levels of MAPK proteins were normalized to the loading control (GAPDH) rather than to their corresponding total protein levels. Although normalization to total protein is commonly applied in signaling studies, the consistent phosphorylation patterns observed across independent experiments, together with functional validation using pharmacological inhibitors (e.g., SP600125), support the interpretation of MAPK pathway involvement. Nevertheless, the present findings should be interpreted as relative changes in phosphorylation status rather than definitive evidence of absolute pathway activation.

Mechanistically, upstream signaling analysis further revealed that combination treatment rapidly increased ASK1 phosphorylation, whereas MKK4 phosphorylation was not detectably increased within the same early time window. This pattern suggests that ASK1-to-JNK transmission may proceed through alternative intermediates (e.g., preferential engagement of other MAP2Ks such as MKK7, or transient kinetics of MKK4 activation

that fall outside the sampling window), and highlights that ASK1 activation alone is not necessarily mirrored by a sustained p-MKK4 signal at the time point examined. Such a “non-parallel” phosphorylation pattern is compatible with the broader view that JNK signaling is highly stimulus-, timing-, and scaffold-dependent, and that upstream kinase readouts can be pathway-selective rather than uniformly propagated across all canonical nodes. Collectively, compared with recent chemosensitization studies emphasizing resistance networks [27,41], our findings add a complementary mechanistic angle by identifying JNK-dependent MAPK signaling as the dominant apoptosis-associated axis under curcumin–doxorubicin co-treatment in osteosarcoma cells, while also distinguishing inducible MAPK signals from permissive basal signaling requirements.

TXNDC5 is known to play a role in redox homeostasis and cellular stress responses, and its dysregulation has been implicated in cancer progression and therapeutic resistance [22,42]. In the present study, combined curcumin and doxorubicin treatment reduced TXNDC5 expression in HOS cells. However, enforced overexpression of TXNDC5 failed to attenuate PARP cleavage induced by the combination treatment, indicating that TXNDC5 downregulation alone is not sufficient to account for apoptotic execution. One possible explanation is that apoptosis induced by the combined treatment is driven by dominant pro-apoptotic signaling pathways that override TXNDC5-mediated protective mechanisms. Consistent with this notion, our data suggest that JNK activation is involved in apoptosis induced by combined curcumin and doxorubicin treatment, indicating that MAPK-dependent apoptotic signaling may predominate over the modulatory effects of TXNDC5. In addition, TXNDC5 may function at an upstream stress-response level, whereas PARP cleavage represents a late-stage apoptotic event. Once the execution phase of apoptosis has been initiated, restoration of TXNDC5 expression may be insufficient to reverse apoptotic progression. This temporal dissociation between stress-associated regulators and apoptotic execution factors has also been described in other models of chemotherapeutic stress. Taken together, these findings suggest that TXNDC5 downregulation is associated with, but not determinative of, apoptosis induced by combined curcumin and doxorubicin treatment. Rather than acting as a central apoptotic regulator, TXNDC5 may serve as a stress-responsive modulator whose effects may be secondary to stronger pro-apoptotic signaling pathways activated by the combination treatment. Consistent with this interpretation, TXNDC5 downregulation accompanies combined curcumin and doxorubicin treatment but does not appear sufficient to counteract apoptosis associated with MAPK-dependent signaling.

While the present findings support the involvement of MAPK signaling in apoptosis induced by combined curcumin and doxorubicin treatment, the mechanistic relationships between individual MAPK pathways, TXNDC5 regulation, and apoptotic execution should be interpreted with caution. Although JNK phosphorylation and inhibitor studies suggest a functional role in apoptosis, the current data do not establish a direct causal relationship at the molecular level. Similarly, although TXNDC5 expression was reduced following treatment, its precise role in regulating apoptotic signaling remains to be further elucidated. Therefore, these findings primarily indicate an association rather than definitive mechanistic linkage.

In addition to JNK-dependent MAPK signaling, our observation that GRP78 was increased by the combination treatment suggests engagement of an endoplasmic reticulum (ER) stress response, which is a common cellular reaction to proteotoxic and oxidative insults induced by chemotherapy. ER stress has been reported to play context-dependent roles in cancer cells, functioning as either a protective mechanism or a contributor to cell death depending on stress intensity and duration [43,44]. Importantly, curcumin has been reported to enhance chemosensitivity by amplifying ER stress signaling in cancer cells. For

example, curcumin was shown to increase cisplatin sensitivity in non-small cell lung cancer cells through activation of ER stress-associated pathways, supporting the concept that ER stress augmentation can contribute to curcumin-mediated chemosensitization [45]. Moreover, mechanistic evidence indicates that curcumin itself can trigger ER stress programs (e.g., ATF6-associated signaling) and promote apoptotic execution, providing a plausible basis for why ER stress markers may rise in our co-treatment setting [46]. Consistently, ER stress activation has also been observed alongside stress-activated kinase signaling (including JNK/p38) in curcumin-treated cancer models, suggesting that ER stress may occur as part of a broader stress-integrated response rather than as an isolated pathway [47,48]. In the present study, because GRP78 was assessed as a marker without functional interrogation of unfolded protein response (UPR) branches (PERK/eIF2 $\alpha$ /ATF4-CHOP, IRE1 $\alpha$ /XBP1, or ATF6) or ER stress dependence, the current data support ER stress engagement as an accompanying stress signature rather than definitive evidence that ER stress is required for apoptosis. Future experiments using pathway-level readouts (e.g., CHOP induction, XBP1 splicing, ATF6 cleavage) and pharmacologic or genetic modulation would be needed to determine whether ER stress is mechanistically upstream of the JNK-dependent apoptotic axis observed here.

Furthermore, the interpretation of increased LC3B-II levels accompanied by reduced p62 expression requires careful consideration, as autophagy is well recognized to exert context-dependent roles in cancer cells under chemotherapeutic or phytochemical stress. In many settings, autophagy is activated as an adaptive or compensatory response that promotes cell survival by alleviating proteotoxic and metabolic stress; however, under conditions of sustained or excessive stress, autophagy may also cooperate with apoptotic signaling or contribute to cell death [49,50]. In the present study, the observed alterations in autophagy-associated markers most plausibly reflect engagement of a stress-responsive autophagy program triggered by combined curcumin and doxorubicin treatment, rather than definitive evidence that autophagy directly drives apoptotic execution.

Consistent with this interpretation, accumulating evidence indicates that curcumin can modulate autophagy through multiple signaling nodes, including the PI3K/Akt/mTOR axis, and that the functional outcome of curcumin-induced autophagy is highly dependent on tumor type, cellular context, and stress intensity [28]. Recent studies have reported that curcumin alone or in combination with conventional chemotherapeutic agents can enhance autophagy-associated responses in cancer cells, often in parallel with apoptotic signaling, supporting the notion that autophagy activation frequently accompanies chemosensitization rather than acting as an isolated death mechanism [45,47]. Nevertheless, because autophagic flux and functional dependence on autophagy were not directly assessed in this study—such as by using lysosomal inhibitors, tandem fluorescent LC3 reporters, or genetic disruption of core autophagy regulators—the present findings should be interpreted as indicative of autophagy modulation as part of a broader cellular stress landscape. Future studies will be required to determine whether autophagy serves a protective, neutral, or contributory role in the apoptotic response elicited by combined curcumin and doxorubicin treatment.

In parallel, changes in additional stress- and survival-related signaling pathways were also observed. Specifically, suppression of NF- $\kappa$ B p65 phosphorylation was observed following combined treatment (Figure 6C). Given the established role of NF- $\kappa$ B signaling in promoting cell survival and chemoresistance, attenuation of NF- $\kappa$ B activity may further contribute to a cellular environment that favors apoptotic signaling. However, as NF- $\kappa$ B signaling was not functionally interrogated in this study, its precise contribution to apoptosis induction warrants further investigation.

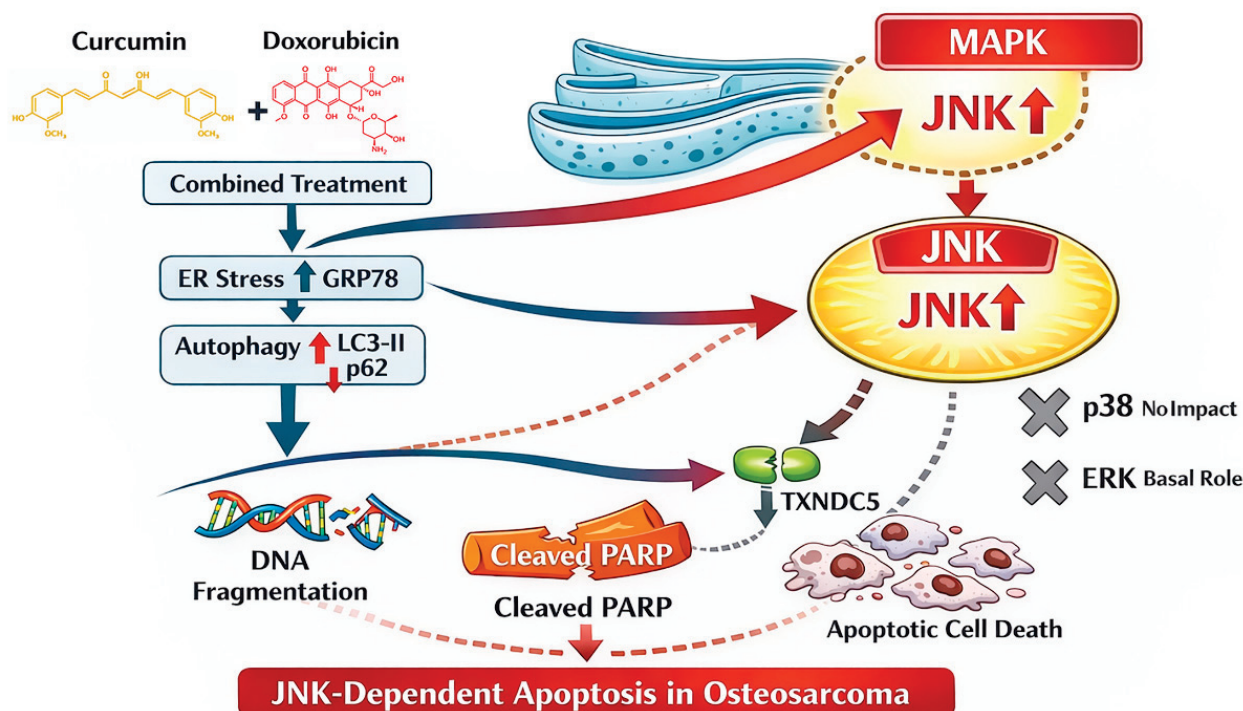
In addition to MAPK-dependent apoptotic signaling, accumulating evidence suggests that curcumin-mediated chemosensitization is closely associated with the induction of oxidative stress and disruption of redox homeostasis. Recent studies have demonstrated that combined curcumin and doxorubicin treatment markedly increases intracellular reactive oxygen species (ROS) levels, leading to mitochondrial dysfunction, ATP depletion, cytochrome c release, and caspase-3 activation. This oxidative stress-driven cytotoxicity is further accompanied by iron accumulation and lipid peroxidation, ultimately contributing to ferroptosis-associated cell death pathways. Importantly, pharmacological inhibition of ROS or ferroptosis has been shown to attenuate apoptosis and DNA fragmentation, supporting a mechanistic link between oxidative stress, ferroptosis, and apoptotic signaling [51]. These findings provide a broader mechanistic framework in which oxidative stress acts as an upstream trigger that integrates multiple stress-response pathways, including MAPK activation, ER stress, and autophagy modulation. In this context, our data support a model in which curcumin–doxorubicin co-treatment induces a multifaceted stress response, while JNK-dependent MAPK signaling serves as the dominant execution-associated pathway driving apoptosis in osteosarcoma cells. Other stress-related events, such as ER stress activation and autophagy-associated changes, may reflect downstream or parallel adaptive responses to oxidative stress rather than primary determinants of apoptotic execution.

Although the p-JNK signal observed in MG-63 cells appeared relatively weaker, the increase in phosphorylation following combined treatment was consistently detected across independent experiments. This variation may reflect differences in protein expression levels or antibody sensitivity between cell lines. These findings provide supportive validation that JNK activation is not restricted to HOS cells, although the mechanistic conclusions of the present study are primarily based on HOS cells.

It is also important to consider the intrinsic differences between osteosarcoma cell lines used in this study. HOS and MG-63 cells differ in their genetic background, differentiation status, and tumorigenic potential. MG-63 cells are generally considered to exhibit a more immature osteoblastic phenotype and distinct proliferative and signaling characteristics compared with HOS cells. These intrinsic differences may influence cellular stress responses and downstream signaling activation, including MAPK pathways. In this context, while MG-63 cells were used to provide supportive validation of the combination effect, the detailed mechanistic findings in the present study are primarily based on HOS cells. Therefore, variations observed between cell lines may reflect cell type-specific signaling properties rather than inconsistencies in experimental outcomes.

Collectively, these observations suggest that combined curcumin and doxorubicin treatment induces a broad cellular stress response involving MAPK signaling, ER stress, autophagy-associated processes, and NF- $\kappa$ B modulation. Among these pathways, JNK-dependent MAPK signaling represents the dominant and functionally required axis mediating apoptotic execution, while other stress-response pathways may serve supportive or context-dependent functions. A schematic model summarizing these findings is presented in Figure 7, highlighting JNK activation as the principal execution-associated pathway, with ER stress, autophagy, NF- $\kappa$ B modulation, and TXNDC5 downregulation representing accompanying stress responses. By distinguishing the primary apoptotic driver from these associated pathways, this study provides mechanistic insight into curcumin-mediated chemosensitization and supports the potential application of curcumin as an adjuvant to enhance the therapeutic efficacy of doxorubicin in osteosarcoma.





**Figure 7.** Schematic model illustrating that combined curcumin and doxorubicin treatment induces apoptosis predominantly through JNK-dependent MAPK signaling, with ER stress, autophagy, NF- $\kappa$ B modulation, and TXNDC5 downregulation as supportive or context-dependent responses.

## 5. Conclusions

This study demonstrates that combined curcumin and doxorubicin treatment effectively induces apoptosis that appears to be primarily associated with JNK-dependent MAPK signaling in human osteosarcoma cells. The enhanced anticancer effect is characterized by reduced cell viability and activation of apoptotic markers, including sub-G1 accumulation, DNA fragmentation, and PARP cleavage. Mechanistically, this apoptotic response is functionally dependent on JNK activation, as pharmacological inhibition of JNK attenuated PARP cleavage. In contrast, p38 activation and ERK signaling were not required as direct apoptotic drivers. Although TXNDC5 expression was reduced following combined treatment, TXNDC5 overexpression failed to suppress apoptosis, indicating a non-determinative role. Collectively, these findings support curcumin as a potential adjuvant agent that enhances doxorubicin-induced apoptosis through JNK-dependent signaling in osteosarcoma cells.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu18081235/s1>, Figure S1: Effects of combined curcumin and doxorubicin treatment on cell viability in MG-63 cells; Figure S2: Induction of apoptosis in MG-63 cells assessed by cleaved PARP expression; Figure S3: Activation of JNK signaling in MG-63 cells following combination treatment.

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## Article

# Olive Leaf Extract (OLE) Anti-Tumor Activities Against Hematologic Tumors: Potential Therapeutic Implications for Pediatric Patients with B-Acute Lymphoblastic Leukemia

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## Abstract

**Background/Objectives:** Several studies reported that olive leaf extract (OLE) may exert potent anti-cancer activities against human solid and hematological tumors. Such effects are mostly related to the polyphenol oleuropein and its derivatives, which are highly concentrated in OLE. Here, we investigated the anti-tumor effects of OLE in vitro against human acute leukemia and lymphoma cells. **Methods:** Cell proliferation and apoptosis have been evaluated by flow cytometry (using CFSE and Annexin-V/7AAD, respectively) in the presence or absence of OLE at different concentrations and in combination with or without chemotherapeutic drugs. Cellular pathways have been analyzed using antibody arrays. **Results:** OLE inhibited cell proliferation and induced apoptosis in B-acute lymphoblastic leukemia (B-ALL) and, to a lesser extent, in lymphomas and acute myeloid leukemia (AML) cell lines. Notably, OLE-induced apoptosis also occurs in primary leukemic blasts from B-ALL patients, both at diagnosis and at relapse, but only marginally in primary AML blasts. The expression and phosphorylation of proteins involved in the induction of apoptosis were modulated by OLE in B-ALL, whereas modest effects were observed in AML. Interestingly, some proteins were modulated in opposite ways in B-ALL and AML, potentially explaining their different responses to OLE. Finally, a synergistic and additive effect was observed for OLE in combination with cytarabine, but not with cyclophosphamide. **Conclusions:** We may envisage that OLE may be used as a food supplement in B-ALL patients treated with cytarabine, taking advantage of the potentiated effect of chemotherapy, without additional side effects.

**Keywords:** olive leaf extract; pediatric leukemias; nutraceuticals

## 1. Introduction

*Olea europaea* L. (Oleaceae) is a characteristic Mediterranean species used to produce olives and oils, representing key components of the traditional Mediterranean diet that includes a high intake of fruit, vegetables, fish, red wine, and extra virgin olive oil (EVOO). Such a diet is widely recognized to have an impact on good health, long life, prevention of

chronic diseases (e.g., cardiovascular and neurodegenerative), and cancers in the Mediterranean population. In addition to EVOO, another phytochemical may be obtained from the olive plant: olive leaf extract (OLE), containing similar biologically active compounds, even at much higher concentrations and a wider variety. OLE is abundant in polyphenolic compounds, broadly categorized into secoiridoids including oleuropein, dimethyloleuropein, and oleuropein-aglycone, simple phenols such as hydroxytyrosol and tyrosol, and flavonoids (e.g., apigenin and luteolin). These polyphenols have been documented to exert anti-microbial [1,2], anti-viral [3–5], hypoglycemic [6], neuro-protective [7,8], anti-inflammatory [9–11] and anti-tumor activities [12–15]. The latter function was reported both in vitro and in vivo against a wide panel of human tumors, including breast, pancreatic, colorectal, and skin cancers, as well as melanoma, glioblastoma, neuroblastoma, and acute leukemias. In the context of prostate and breast cancers, oleuropein and hydroxytyrosol were the main molecules endowed with anti-tumor properties; noteworthy, it was reported that they were able to discriminate between cancer and normal cells by inhibiting proliferation and inducing apoptosis only in the former [16–18]. In acute leukemias, the ability of OLE to counteract leukemia cell growth was evaluated exclusively against cell lines of myeloid origin (i.e., K562 and HL-60), but not in the B-acute lymphoblastic leukemias (ALL). In addition, B-cell lymphomas have not yet been investigated. The anti-tumor activity may be exerted through different and complementary mechanisms [15] mainly related to the (i) inhibition of genotoxicity and mutagenesis, (ii) induction of apoptosis, (iii) inhibition of cell proliferation, (iv) selective cytotoxicity, (v) regulation of oxidative stress through reactive oxygen species (ROS), and (vi) inhibition of angiogenesis, tumor cell migration and metastasis. Thus, olive polyphenols can target all phases of chemoprevention from primary prevention by inhibiting cancer initiation, to secondary prevention by inhibiting cancer promotion, and therapeutic intervention by inhibiting cancer progression. The broad beneficial effects of OLE are of particular relevance in the context of oncologic pediatric patients, especially those with poor prognoses, receiving debilitating treatments that affect the patients' quality of life.

With this background, we investigated whether OLE may function as an anti-tumor agent against pediatric B-ALL, acute myeloid leukemias (AML), and human B-cell lymphomas, investigating the cellular pathways involved. B-ALL is the most common hematological cancer in children caused by an abnormal clonal proliferation of B-cell progenitors, thus manifesting as the accumulation of malignant and poorly differentiated lymphoid cells within the bone marrow, peripheral blood, and some extra-medullary sites. Despite the optimization of treatment protocols for pediatric B-ALL, approximately 15% of patients experience a relapse, and the overall survival of these patients is less than 10%. Moreover, other patients are refractory to the treatment and do not achieve remission. Hematopoietic stem cell transplantation is almost inevitably part of salvage treatment after relapse, but transplant-related mortality and long-term morbidity remain a pitfall. The prognosis of AML, which represents the second most common hematologic malignancy in children, remains less favorable compared to ALL, and approximately 30% of AML patients experience relapse and ultimately die from disease-related complications or treatment side effects. Finally, B-cell lymphoma is one of the more common cancers among children, teens, and young adults that starts in early forms of B lymphocytes. The overall 5-year relative survival rate is approximately 74% and patients, as for leukemias, undergo intensive chemotherapy and radiotherapy, surgery, hematopoietic stem cell transplantation, and immunotherapy.

Thus, novel therapeutic approaches that are less toxic, selectively target malignant cells, and potentially strengthen the immune responses of oncologic pediatric patients affected by leukemias or lymphomas are needed.

## 2. Materials and Methods

### 2.1. OLE Composition

Aqueous OLE, whose composition has been previously reported [12], was used throughout the study and was kindly provided by Evergreen Life, San Giovanni al Natisone, Italy. The manufacturer has implemented a patented method for the extraction at very low temperatures of different active compounds, mainly polyphenols, from olive leaves. The mean phytochemical compounds and their concentrations contained in OLE were the following: Oleuropein 2656 mg/L, Hydroxytyrosol 213 mg/L, Tyrosol 174 mg/L, Elenolic Acid 1393 mg/L, and Rutin 237 mg/L. Two different batches of OLE have been used throughout this study, with minimal variations in the composition, as disclosed by the manufacturer.

### 2.2. Cell Lines and Primary Leukemias from Pediatric Patients

The following human cell lines were used: NALM-6 and 697 B-ALL; K562, THP-1, and RPMI8866 myeloid leukemias; SUDHL-4, U937, and RAJI lymphomas (ATCC and Biobank at Ospedale San Martino, Genova, Italy). Cells were cultured in RPMI-1640 (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, by Euroclone, Milano, Italy), 50 IU/mL penicillin, 50 g/mL streptomycin sulphate, and 2 mM L-glutamine (Euroclone, Milano, Italy). Fresh medium was replaced every three days.

Leukemia cells (five B-ALL and three AML) were derived from patients' heparinized bone marrow aspirates (>90% blast cells) at diagnosis and, in patients experiencing disease recurrence, also at relapse, following written informed consent according to Helsinki Declaration. Leukemia cells were isolated by Ficoll-Hypaque Lympholyte<sup>®</sup> (Cedarlane, Burlington, ON, Canada) density gradient centrifugation, then cryopreserved in FBS (Euroclone SPA, Milan, Italy), with 10% DMSO (AL.CHI.MI.A S.R.L., Padua, Italy) for later use. Characteristics of patients are summarized in Table 1.

**Table 1.** Characteristics of patients. Leukemia blasts were obtained from patients (Pt) at the time of diagnosis, at onset, and, in cases, at relapse.

| Patients | Disease | Age (Years) | Leukemia Blasts |
|----------|---------|-------------|-----------------|
| Pt #1    | B-ALL   | 17          | diagnosis       |
|          |         |             | relapse         |
| Pt #2    | B-ALL   | 5           | diagnosis       |
|          |         |             | relapse         |
| Pt #3    | B-ALL   | 9           | diagnosis       |
|          |         |             | relapse         |
| Pt #4    | B-ALL   | 8           | diagnosis       |
| Pt #5    | B-ALL   | 4           | diagnosis       |
| Pt #6    | AML     | 12          | diagnosis       |
| Pt #7    | AML     | 10          | diagnosis       |
| Pt #8    | AML     | 2           | diagnosis       |

### 2.3. Cell Proliferation

Since oleuropein was the most effective and the most concentrated polyphenol in OLE, the concentration of OLE reported throughout the study was referred to the final concentration of oleuropein. Leukemia and lymphoma cell proliferation was assessed



by flow cytometry using a CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, cell lines were resuspended in RPMI-1640 without serum, stained with Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) at a final concentration of 2  $\mu$ M, and incubated at 37 °C for 15 min. After washing in RPMI 10% FBS, cells were resuspended in RPMI 10% FBS, seeded in a 96-well plate ( $10^5$ /well), and treated with OLE at the final concentrations of 50, 100, 200, and 400  $\mu$ M. After 6 days of culture, cells were washed and analyzed for cell proliferation, as witnessed by CFSE dilution, by flow cytometry. Cells cultured for 6 days in the absence of FBS were used as negative controls. Cells were harvested, washed, and resuspended in 300  $\mu$ L of MACS buffer (PBS supplemented with 2  $\mu$ M EDTA and 0.5% BSA). Samples were then run on a Gallios® flow cytometer (Beckman Coulter, Brea, CA, USA), acquiring at least  $3 \times 10^4$  events, and data were analyzed using Kaluza® analysis software version 2.0 (Beckman Coulter, Brea, CA, USA). The results were expressed as CFSE mean fluorescence intensity (MFI).

#### 2.4. Leukemia and Lymphoma Cell Apoptosis

Leukemia and lymphoma cell lines, as well as primary samples from pediatric leukemia patients, were analyzed for induction of apoptosis driven by OLE. Cells were seeded at 105/well in 96-well plates, treated for 24, 48, and 72 hours (h) with OLE 50, 100, 200, and 400  $\mu$ M, and tested for apoptosis by flow cytometry using the Annexin V-FITC/7AAD kit (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. Briefly,  $10^5$  cells were washed with PBS and resuspended in 100  $\mu$ L of binding buffer 1X. Next, 7-AAD and FITC-conjugated Annexin V were added, and cells were incubated for 15 min on ice. Next, 300  $\mu$ L of binding buffer 1X was added to each sample, and cells were run on a Gallios® flow cytometer (Beckman Coulter, Brea, CA, USA), acquiring at least  $3 \times 10^4$  events. Data were analyzed using Kaluza® analysis software (Beckman Coulter, Brea, CA, USA). Annexin V<sup>+</sup> staining identified early apoptotic cells, 7AAD<sup>+</sup>/Annexin<sup>+</sup> the late apoptotic cells, and 7AAD<sup>+</sup>/Annexin<sup>−</sup> dead cells. B-ALL and AML primary samples were treated for 24 h with 100 and 200  $\mu$ M OLE and tested for apoptosis.

#### 2.5. OLE Molecular Pathways in Leukemia Cells

To investigate the molecular pathways involved in the apoptosis induced by OLE in leukemia cells, we selected the NALM-6 cells, due to their high response to OLE in terms of inhibition of cell proliferation and induction of apoptosis, and the K562 cell line for its low response to OLE treatment. Cells were treated in the presence or absence of 200  $\mu$ M OLE for 24 h, and protein extracts were obtained following the protocol reported in each human proteome profiler kit. Proteins were quantified using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) and Tecan Infinite 200 Pro Bioreader (Tecan, Männedorf, Switzerland) (absorbance at 555 nm). A total mass of 200  $\mu$ g of each protein lysate were assessed on 4 different human proteome profiler kits following the manufacturer's instructions (Bio-Techne, Minneapolis, MN, USA): (i) apoptosis array detecting the relative levels of expression of 35 apoptosis-related proteins, (ii) NF $\kappa$ B pathway array analyzing 41 different proteins and 4 serine or tyrosine phosphorylation sites antibodies, (iii) phospho-kinase array with 37 different kinases and 2 related total proteins, and (iv) cell stress array analyzing 26 different stress-related proteins. The relative amount of each individual protein (corresponding to each spot tested in duplicate) was calculated using Image Lab software (Bio-Rad, version 6), upon normalization to internal controls. The fold increase was calculated for each protein as the ratio between values obtained in treated versus untreated cells. A fold increase of 1 indicated no modulation, whereas positive



values indicated an up-regulation and negative values indicated a down-regulation. An arbitrary cut-off value for fold increase (1.8) was established, and only proteins with a fold increase  $>1.8$  or  $>-1.8$  are shown.

## 2.6. Combined Effect of OLE and Chemotherapeutic Agents on B-ALL Cell Lines

NALM-6 and 697 B-ALL cell lines were treated for 24 and 48 h with a combination of 50 or 100  $\mu\text{M}$  OLE and cytarabine (Accord, 0.5 and 1  $\mu\text{M}$ ) or cyclophosphamide (Endoxan, Baxter, 0.5 and 1  $\mu\text{g/mL}$ ), obtained as a spare aliquot after therapeutic use. The percentage of live cells (7AAD<sup>−</sup> / Annexin V<sup>−</sup>) was analyzed, as described above. The combined effect of OLE with the other drugs was analyzed using ZIP and Bliss models for additive effects and HSA and Loewe models for synergistic effects. These analyses were carried out using the SynergyFinder+ web application (<https://synergyfinder.org>) [19–23].

## 2.7. Statistical Analysis

Statistical analysis was carried out using Prism 5.03 software (GraphPad Inc., Boston, MA, USA). Data distribution was analyzed using the KS normality test and the D'Agostino and Pearson omnibus normality test. Comparison of data sets was analyzed using a *t*-test or Mann–Whitney test, depending on data distribution. *p*-values  $< 0.05$  were considered statistically significant.

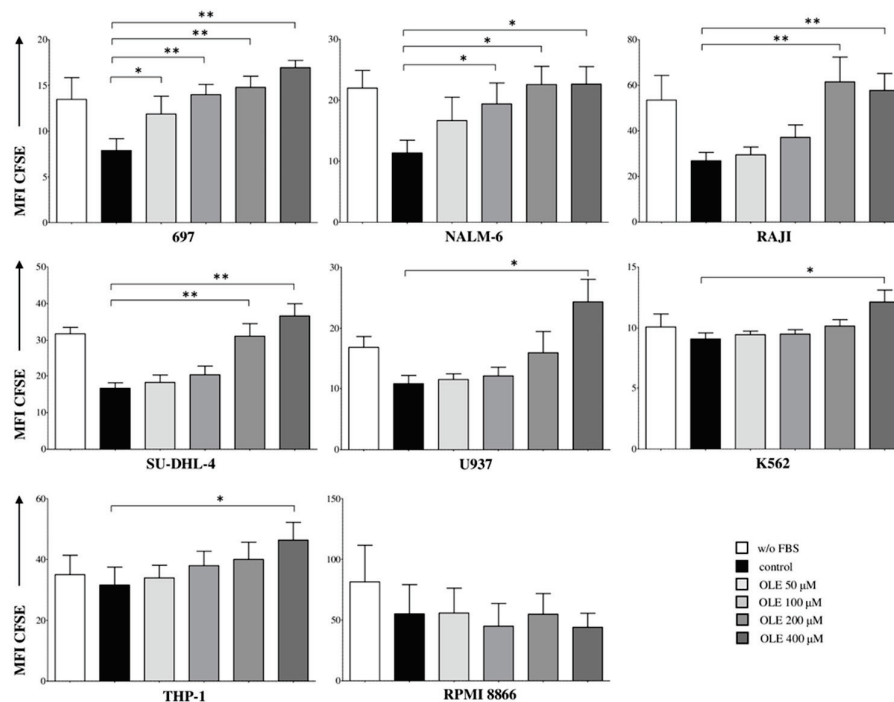
# 3. Results

## 3.1. OLE Inhibits the Proliferation of Leukemia/Lymphoma Cell Lines

First, we tested whether OLE could affect the proliferation of leukemia/lymphoma cells. To this end, we analyzed a panel of cell lines, including B-ALL (697 and NALM-6), Burkitt's lymphoma (RAJI), myeloid leukemia (K562, RPMI8866, and THP-1), and histiocytic lymphoma (SU-DHL-4 and U937), treated for 48 h with different concentrations of OLE. As shown in Figure 1, the proliferation of B-ALL cell lines was significantly inhibited by OLE at different concentrations, as witnessed by the increase in MFI CFSE. In detail, the proliferation of the 697 cell line (CFSE MFI mean  $\pm$  SE  $7.88 \pm 1.29$ ) was significantly reduced by OLE at 50  $\mu\text{M}$  ( $11.87 \pm 1.95$ ,  $p = 0.03$ ), 100  $\mu\text{M}$  ( $13.98 \pm 1.12$ ,  $p = 0.0076$ ), 200  $\mu\text{M}$  ( $14.79 \pm 1.22$ ,  $p = 0.0043$ ), and 400  $\mu\text{M}$  ( $16.94 \pm 0.79$ ,  $p = 0.0011$ ). Similarly, the proliferation of the NALM-6 cell line ( $11.36 \pm 2.08$ ) was significantly inhibited by OLE at 100  $\mu\text{M}$  ( $19.38 \pm 3.44$ ,  $p = 0.01$ ), 200  $\mu\text{M}$  ( $22.58 \pm 2.97$ ,  $p = 0.01$ ), and 400  $\mu\text{M}$  ( $22.64 \pm 2.87$ ,  $p = 0.0011$ ), but not at 50  $\mu\text{M}$  ( $16.67 \pm 3.82$ ).

The effect of OLE on the proliferation of other cell lines was more limited at the same time point and concentrations. In particular, RAJI cell line proliferation ( $26.84 \pm 3.67$ ) was found to be significantly reduced by OLE only at 200  $\mu\text{M}$  ( $61.46 \pm 10.9$ ,  $p = 0.0079$ ) and 400  $\mu\text{M}$  ( $57.74 \pm 7.4$ ,  $p = 0.004$ ). Other OLE concentrations proved less effective. Similar results were obtained in histiocytic lymphoma cell lines SU-DHL-4 (CTR  $16.63 \pm 1.47$ ; OLE 200  $\mu\text{M}$   $31.1 \pm 3.41$ ,  $p = 0.0076$ ; OLE 400  $\mu\text{M}$   $36.62 \pm 3.35$ ,  $p = 0.0011$ ), and U937 (CTR  $10.78 \pm 1.34$ ; OLE 400  $\mu\text{M}$   $24.32 \pm 3.69$ ,  $p = 0.01$ ) cell lines.

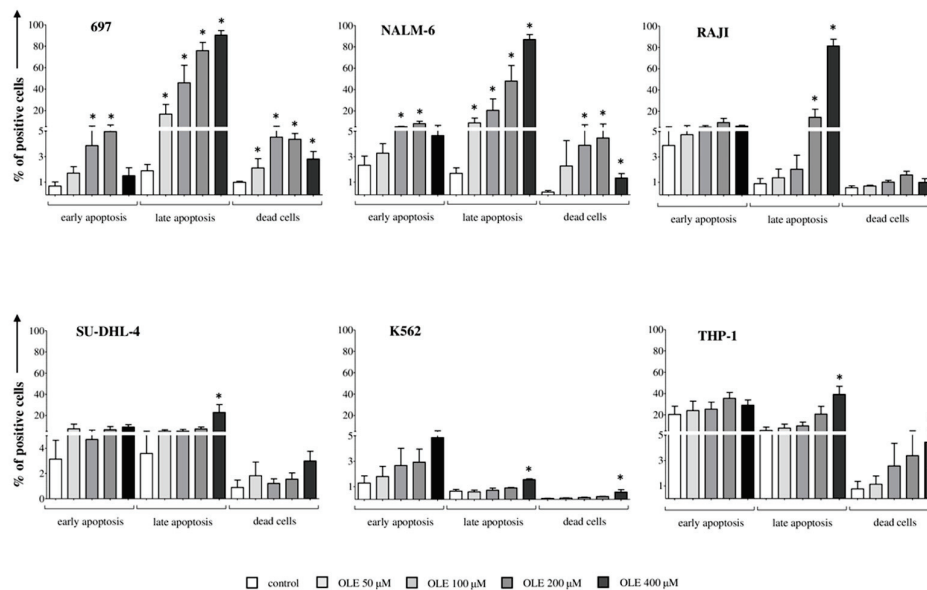
Myeloid leukemia cells lines resulted the most resistant to OLE treatment, in particular, proliferation of K562 and THP-1 cell lines was inhibited only at the highest dose of OLE (K562: CTR  $9.1 \pm 0.5$ ; OLE 400  $\mu\text{M}$   $12.13 \pm 0.98$ ,  $p = 0.01$ ; THP-1: CTR  $31.59 \pm 5.91$ ; OLE 400  $\mu\text{M}$   $46.38 \pm 5.82$ ,  $p = 0.04$ ), whereas no modulation of cell proliferation was observed in RPMI8866 cell at any OLE concentration tested.



**Figure 1.** Anti-proliferative effects of OLE on leukemia and lymphoma cell lines. Different cell lines, namely 697 and NALM-6 (B-ALL), RAJI, SUDHL-4, and U937 (lymphomas), K562, THP-1 and RPMI8866 (myeloid leukemias) were stained with CFSE and cultured for 6 days in RPMI without FBS (white bars) or supplemented with 10% FBS (black bars) in the presence or absence of OLE at different concentrations (50, 100, 200 and 400  $\mu$ M, grey bars). Cell proliferation was assessed by flow cytometry as CFSE dilution. Results are indicated as MFI for CFSE. Mean of six different experiments + SE is shown. Asterisks indicate a statistically significant difference (\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ).

### 3.2. OLE-Induced Apoptosis in Leukemia/Lymphoma Cell Lines

Next, we investigated the ability of OLE to induce apoptosis of leukemia/lymphoma cells, at the same concentrations used for proliferation studies, after 24, 48, and 72 h of treatment. OLE-induced cell apoptosis at any time point tested, and data from 48 h treatment (i.e., intermediate time) are reported in Figure 2. On the basis of the results obtained on cell proliferation, we first analyzed the B-ALL cell lines. The percentage of 697 cells in early apoptosis after 48 h of culture (% of Annexin V<sup>+</sup>/7AAD<sup>−</sup> cells, mean  $\pm$  SE  $0.68 \pm 0.32$ ) was significantly increased by OLE at 100  $\mu$ M ( $3.86 \pm 1.75$ ,  $p = 0.05$ ) and 200  $\mu$ M ( $4.95 \pm 1.66$ ,  $p = 0.05$ ). In addition, the percentage of 697 cells in late apoptosis (Annexin V<sup>+</sup>/7AAD<sup>+</sup> cells,  $1.88 \pm 0.49$ ) was significantly increased in the presence of OLE at the concentration of 50  $\mu$ M ( $16.72 \pm 9$ ,  $p = 0.05$ ), 100  $\mu$ M ( $45.88 \pm 16.44$ ,  $p = 0.05$ ), 200  $\mu$ M ( $75.97 \pm 7.61$ ,  $p = 0.05$ ), and 400  $\mu$ M ( $90.36 \pm 4.28$ ,  $p = 0.05$ ). The percentage of dead cells (Annexin V<sup>−</sup>/7AAD<sup>+</sup> cells,  $0.98 \pm 0.07$ ) was also significantly increased by OLE at 50  $\mu$ M ( $2.1 \pm 0.73$ ,  $p = 0.05$ ), 100  $\mu$ M ( $4.52 \pm 0.89$ ,  $p = 0.05$ ), 200  $\mu$ M ( $4.36 \pm 0.44$ ,  $p = 0.05$ ), and 400  $\mu$ M ( $2.8 \pm 0.59$ ,  $p = 0.05$ ). Similar results were obtained by analyzing the NALM-6 cell line, where the percentage of cells in early apoptosis after 48 h of culture ( $2.32 \pm 0.72$ ) was significantly increased by OLE at 100  $\mu$ M ( $5.1 \pm 0.45$ ,  $p = 0.05$ ) and 200  $\mu$ M ( $8.12 \pm 2.15$ ,  $p = 0.05$ ). In addition, the percentage of cells in late apoptosis ( $1.69 \pm 0.43$ ) was significantly increased by OLE at 50  $\mu$ M ( $8.79 \pm 4.52$ ,  $p = 0.05$ ), 100  $\mu$ M ( $20.6 \pm 10.6$ ,  $p = 0.05$ ), 200  $\mu$ M ( $47.99 \pm 14.51$ ,  $p = 0.05$ ), and 400  $\mu$ M ( $86.83 \pm 4.66$ ,  $p = 0.05$ ), as well as of dead cells ( $0.21 \pm 0.12$ ) that was significantly increased by OLE at 100  $\mu$ M ( $3.9 \pm 3.14$ ,  $p = 0.05$ ), 200  $\mu$ M ( $4.47 \pm 3.2$ ,  $p = 0.05$ ), and 400  $\mu$ M ( $1.32 \pm 0.35$ ,  $p = 0.05$ ).



**Figure 2.** Pro-apoptotic effects of OLE on leukemia and lymphoma cell lines. The same leukemia/lymphoma cell lines tested in proliferation assay were analyzed for apoptosis upon treatment with OLE. Cells were cultured for 48 h in RPMI supplemented with 10% FBS (white bars) in the presence or absence of OLE at different concentrations (50, 100, 200 and 400 μM, grey bars). Apoptosis was assessed by staining with 7AAD and Annexin-V FITC and flow cytometric analysis. Results are indicated as % of Annexin V<sup>+</sup> cells (early apoptosis), Annexin V<sup>+</sup>/7AAD<sup>+</sup> cells (late apoptosis) and 7AAD<sup>+</sup> cells (dead cells). Mean of six different experiments + SE is shown. Asterisks indicate a statistically significant difference ( $p \leq 0.05$ ).

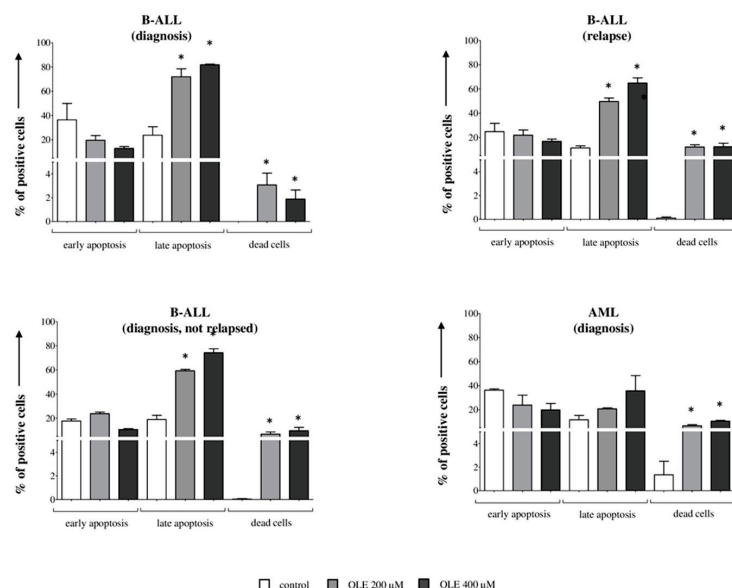
Similarly to that reported in cell proliferation studies, limited effects were observed in terms of induction of apoptosis in RAJI and SU-DHL-4 lymphoma cell lines, where we observed only an increased percentage of cells in late apoptosis ( $0.88 \pm 0.4$  and  $3.59 \pm 1.62$ , respectively). Such an increase was observed in RAJI cells in the presence of OLE at 200 μM ( $14.32 \pm 7.62$ ,  $p = 0.05$ ) and 400 μM ( $81.31 \pm 6.45$ ,  $p = 0.05$ ) and in the SU-DHL-4 cell line at 400 μM OLE ( $22.94 \pm 7.3$ ,  $p = 0.05$ ). We obtained similar results also in RPMI8866 and U937 cells.

Finally, we analyzed the myeloid leukemia cell lines. As expected, we did not observe any induction of early apoptosis in the K562 cell line, but only a slight increase of cells in late apoptosis ( $0.64 \pm 0.13$ ) and of dead cells ( $0.07 \pm 0.01$ ) in the presence of 400 μM OLE ( $1.55 \pm 0.06$  and  $0.58 \pm 0.18$ , respectively,  $p = 0.05$ ). Superimposable results were obtained by analyzing the THP-1 cell line, with an increase in cells in late apoptosis ( $5.32 \pm 3.16$ ) and dead cells ( $0.76 \pm 1.95$ ) with 400 μM OLE ( $39.23 \pm 7.53$  and  $4.44 \pm 2.78$ , respectively,  $p = 0.05$ ).

### 3.3. OLE-Induced Apoptosis in Primary Blasts from Leukemia Patients

To validate the results obtained with cell lines, we analyzed apoptosis of leukemic blasts obtained from patients with B-ALL and AML after 24 h of culture, in the presence or absence of OLE (200 and 400 μM). As shown in Figure 3, the percentage of leukemic blasts from patients with B-ALL at diagnosis in late apoptosis (% of Annexin V<sup>+</sup>/7AAD<sup>−</sup> cells, mean ± SE  $23.81 \pm 6.99$ ) was significantly increased by OLE at 200 μM ( $72 \pm 6.53$ ,  $p = 0.05$ ) and 400 μM ( $81.91 \pm 0.56$ ,  $p = 0.05$ ). The percentage of dead cells (% 7AAD<sup>+</sup> cells, mean ± SE  $0.1 \pm 0.1$ ) was also significantly increased by OLE at 200 μM ( $3.08 \pm 0.98$ ,  $p = 0.05$ ) and 400 μM ( $1.89 \pm 0.75$ ,  $p = 0.05$ ). We obtained superimposable results from the same B-ALL patients at relapse, with a significantly higher percentage of both apoptotic cells ( $11.47 \pm 1.69$ ) and dead cells ( $0 \pm 0.1$ ) in the presence of OLE at 200 μM ( $49.74 \pm 2.84$ ,

$p = 0.05$  and  $12.2 \pm 1.8$ ,  $p = 0.05$ , respectively) and  $400 \mu\text{M}$  ( $64.94 \pm 4.14$ ,  $p = 0.05$  and  $12.47 \pm 2.87$ ,  $p = 0.05$ , respectively). Regarding the leukemic blasts from patients who maintained disease remission, the percentage of blasts in late apoptosis ( $19.03 \pm 3.42$ ) was significantly increased by OLE at  $200 \mu\text{M}$  ( $59.38 \pm 1.2$ ,  $p = 0.05$ ) and  $400 \mu\text{M}$  ( $74.3 \pm 3.2$ ,  $p = 0.05$ ), as well as the percentage of dead cells (ctr:  $0.04 \pm 0.04$ ; OLE  $200 \mu\text{M}$ :  $6.9 \pm 1.7$ ,  $p = 0.05$ ; OLE  $400 \mu\text{M}$ :  $9.74 \pm 2.74$ ,  $p = 0.05$ ).



**Figure 3.** Pro-apoptotic effects of OLE on primary leukemia cells. Leukemic blasts from B-ALL patients at onset, relapse, onset with good prognosis (retrospectively not relapsed) and from AML patients at onset were cultured for 24 h in RPMI supplemented with 10% FBS (white bars) in the presence or absence of OLE at different concentrations (200 and  $400 \mu\text{M}$ , grey and black bars, respectively). Apoptosis was assessed by flow cytometry by staining with 7AAD and Annexin-V FITC. Results are indicated as % of Annexin V<sup>+</sup> cells (early apoptosis), Annexin V<sup>+</sup>/7AAD<sup>+</sup> cells (late apoptosis) and 7AAD<sup>+</sup> cells (dead cells). Mean of experiments performed on three different patients for each group + SE is shown. Asterisks indicated a statistically significant difference ( $* p \leq 0.05$ ).

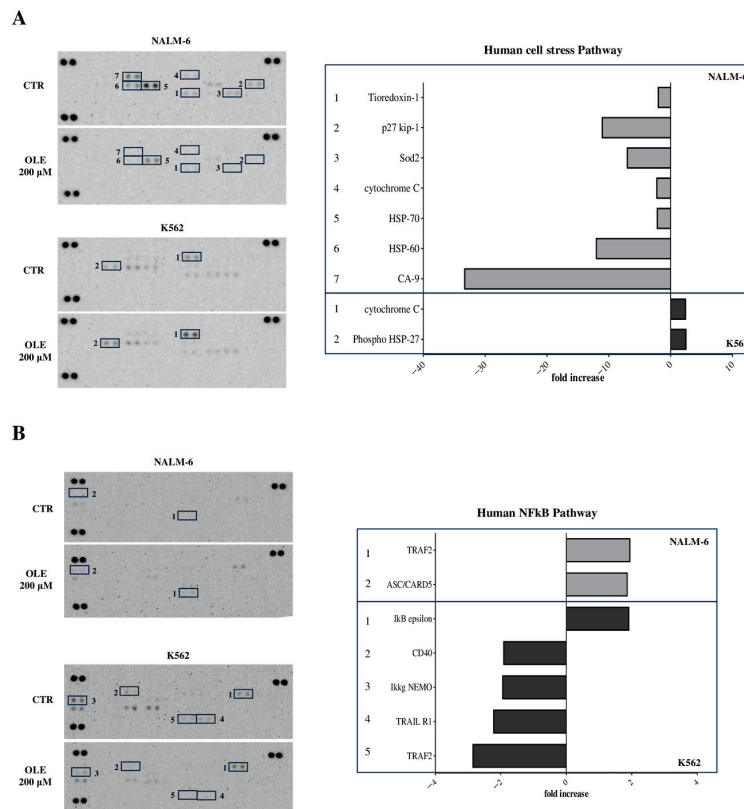
Next, we analyzed leukemic blasts from patients with AML. In contrast to ALL, but consistent with that reported for AML cell lines, OLE did not induce a significant increase in apoptotic cells, but only a slight increase in dead cells ( $1.35 \pm 1.15$ ) either at  $200 \mu\text{M}$  ( $6.7 \pm 0.9$ ,  $p = 0.05$ ) and  $400 \mu\text{M}$  ( $10.6 \pm 0.62$ ,  $p = 0.05$ ).

These results were in accordance with those obtained with B-ALL and AML cell lines and confirm that B-ALL is the most sensitive acute leukemia to OLE treatment.

### 3.4. OLE Differentially Modulated Cellular Pathways in B-ALL and AML Cell Lines

To determine which cellular pathways may be affected in response to OLE treatment, we performed antibody array analyses on cellular lysates from the NALM-6 and K562 cell lines, the most treatment-sensitive and resistant, respectively. To this end, cells were cultured in the presence of OLE  $200 \mu\text{M}$  for 24 h. The arrays selected included 139 molecules involved in cellular stress and apoptosis.

First, we investigated the expression of different proteins involved in cellular stress. As shown in Figure 4A, the expression of different proteins was down-modulated in the NALM-6 cell line after OLE treatment, including Carbonic Anhydrase (CA-9,  $-33.3$ ), Heat Shock Protein (HSP)-60 ( $-12.2$ ), p27/kip-1 ( $-11.06$ ), Superoxide Dismutase (SOD)2 ( $-7.01$ ), cytochrome C ( $-2.23$ ), HSP-70 ( $-2.17$ ), and Tioredoxin-1 ( $-2.01$ ). On the contrary, a limited effect of OLE was observed in K562 cells, with a moderate increase of cytochrome C (fold increase: 2.41) and phospho-HSP-27 (2.46) proteins.

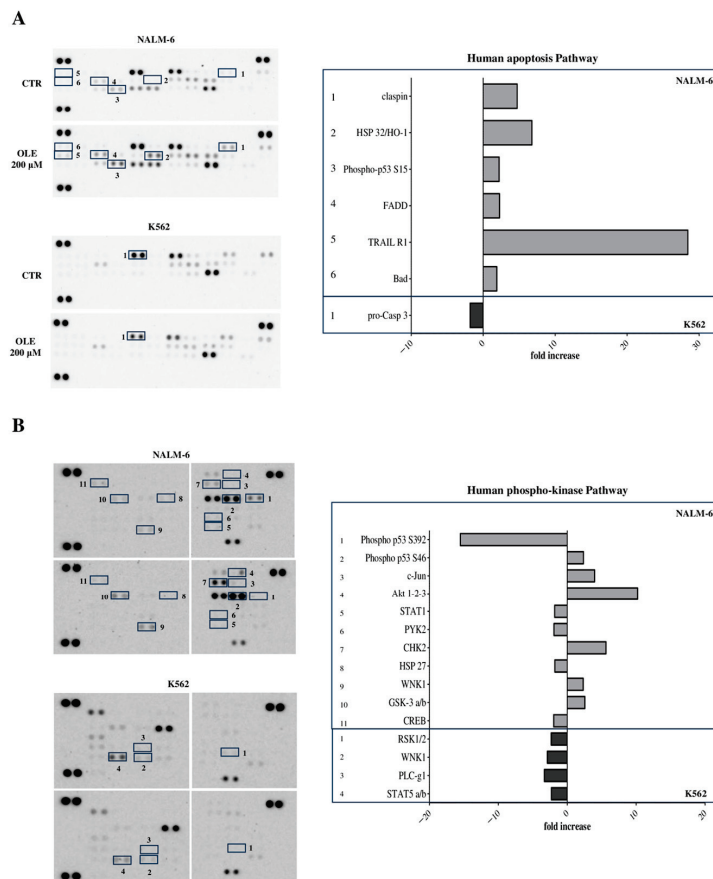


**Figure 4.** Modulation of molecules involved in the cellular stress pathways by OLE in B-ALL and AML cell lines. Antibody array was performed to analyze cellular stress (**A**) and NFkB (**B**) pathways on cellular lysates from NALM-6 and K562 cell lines cultured for 24 h in RPMI supplemented with 10% FBS in the presence or absence of OLE at 200  $\mu$ M. Raw blots are shown in the left panels, where spots for each protein (in duplicate) are numerated. On the right panels fold increase ( $>1.8$ ) and decrease ( $<-1.8$ ) are shown for each individual protein, as numerated in the left panels. Grey bars indicated proteins modulated in NALM-6 cell line, whereas black bars indicated proteins modulated in K562 cell line.

The analysis of the NFkB pathway revealed that OLE-induced up-regulation of a few molecules, including TRAF2 (1.94) and Apoptosis-associated Speck-like protein containing CARD (ASC/CARD5, 1.86) in the NALM-6 cell line (Figure 4B), whereas a higher number of molecules were modulated by OLE in K562 cells. In particular, the expression of IκB epsilon was increased (1.91), and that of Tumor Necrosis Factor Receptor Associated Factors (TRAF)2 ( $-2.85$ ), TNF-Related Apoptosis-Inducing Ligand Receptor (TRAIL R)1 ( $-2.22$ ), CD40 ( $-1.91$ ), and Iκg/NEMO ( $-1.94$ ) was down-regulated.

Next, we analyzed the apoptotic pathway. As shown in Figure 5A, the expression of multiple proteins was up-regulated by OLE in NALM-6 cells, including TRAIL R1 (28.43), Heme Oxygenases (HO)-1/HSP-32 (6.77), clapsin (4.72), phospho-p53 S15 (2.22), Fas Associated via Death Domain (FADD, 2.27), and Bad (1.89), whereas in K562 cell line we observed only a slight down-modulation of pro-caspase 3 ( $-1.81$ ). Finally, we analyzed the phospho-kinase pathway and, again, the expression of different protein was affected by OLE in NALM-6, with an increased phosphorylation of Akt 1-2-3 (10.22), CHK-2 (5.62), WNK1 (2.32), Glycogen Synthase Kinase (GSK)-3  $\alpha/\beta$  (2.55), c-jun (3.97), and phospho-p53 s46 (2.36) and decreased phosphorylation of phospho-p53 s392 ( $-15.51$ ), STAT1 ( $-1.82$ ), PYK2 ( $-1.92$ ), HSP-27 ( $-1.8$ ), and cAMP Response Element-binding protein (CREB, 1.97). In contrast, the phosphorylation of only a few kinases was decreased by OLE in K562 cells, including RSK1/2 ( $-2.33$ ), WNK1 ( $-2.88$ ), PLC- $\gamma$ 1 ( $-3.32$ ), and STAT5  $\alpha/\beta$  ( $-2.3$ ).





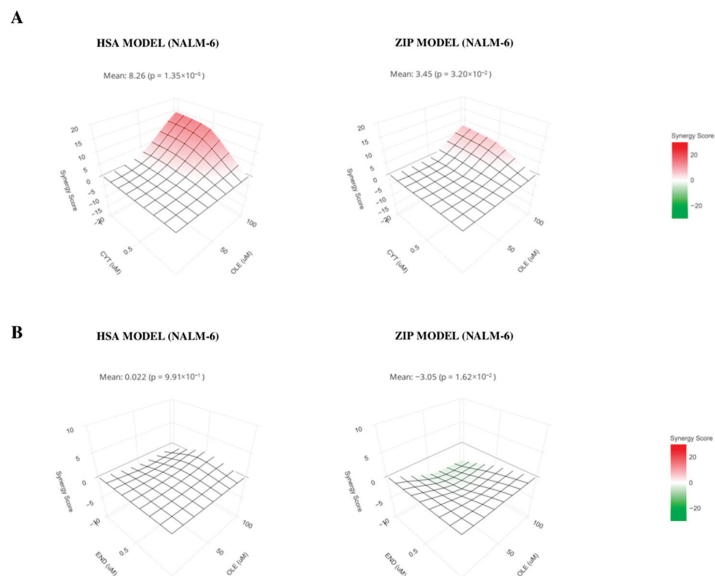
**Figure 5.** Impact of OLE on apoptosis pathways and phosphorylation of kinases in B-ALL and AML cell lines. Antibody array was performed to analyze apoptosis (A) and phosphokinase (B) pathways on cellular lysates from NALM-6 and K562 cells cultured for 24 h in RPMI supplemented with 10% FBS in the presence or absence of OLE at 200  $\mu$ M. Raw blots are shown in the left panels, where spots for each protein (in duplicate) are numerated. On the right panels fold increase ( $>1.8$ ) and decrease ( $<-1.8$ ) are shown for each individual protein, as numerated in the left panels. Grey bars indicated proteins modulated in NALM-6 cell line, whereas black bars indicated proteins modulated in K562 cell line.

### 3.5. Induction of Apoptosis in B-ALL Cells by OLE in Combination with Chemotherapeutic Drugs

On the basis of previous results, we focused on B-ALL cells to assess the potential additive and/or synergistic pro-apoptotic activity of OLE in combination with chemotherapeutic drugs commonly used to treat leukemia patients. To this end, we selected cytarabine and cyclophosphamide/endoxan as chemotherapeutic agents that, in our *in vitro* experiments, were used at concentrations of 0.5 and 1  $\mu$ M and of 0.5 and 1  $\mu$ g/mL, respectively. At the same time, NALM-6 and 697 cells were treated alone or in combination with OLE at 50 or 100  $\mu$ M. The percentage of 7AAD-/Annexin V-alive cells was evaluated by flow cytometry for each experimental condition, and data were analyzed using the SynergyFinder+ web application. The additive and synergistic effects were analyzed by the ZIP and HSA models, respectively.

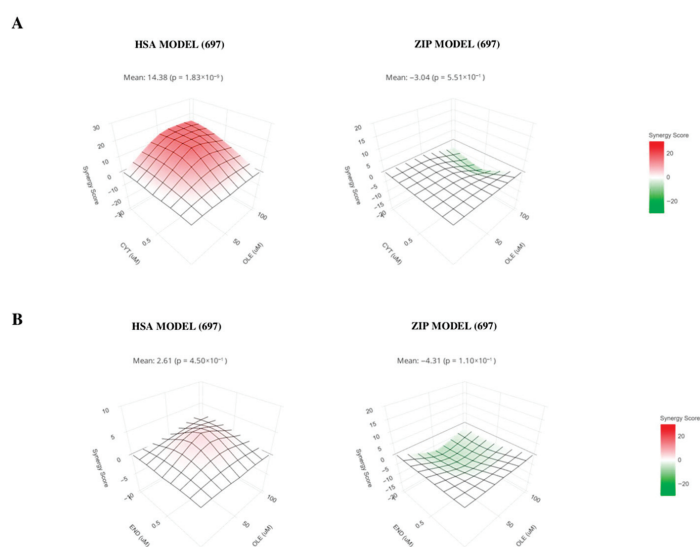
As shown in Figure 6A, the HSA model highlighted a synergistic effect of OLE in combination with cytarabine on NALM-6 cells (left panel, mean score: 8.26,  $p = 1.35 \times 10^{-5}$ ). A significant additive effect was also observed using the ZIP model (right panel, score: 3.45,  $p = 0.03$ ). Conversely, experiments performed with OLE and cyclophosphamide/endoxan revealed no synergistic effects (Figure 6B) using the HSA model (left panel, score 0.022,  $p = 0.99$ ), whereas the ZIP model suggested an antagonistic activity (right panel, score  $-3.05$ ,  $p = 0.016$ ).





**Figure 6.** OLE effects on B-ALL in combination with chemotherapeutic drugs. NALM-6 cell line was cultured 48 h in the presence or absence of OLE (50 and 100  $\mu\text{M}$ ), cytarabine (cyt 0.5 and 1  $\mu\text{M}$ ) and cyclophosphamide/endoxan (end 0.5 and 1  $\mu\text{g/mL}$ ) and the percentage of alive cells was evaluated for each experimental combination. Synergistic effects (left panels) were analyzed by HSA model for OLE/cytarabine (A) and OLE/end (B). Additive effects (right panels) were analyzed using the ZIP model for OLE/cytarabine (A) and OLE/end (B). Means and  $p$  values are shown.

As shown in Figure 7A, similar results were obtained with the 697 cells, since a synergistic effect emerged with a combined treatment of OLE and cytarabine (left panel, HSA model mean score: 14.38,  $p = 1.83 \times 10^{-9}$ ) and no significant additive activity (right panel, ZIP model, score: -3.04,  $p = 0.55$ ). No synergistic or additive effect emerged from the combined treatment with OLE and cyclophosphamide/endoxan (Figure 7B; HSA model score 2.61,  $p = 0.45$  on the left; ZIP model score -4.31,  $p = 0.11$ ).



**Figure 7.** OLE anti-tumor activity against AML in combination with chemotherapeutic drugs. 697 cell line was cultured 48 h in the presence or absence of OLE (50 and 100  $\mu\text{M}$ ) in combination with cytarabine (cyt 0.5 and 1  $\mu\text{M}$ ) or cyclophosphamide/endoxan (end 0.5 and 1  $\mu\text{g/mL}$ ) and the percentage of alive cells was evaluated for each experimental combination. Synergistic effects (left panels) were analyzed by HSA model for OLE/cytarabine (A) and OLE/end (B). Additive effects (right panels) were analyzed using the ZIP model for OLE/cytarabine (A) and OLE/end (B). Means and  $p$  values are shown.

Similar results were obtained using Bliss and Loewe models to analyze synergistic or additive effects, respectively (Supplementary Figures S1 and S2), as shown in the heat maps reported in Supplementary Figures S3–S6.

#### 4. Discussion

OLE has attracted much interest in recent years, since different studies demonstrated its efficacy in anti-cancer treatment [12,24–31]. Indeed, OLE contains a high concentration of several molecules with a direct activity against solid and hematological tumors, and/or indirect anti-tumor effects via an increase in chemotherapy efficacy, such as oleuropein [32–42], rutin [43–49], hydroxytyrosol [50–54], and tyrosol [55,56]. Among them, oleuropein represents the main polyphenol and the most effective molecule against tumor cells [31]. The concentration of these compounds in OLE used in this study is reported in Section 2, and the variability of these concentrations is very low among different batches. However, the standardization of the extract is crucial for future preclinical and clinical studies. So far, only a few studies have investigated the effects of OLE or oleuropein in the context of human leukemias [57–61]. Here, we reported that OLE has anti-proliferative and pro-apoptotic effects *in vitro* on B-ALL cell lines, whereas such activities were limited in AML and lymphoma cell lines. Furthermore, the induction of apoptosis and cell death in both B-ALL and AML cell lines was dose-dependent. More importantly, similar pro-apoptotic effects were also observed in primary leukemic blasts from patients with B-ALL but not in those from AML patients.

To clarify the molecular mechanisms underlying the anti-proliferative and pro-apoptotic effects of OLE against acute leukemias, we investigated the involvement of different cellular pathways using a panel of antibody arrays assessing the modulation of 102 molecules involved in the cellular stress, NF $\kappa$ B pathway, and apoptosis, as well as 37 phosphorylated kinases. In all of these pathways, we found a different OLE-mediated modulation between B-ALL and AML cell lines, which may explain the different and selective effects observed on proliferation and apoptosis. Indeed, B-ALL cells, more than AML cells, responded to OLE and showed greater inhibition of cell proliferation and induction of apoptosis. Such biological effects were paralleled by an increase in 6/35 and a decrease in 1/35 molecules involved in apoptotic processes in the B-ALL NALM-6 and in the AML K562 cells, respectively. In particular, the strongest effect observed in NALM-6 may be ascribed to the reduction of proteins known to be associated with the inhibition of proliferation and induction of apoptosis of cancer cells [62,63]. CA-9 is one of the most important proteins promoting different phases of cancer development, which is overexpressed in response to tumor hypoxia in many cancers. It plays a critical role in hypoxia-associated tumor acidosis and development of the metastatic phenotype, thus representing an attractive and promising target for systemic anticancer therapy [64]. Interestingly, it has recently been reported that a close relationship exists between CA-9, GSK3 $\beta$ , and AKT, which we found all modulated in the B-ALL NALM-6 cells [65]. The authors investigated the mechanism of action of ibuprofen on hypoxic cancer cells and whether ibuprofen may influence CA-9. They demonstrated that the drug down-regulated CA-9 and that such an effect was a consequence of the increased phosphorylation of AKT that led to increased inhibitory phosphorylation of GSK3 $\beta$  at Serine 9 (pS9-GSK3 $\beta$ ) [65]. Since these modulations have also been observed in our study, we may speculate that the same pathway occurred in the induction of apoptosis and cell death of B-ALL cells upon treatment with OLE. Nonetheless, it is conceivable that additional molecules participated in the final anti-tumor effects driven by OLE. Indeed, four additional proteins related to cellular stress were down-regulated by OLE, namely HSP-60 and -70, SOD2, and Tioredoxin-1. HSP-60 and -70 are abundantly present in cancer, providing malignant cells a selective advantage through the suppression

of apoptotic pathways, by regulating necrosis, interfering with tumor immunity, promoting angiogenesis, and supporting metastasis [66,67]. HSP-60 inhibits apoptosis mainly through a mitochondrial signal peptide [68], whereas HSP-70 prevents the activation of caspase [69]. Furthermore, the loss of these HSPs in normal cells, or their down-modulation in tumor cells, increases p53 expression and activates p53-dependent apoptosis, and finally, cell death [67,70]. Notably, in our study, we found that OLE modulated the phosphorylation of p53 (see below).

SOD2 is a member of the SOD family of antioxidants, which play an essential role in cellular protection against mitochondrial oxidative damage [71]; thus, its inhibition may impair the mitochondrial functions [72], increasing ROS and finally inducing apoptosis. In this context, HO-1/HSP32 and Bad, two molecules associated with oxidative stress and consequent apoptosis [73], were up-regulated by OLE, while not increasing the amount of cytochrome C. However, it is worth mentioning that we assessed the modulation of all these molecules in total cellular extracts; thus, we cannot exclude that the subcellular compartmentalization of cytochrome C changed upon OLE treatment and, in particular, its release from mitochondria. Thioredoxin-1 physiologically inhibits apoptosis by regulating the activities of caspases and procaspases through S-nitrosylation, and its inhibition was associated with the induction of apoptosis of tumor cells [74]. Controversial effects may be ascribed to p27/Kip1, down-modulated by OLE in NALM-6 cells, which was identified as a pivotal player during metabolic stress. Accordingly, p27 is known to be involved in pathways correlated with autophagy and apoptosis [75], although with dual effects depending on the cellular context. Notably, Russo et al. [76] investigated the mechanisms by which polyphenols interact with cellular processes by modulating the signal transduction pathways. They reported that the majority of polyphenols induced a p27 increase in a panel of tumor cell lines (e.g., promyelocytic leukemia, ovarian carcinoma, and multiple myeloma), but not in pulmonary mesothelioma and breast carcinoma. Nonetheless, the final result was the induction of apoptosis and cell cycle arrest in all cell lines.

Six out of the 35 proteins analyzed and associated with the induction of apoptosis have been found to be up-regulated in B-ALL upon OLE treatment, whereas only one was modulated in K562 cells. Among them, a strong up-regulation of TRAIL R1 was paralleled by the increase of FADD and TRAF2. The apoptosis induced by the TRAIL-R1 pathway needs the recruitment of the adaptor protein FADD to the cytoplasmic region of the receptor, followed by recruitment of pro-caspase-8 or pro-caspase-10. The formation of this death-inducing signaling complex triggers cleavage and activation of caspase-8 or caspase-10, which in turn activate downstream caspase-3, -6, and -7, and apoptosis [77]. Although we have not studied the modulation of caspases in the present work, we may hypothesize that this apoptotic pathway may also be utilized by OLE. In addition, the observed up-regulation of TRAIL R1 may render B cells sensitive to TRAIL-induced apoptosis [78].

The NFκB pathway was studied since we have previously reported that OLE-induced apoptosis in neuroblastoma cell lines by activating NFκB through phosphorylation of the p65 subunit [12]. In B-ALL cells, we did not observe this modulation, whereas we observed an increase in TRAF2 and ASC/CARD5. TRAF2, which is a key player in cancer cell survival, has been described as a tumor promoter, but may also act as a tumor suppressor [79]. Thus, it is conceivable that the regulation of TRAF2 induced by OLE may be implicated in the inhibition of proliferation and induction of apoptosis in our experimental setting. ASC/CARD5, which is an adaptor protein involved in the activation of apoptosis, was also up-regulated, as well as FADD, another crucial adaptor protein connecting death receptors to caspases [80]. Notably, upon OLE treatment of the K562 AML cell line, we highlighted a negative modulation of five different molecules, all involved in

the TRAF2 signaling (i.e., TRAF2, TRAIL R1, I $\kappa$ B epsilon, CD40, and I $\kappa$ kg/NEMO) [81,82]. These data are consistent with the opposite effects driven by OLE on B-ALL and AML cells.

Regarding the phosphorylation pattern, we detected a peculiar modulation of p53, a key regulator of apoptosis, affected by OLE in B-ALL, with enhanced phosphorylation at S15 and S46 and decreased phosphorylation at S392. It has been reported that the phosphorylation of the S46 (and also S15) residue is crucial for p53 activation and its binding to DNA and transcription of apoptotic target genes [83–85]. In contrast, phosphorylation of S392 is required for the mitochondrial localization and transcription-independent apoptotic function of p53 [86]. Thus, the down-modulation of the p53 phosphorylation at this site suggested that in our experimental setting, p53 predominantly acts in the nucleus through the activation of transcription of apoptosis-related genes. In addition, OLE induced the phosphorylation (and thus activation) of CHK2, which is a tumor suppressor involved in the induction of apoptosis [87]. Two additional factors that may lead either to apoptosis or survival of cancer cells have been found to increase, and are STAT1 [88] and WNK1 [89]. Conversely, the phosphorylation of CREB and HSP-27, which promote cell survival and inhibit apoptosis, was reduced by OLE [90,91].

We also found increased phosphorylation of c-jun and AKT upon OLE treatment. These results are difficult to explain, since the phosphorylation of c-jun and AKT usually promotes cell survival [92,93]. We may hypothesize that B-ALL cells reacted to OLE through the phosphorylation of these two factors in order to counterbalance the induction of apoptosis. Furthermore, the phosphorylation of PYK2, which mediated anti-apoptotic signaling of AKT, is decreased by OLE in B-ALL. Beyond the abovementioned relationship between CA-9, GSK3 $\beta$ , and AKT, it is to be taken into account that all these pathways underlying apoptosis and cell death are very complex, with an impressive number of molecules involved. Thus, it is conceivable that OLE triggers apoptosis as a result of a balance of several modulated molecules, which interact with each other. Here, we demonstrated for the first time to our knowledge that OLE, similarly to what we previously reported in neuroblastoma cells, acts as direct anti-tumor agent in B-ALL and with a lesser extent in AML cells, highlighting the potential molecular mechanisms involved, which are related to the induction of cellular stress and apoptosis, and are conceivably mediated by the extrinsic pathway, as witnessed by the highest modulation of TRAIL R1. Nonetheless, to unambiguously demonstrate such a conclusion, additional studies are required involving analysis of caspases and/or experiments focused on blocking death receptors such as TRAIL. The impact of OLE on these cellular pathways was modest in AML, resulting in a greater resistance to induction of apoptosis and inhibition of cell proliferation. Indeed, most of the proteins in AML were modulated in an opposite way compared to what was observed in B-ALL.

In summary, the effects of OLE on B-ALL cells are mainly related to (i) down-regulation of proteins involved in cancer progression, antioxidants, and heat-shock proteins, (ii) up-regulation of proteins related to apoptosis, and (iii) alteration of protein phosphorylation. The final effects based on these pathways are the inhibition of cell proliferation and induction of apoptosis. Thus, the resistance of AML cells to OLE is conceivably related to the minimal or absent alteration of these molecular pathways and, in addition, to a modulation of key proteins in the opposite way.

Interesting and promising data were obtained in experiments performed with OLE in combination with chemotherapeutic drugs. We selected two standard-of-care chemotherapeutic agents used for the management of acute leukemias and tested clinical-grade cytarabine and cyclophosphamide, as spare aliquots after therapeutic use in pediatric leukemic patients. In particular, cytarabine is currently used to treat patients with relapse/refractory disease [94,95], whereas cyclophosphamide represents a standard of care

treatment for B-ALL patients [96]. Our data suggested that OLE may be used as a food supplement for these patients in combination with the drug to implement its anti-tumor efficacy. Notably, the presence or absence of synergistic and additive effects of OLE with cytarabine and cyclophosphamide, respectively, may conceivably be related to their different mechanisms of action. Indeed, cyclophosphamide acts as an alkylating agent, interfering with DNA replication and transcription of mRNA [97]. In contrast, cytarabine induces DNA damage through the incorporation of similar pyrimidine and purine nucleosides, leading to the activation of two kinase pathways, namely ataxia telangiectasia mutated and Rad3-related Checkpoint kinase 1 (ATR-CHK1) and ataxia telangiectasia mutated Checkpoint kinase 2 (ATM-CHK2), which may lead to DNA repair or apoptosis [98,99]. Due to the finding that OLE upregulated CHK2 in ALL (but not in AML) cell lines, we may speculate that the synergistic/additive effects observed with cytarabine may be related to the concomitant induction of CHK2 in target cells.

Pharmacokinetics and/or bioavailability of the different compounds present in OLE must be taken into account in view of clinical perspectives. Previous studies have addressed this issue, demonstrating that oleuropein and hydroxytyrosol are stable after digestion and reach their peak in the plasma of subjects consuming OLE within 20–75 min, and are still present 2–3 h after ingestion [100,101].

## 5. Conclusions

We here showed that exclusively the combination of OLE with cytarabine led to induction of apoptosis with effects ranging from synergy to addiction, whereas no synergic nor additive effects were observed when OLE was tested in combination with cyclophosphamide. These findings suggest that OLE not only has autonomous anti-tumor potential but can also increase the effectiveness of existing treatments *in vitro*. Although these data represent a proof-of-concept, we believe that a deep mechanistic insight aimed at validating the data raised from this study should be addressed in the future. Furthermore, our results highlighted a potential use of OLE in the clinical setting and management of pediatric B-ALL patients. Notably, recent technologies delineate areas for new interactions between food engineering and the medical field to aid in the best efficacy of cancer drugs, and to ensure more attractive and proper nutrition for cancer patients. In this view, it is to be taken into mind that OLE is an aqueous nutraceutical that may be easily administered as a food integrator without any side effects. Thus, we may envisage clinical settings with the use of OLE as an adjuvant compound during chemotherapy with cytarabine in pediatric oncology, taking advantage of its anti-proliferative and pro-apoptotic effects on B-ALL cells described here and of the already known anti-inflammatory properties. However, this hypothesis needs to be validated in future clinical studies to confirm the potential effectiveness of OLE as a food supplement in combination with existing therapies for B-ALL patients.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu18010015/s1>, Figure S1: OLE displays synergistic effects with cytarabine; Figure S2: OLE did not show any synergy or additive effects with cyclophosphamide; Figure S3: Heat maps of OLE/cytarabine in NALM-6 cells; Figure S4: Heat maps of OLE/cyclophosphamide synergistic effects in NALM-6 cells; Figure S5: Heat maps of OLE/cytarabine synergistic effects in 697 cells; Figure S6: Heat maps of OLE/cyclophosphamide synergistic effects in 697 cells.

**Author Contributions:** Conceptualization: F.M.; Data curation: I.A., L.C. and F.M.; Formal analysis: I.A., L.C. and F.M.; Funding acquisition: I.A. and F.M.; Investigation: I.A., L.C. and F.M.; Methodology: I.A., F.M., C.B. and E.C.; Project administration: F.M.; Resources: F.M., D.M. and I.A.; Software: F.M., I.A. and C.B.; Supervision: F.M.; Validation: F.M. and I.A.; Writing—original draft: I.A., C.B., D.M.



and F.M.; Writing review and editing: I.A., C.B., D.M., L.C., E.C. and F.M. All authors have read and agreed to the published version of the manuscript.

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

## Abbreviations

The following abbreviations are used in this manuscript:

|       |                                                   |
|-------|---------------------------------------------------|
| OLE   | Olive leaf extract                                |
| B-ALL | B-acute lymphoblastic leukemias                   |
| ROS   | Reactive oxygen species                           |
| AML   | Acute myeloid leukemias                           |
| CFSE  | Carboxyfluorescein diacetate N-succinimidyl ester |

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## Article

# Antitumor, Antioxidant, and Hepatoprotective Effects of Grape Seed Oil Nanoemulsion as a Dietary Phytochemical Intervention in Ehrlich Solid Tumors

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**Abstract: Background/Objectives:** Grape seed oil (GSO) is a potent source of dietary phytochemicals, particularly polyphenols and flavonoids, known for their health-promoting properties. This study aims to investigate the anticancer and hepatoprotective effects of a nanoemulsion formulation of grape seed oil (GSONE), to enhance the efficacy and bioavailability of its phytochemical constituents against solid tumors. **Methods:** Ninety female Swiss albino mice were divided into six groups: control, alone, GSONE alone, Ehrlich solid tumor (EST), EST treated with GSO, and EST treated with GSONE. Tumor development, growth performance, serum biochemistry, antioxidant status, hepatic histopathology, apoptotic gene expression, and flow cytometry analyses were assessed following 30 days of daily oral treatment. **Results:** GSONE significantly reduced tumor weight and volume (52.9%) and more effectively counteracted tumor-induced body weight loss than crude GSO. Treatment with GSONE normalized serum protein levels and improved liver function markers (AST, ALT, ALP, total bilirubin) to near-control values. Tumor markers (AFP, CEA) and oxidative stress indices (MDA, 8-OHdG) were markedly decreased, while activities of hepatic antioxidants (SOD, CAT, GPx, GSH) were restored. GSONE enhanced gene expression of pro-apoptotic markers (Bax, TP53, caspase-3, caspase-9), suppressed anti-apoptotic Bcl-2, and significantly increased the proportion of p53- and cleaved caspase-3-positive tumor cells. Liver histopathology and ultrastructure demonstrated normalized morphology and reduced damage in GSONE-treated mice. Multivariate analyses confirmed GSONE's restorative effect compared to raw GSO. **Conclusions:** The delivery of dietary phytochemicals via nanoemulsion significantly enhances antitumor and hepatoprotective actions in a preclinical solid tumor model. These findings support the potential of phytochemical-rich edible oils, enhanced by nanotechnology, for dietary prevention and adjunctive management of cancer.



**Keywords:** grape seed oil; nanoemulsion; Ehrlich solid tumor; antioxidant activity; antitumor activity

## 1. Introduction

Dietary phytochemicals, naturally occurring bioactive compounds in plant-derived foods, have gained increasing recognition for their role in cancer chemoprevention and therapy [1]. Polyphenols, flavonoids, and related compounds modulate redox homeostasis, regulate apoptosis, and influence multiple molecular pathways involved in carcinogenesis [2]. Accumulating evidence supports that phytochemical-rich diets may lower cancer risk and improve disease outcomes [3]. Recent advances in nutrition and pharmaceutical technology have underscored the importance of optimizing the delivery, stability, and bioavailability of these compounds to maximize their therapeutic impact [4,5].

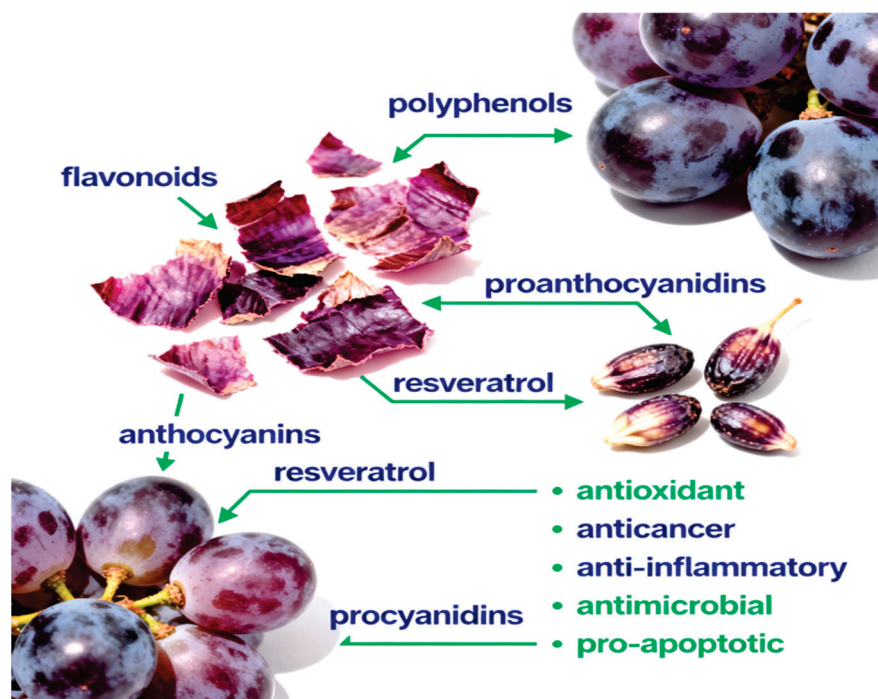
Grape seeds and their extracted oil are valuable agricultural by-products of grape processing, recognized for their high nutritional value and rich phytochemical composition [6]. Grape seed oil (GSO) is obtained mainly by cold pressing or solvent extraction of *Vitis vinifera* seeds, which contain approximately 8–20% oil. This oil is particularly valued for its delicate flavor, high smoke point, and balanced lipid composition, making it useful for both culinary and therapeutic applications [7,8].

Nutritionally, grape seed oil is rich in polyunsaturated fatty acids (PUFAs), which account for nearly 85–90% of its fatty acid content. Linoleic acid (C18:2, *n*-6) constitutes around 68–76%, while oleic acid (C18:1, *n*-9) represents 14–15%, accompanied by small fractions of alpha-linolenic, palmitic, and stearic acids [9]. This lipid profile contributes to maintaining cardiovascular and metabolic health by promoting favorable lipid metabolism and supporting membrane fluidity [7]. GSO is also an exceptional natural source of vitamin E (1–53 mg/100 g), mainly in the form of  $\gamma$ -tocopherol and tocotrienols, compounds known for their potent antioxidant and anti-aging properties [10].

In addition to lipophilic antioxidants, grape seed oil contains trace hydrophilic bioactives such as polyphenols (catechins, epicatechins, procyanidins, and resveratrol derivatives) and phytosterols ( $\beta$ -sitosterol, campesterol, and stigmasterol) [11]. These components contribute synergistically to its broad spectrum of biological activities, including antioxidant, anti-inflammatory, anti-obesity, hepatoprotective, and antitumor effects demonstrated in both in vitro and in vivo models [12,13]. Figure 1 provides an overview of the main classes of bioactive compounds present in grape skins and seeds, and summarizes their documented health-promoting actions.

Beyond its dietary importance, grape seed oil has been incorporated into several pharmaceutical and cosmeceutical formulations, including (a) nanoemulsions and liposomal carriers, which enhance solubility, stability, and absorption of natural antioxidants [14,15], and (b) topical and oral nutraceutical preparations for skin protection, wound healing, lipid regulation, and systemic antioxidant support [16–18]. These formulations aim to overcome the poor aqueous solubility and oxidative instability associated with the native oil, thereby improving its therapeutic performance [19,20].

Despite GSO's diverse health-promoting constituents, its high *n*-6 PUFA content predisposes it to oxidation, limiting its direct storage and industrial use [21]. Therefore, nanoformulation approaches, such as emulsions and encapsulated systems, have attracted substantial attention for enhancing bioavailability and shelf stability [20,22,23]. In this context, grape seed oil nanoemulsion (GSONE) represents a novel strategy to potentiate the therapeutic effects of dietary phytochemicals while maintaining biocompatibility and safety [24].



**Figure 1.** The phytochemical composition of grape skins and seeds includes polyphenols, flavonoids, proanthocyanidins, anthocyanins, resveratrol, and procyanidins. These compounds exhibit diverse biological activities, including antioxidant, anticancer, anti-inflammatory, antimicrobial, and pro-apoptotic effects, which contribute to their potential to promote health and prevent disease.

Experimental cancer models remain indispensable for preclinical testing of such nutritional interventions. The Ehrlich solid tumor (EST) model, derived from murine mammary adenocarcinoma, closely mimics the clinical and histopathological features of aggressive human breast tumors, such as rapid proliferation, high malignancy, short biological cycles, and lack of tumor-specific transplantation antigens [25]. Both solid and ascitic forms of Ehrlich carcinoma are routinely used to investigate tumor biology and assess novel antitumor strategies [26–28]. Tumor progression in the EST model is known to trigger systemic metabolic imbalances and organ dysfunction, notably hepatic and renal oxidative stress, DNA damage, and cellular injury [29]. Such alterations highlight the pivotal role of reactive oxygen species (ROS) in carcinogenic processes and reinforce the protective role of natural antioxidants in maintaining redox balance and genomic stability [30,31]. Dietary antioxidants thus represent a physiologically relevant and ethically sustainable avenue for developing biocompatible cancer-preventive adjuvants [32].

The present study aimed to develop a GSONE to overcome the intrinsic limitations of native GSO and to investigate its chemopreventive, antitumor, and hepatoprotective properties in a murine EST model. We further sought to elucidate the underlying molecular mechanisms by assessing apoptosis-related gene expression, hepatic redox indices, and histopathological alterations following chronic treatment.

## 2. Materials and Methods

### 2.1. Preparation of Grape Seed Oil Nanoemulsion

Organic grape seed oil (GSO) was obtained from AB Chem Company (Mansoura City, Egypt). A monolayer nanoemulsion was formulated using 20% GSO. Briefly, the oil phase was gradually mixed with 68 mL of an aqueous phase containing 10% Tween 80 (surfactant) and 2% ethanol (co-surfactant) under gentle stirring at 25 °C. Water was added at a controlled rate of 1.0 mL/min to maintain uniform emulsification. The emulsion was

subjected to ultrasonic bath treatment for 30 min, followed by final homogenization with a probe-type ultrasonic homogenizer (Sonics Vibra-Cell, VC 505, Newtown, CT, USA) at 60% amplitude for 5 min, with a one-second on and one-second off cycle. This process yielded a stable GSO nanoemulsion (GSONE).

For morphological analysis, freshly prepared GSONE samples were visualized using a 160 kV transmission electron microscope (JEOL JEM-2100, Akishima, Japan). Image acquisition and processing were performed with Soft Imaging Viewer and Digital Micrograph (Gatan Microscopy Suite, version 2.11.1404.0). Particle size distribution (Z-average), polydispersity index (PDI), and zeta potential were determined using a Zeta sizer Nano ZS analyzer (Malvern Instruments, Malvern, UK).

## 2.2. Induction of EAC in Mice

The Ehrlich Ascites Carcinoma (EAC) cell line was obtained from the National Cancer Institute (NCI), Cairo, Egypt. To preserve cell viability, EAC cells were serially passaged *in vivo* by intraperitoneal injection of  $2.5 \times 10^6$  cells into female Swiss albino mice every 10 days, following the protocol described by Yilmaz et al. [33].

Only female mice were selected for this study because the EAC model originates from murine mammary tissue and most closely simulates the biological behavior, treatment response, and progression patterns of breast cancer seen in women. Utilizing females not only reflects the clinical distribution of breast cancer but also conforms to standard EAC experimental practices, thereby enhancing the translational relevance and scientific rigor of antitumor investigations [25,34].

Mice were anesthetized with intraperitoneal administration of ketamine (80 mg/kg) and xylazine (10 mg/kg). Ascitic fluid was collected from EAC-bearing donor mice on days 7 or 8 post-inoculation under identical anesthesia conditions. Cell viability was assessed using a hemocytometer and 0.4% trypan blue dye exclusion, as described previously [35]. The viable cell suspension was then adjusted to  $2.5 \times 10^6$  cells in 0.2 mL phosphate-buffered saline (PBS) for subsequent inoculation into experimental mice.

## 2.3. Animal Handling and Experimental Protocol

A total of ninety healthy female Swiss albino mice (*Mus musculus*; mean body weight: 21.61 g) were provided by the Faculty of Medicine, Mansoura University, Egypt. Animals were housed in plastic cages under controlled laboratory conditions (25 °C, 45% relative humidity, 12 h light/dark cycle) with *ad libitum* access to food and water. Mice were allowed to acclimate for one week under these standardized conditions. The overall experiment timeline is summarized in Figure 2.

| Phase                    | Procedure                                                                                                                               | Duration              |
|--------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Acclimatization          | Housing, daily monitoring                          | 7 days                |
| Tumor induction          | EAC inoculation (0.2 mL, $2.5 \times 10^6$ cells)  | Day 1                 |
| Treatment administration | Oral GSO/GSONE (4 mL/kg/day)                        | 30 days               |
| Monitoring               | Body weight, tumor growth, health status                                                                                                | Throughout experiment |
| Sample collection        | Euthanasia and tissue collection (see below for details)                                                                                | Day 30                |

**Figure 2.** The overall study timeline and protocol were followed in this experiment.

All animal care and procedures were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals (8th edition, NRC, 2011) and the International Guiding Principles for Biomedical Research Involving Animals (1985).” All animal procedures were reviewed and approved by the “Research Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt (approval code: MU-ACUC; VM.R.25.10.247).”

A preliminary pilot study was conducted to assess the safety margin of grape seed oil (GSO) in healthy mice, with oral doses of 1, 2, 3, and 4 mL/kg/day administered for 30 consecutive days. The upper dose limit was selected based on previous reports of hepatoprotective efficacy in mice [36]. Throughout, animals were monitored for mortality, behavior, and toxicity. As no adverse effects were observed at 4 mL/kg/day, this dose was used for all subsequent experiments.

Mice were randomly allocated into six groups ( $n = 15$  each):

- Group 1 (Control): received no treatment.
- Group 2 (GSO): received GSO orally at 4 mL/kg/day for 30 days.
- Group 3 (GSONE): received GSO nanoemulsion (GSONE) orally at the same dose.
- Group 4 (EST): inoculated intramuscularly with 0.2 mL of EAC cell suspension ( $2.5 \times 10^6$  cells) in the right thigh on day 1 and left untreated thereafter.
- Group 5 (EST + GSO): received daily oral GSO (4 mL/kg) for 30 days following EAC inoculation.
- Group 6 (EST + GSONE): received daily oral GSONE (4 mL/kg) for 30 days following EAC inoculation.

The sample size for each group was determined with reference to previous EAC murine studies, in which group sizes of 10–12 animals were found sufficient to detect biological and tumor-related differences. To achieve a statistical power of at least 80% for pairwise group comparisons at a significance level of  $\alpha = 0.05$ , and to accommodate potential attrition due to post-inoculation mortality (anticipated at 10–20%), the group size was set at 15. This design provides adequate power for one-way ANOVA analysis across six groups (total  $n = 90$ ), aligning with established international standards for rigor and ethical animal use in preclinical research [37,38]. No specific pre-established inclusion or exclusion criteria were applied in this study; all animals enrolled in the study completed the experimental protocol.

#### 2.4. Assessment of Body Weight, Tumor Weight, and Tumor Volume

Body weights were recorded for all animals at baseline (day 0) and at the conclusion of the experimental period. Net final body weight was determined by subtracting the excised tumor mass from the final body weight. Tumor volume was measured at regular intervals from day 8 to day 30 in the EST, EST + GSO, and EST + GSONE groups using digital Vernier calipers. Tumor dimensions (major axis, B; minor axis, A) were used to calculate volume according to the formula:  $TV \text{ (mm}^3\text{)} = 0.52 \times A \times B^2$  [39]. On day 30, animals were euthanized, and tumors were excised and weighed to determine the final tumor weight.

#### 2.5. Sample Collection

At the end of the experimental period, 10 randomly selected mice from each group were fasted for 10 h and anesthetized with inhaled tetrahydrofuran to minimize distress and ensure consistent blood collection. The remaining mice ( $n = 5$  for each group) served as contingency reserves in case of mortality, unavailable samples, or insufficient tissue/RNA yields. Animals were euthanized by cervical dislocation. Blood was collected from the retro-orbital venous sinus into non-heparinized tubes, allowed to clot at room temperature,

and centrifuged at  $3000 \times g$  for 10 min. Serum was separated and immediately stored in 0.5 mL Eppendorf tubes for subsequent biochemical analyses.

## 2.6. Serum Biochemical Analysis

Serum biochemical parameters were measured using commercially available diagnostic kits, according to the manufacturers' protocols. Analytes included total protein (TP; CBP007-K), albumin (MET-5017), total bilirubin (TB; BR 1001), alkaline phosphatase (ALP; CBA-301), alanine aminotransferase (ALT; MET-5123), and aspartate aminotransferase (AST; MET-5127); globulin was calculated as the difference between total protein and albumin. Alpha-fetoprotein (AFP) was quantified using the mini-VIDAS<sup>®</sup> automated ELFA system (Biomérieux, Marcy-L'Étoile, France), while carcinoembryonic antigen (CEA) was measured with a Mouse CEA ELISA Kit (MyBioSource, San Diego, CA, USA), both per manufacturers' instructions.

## 2.7. Assessment of Antioxidant Status and DNA Oxidative Damage

Liver samples were washed in 1.15% ice-cold potassium chloride to remove blood residue. Specimens destined for biochemical assays were rapidly frozen at  $-20^{\circ}\text{C}$ , and others were fixed in 10% neutral-buffered formalin for microscopy. Homogenization was performed in ice-cold phosphate buffer (pH 7.4), followed by centrifugation at 4000 rpm ( $4^{\circ}\text{C}$ , 15 min). Supernatants were used to determine activities of superoxide dismutase (SOD; Cat. No. SD 2521), catalase (CAT; CA 2517), glutathione peroxidase (GSH-Px; GP 2524), and reduced glutathione (GSH; GR 2511). Lipid peroxidation was measured as malondialdehyde (MDA) by TBARS assay (MDA; MD 2529). DNA oxidative damage was assessed by quantifying 8-hydroxy-2'-deoxyguanosine (8-OHdG) using a competitive ELISA kit (Trevigen, Gaithersburg, MD, USA). Absorbance was measured at 450 nm, and results were calculated from a standard curve, expressed as nanograms per milligram of tissue protein.

## 2.8. Gene Expression Analysis of Apoptotic Markers

Total RNA was extracted from tumor tissues of mice bearing EST using the GeneJET<sup>™</sup> RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration and purity were quantified by spectrophotometry (NanoDrop, Wilmington, DE, USA), and integrity was confirmed by agarose gel electrophoresis, which showed distinct 28S and 18S rRNA bands with a 2:1 intensity ratio. First-strand complementary DNA (cDNA) was synthesized from 5  $\mu\text{g}$  of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Gene-specific primers for *Bax*, *Bcl-2*, *TP53*, *Caspase-3*, *Caspase-9*, and the housekeeping gene Glyceraldehyde phosphate dehydrogenase (*GAPDH*) (Table 1) were designed with NCBI Primer-BLAST and synthesized by Invitrogen (Carlsbad, CA, USA). *GAPDH* served as the internal control for normalization. Primer sequences were based on methodologies reported by Mohamed et al. [40] and Gencer et al. [41].

**Table 1.** Sequences of Primers Employed in qRT-PCR for Target Gene Amplification.

| Gene         | Sequences (5'-3')                                    | Length (bp) |
|--------------|------------------------------------------------------|-------------|
| <i>Bax</i>   | F: GTCTCCGGCGAATTGGAGAT<br>R: ACCCGGAAGAAGACCTCTCG   | 100         |
| <i>Bcl-2</i> | F: CATCGCCCTGTGGATGACTG<br>R: GGCCATATAGTTCCACAAAGGC | 95          |



Table 1. Cont.

| Gene             | Sequences (5'-3')                                      | Length (bp) |
|------------------|--------------------------------------------------------|-------------|
| <i>TP53</i>      | F: CCCCTGTCATCTTTTGTCCCT<br>R: AGCTGGCAGAATAGCTTATTGAG | 137         |
| <i>Caspase-3</i> | F: ACTGGAATGTCAGCTCGCAA<br>R: GCAGTAGTCGCCTCTGAAGA     | 270         |
| <i>Caspase-9</i> | F: ACGTGAACCTTCTGCCCTTCC<br>R: GGTCGTTCTTCACCTCCACC    | 117         |
| <i>GAPDH</i>     | F: GTATCGGACGCCTGGTTAC<br>R: CTTGCCGTGGGTAGAGTCAT      | 128         |

F: Forward; R: Reverse; bp: base pair; *Bax*: Bcl-2-associated X protein; *Bcl-2*: B-cell lymphoma 2; *TP53*: Tumor Protein 53; *Caspase-3/9*: Cysteine-aspartic acid protease-3/9; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

### 2.9. Flow Cytometric Analysis of p53 and Caspase-3 Expression

Single-cell suspensions were prepared from EST of control and treated mice (GSO, GSONE) to assess apoptotic pathway activation by quantifying Tp53 and cleaved caspase-3 expression. Excised tumor tissues were minced under sterile conditions, enzymatically dissociated in 1 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) in PBS (pH 7.4) at 37 °C with gentle agitation for 30 min, and then filtered through a 70 µm nylon mesh. Cells were washed twice in ice-cold PBS (centrifuged at 300× g for 5 min), then fixed and permeabilized using the Cytfix/Cytoperm kit (BD Biosciences, Franklin Lakes, NJ, USA). For p53 detection, permeabilized cells were incubated with an Alexa Fluor 488-conjugated anti-p53 antibody (Abcam, ab225239) according to the manufacturer's protocol. Cleaved caspase-3 was detected with a phycoerythrin (PE)-conjugated anti-cleaved caspase-3 antibody (Abcam, ab32042).

To ensure analytical rigor and reproducibility, cell viability was monitored throughout the sample preparation process. Appropriate controls, including unstained, isotype-matched, and single-color compensation controls, were used to gate the data and confirm antibody specificity accurately. Instrument calibration was performed daily using standardized fluorescent beads, and acquisition and compensation settings were standardized for all samples. Each analysis included at least 10,000 single, viable cell events, and doublet discrimination was routinely applied during gating. Data were collected on a BD FACSCanto™ II flow cytometer (BD Biosciences) and analyzed with FlowJo™ software (version 10.8.1). Mean fluorescence intensity (MFI) and the percentage of positively stained cells were determined for each marker, enabling comparisons among the study groups.

### 2.10. Histopathological Analysis

After fixation in 10% neutral-buffered formalin, liver tissues were dehydrated using a graded ethanol series (70%, 80%, 90%, and 100%, one hour per step), then cleared in two successive xylene baths before paraffin embedding. Specimen identity was carefully maintained throughout processing, and all samples were checked at receipt for correct labeling and presence of adequate fixative to avoid pre-analytic errors. Paraffin blocks were sectioned using a rotary microtome at an approximate thickness of 5 µm. Serial sections were mounted, stained with hematoxylin and eosin (H&E), and examined microscopically. Each batch of staining included an established normal liver tissue section as an internal control to ensure consistency of stain quality and interpretation.

Microscopic evaluation was performed using a high-resolution digital camera to document representative fields of interest. For semi-quantitative assessment, liver lesions were graded in three mice per group, analyzing three sections per slide and four randomly selected fields per section at 400× magnification (totaling 12 fields per animal). All



fields were scored independently by a blinded observer using a standardized four-grade histopathological scoring system (0 = none, 1 = mild, 2 = moderate, 3 = severe; see Table 2), and the mean lesion score for each mouse was calculated by averaging across all examined fields. Quality of tissue sections and staining was monitored throughout, and any slide with a technical artifact or inadequate staining was reprocessed and reassessed to maintain high analytical standards.

**Table 2.** Semi-quantitative scoring of hepatic damage was based on a four-point grading scale.

| Score        | Hepatic Lesions Were Graded Using an Integrated Semi-Quantitative Scoring System.                                                                                                                                            |
|--------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 0 (none)     | Histological analysis revealed normal architecture.                                                                                                                                                                          |
| 1 (mild)     | Hepatocellular degeneration and necrosis were occasionally observed, accompanied by minimal or absent inflammatory infiltration and rare vascular congestion.                                                                |
| 2 (moderate) | Moderate vacuolar degeneration, scattered inflammatory cell infiltration, multifocal hepatocellular necrosis, and mild blood vessel congestion were shown in the hepatic tissue.                                             |
| 3 (severe)   | Diffuse and severe degenerative alterations were shown in hepatic tissue, along with widespread infiltration of leukocytes, extensive hepatocellular necrosis, and moderate to severe congestion of the hepatic vasculature. |

#### 2.11. Transmission Electron Microscopy (TEM)

Liver samples were fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) at 4 °C for 24 h to preserve their ultrastructure. Following thorough rinsing, specimens were post-fixed in 1% osmium tetroxide for 2 h. Tissues were dehydrated through a graded ethanol series (50–100%) and cleared in acetone before being embedded in epoxy resin. Ultrathin sections (60–70 nm) were cut using a diamond knife on an ultramicrotome. Sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 2100 transmission electron microscope (Japan) operated at 160 kV. Each processing batch included internal controls, and section quality was confirmed before imaging. Representative regions were selected for examinations, and digital images were acquired to minimize operator bias.

#### 2.12. Statistical Analysis

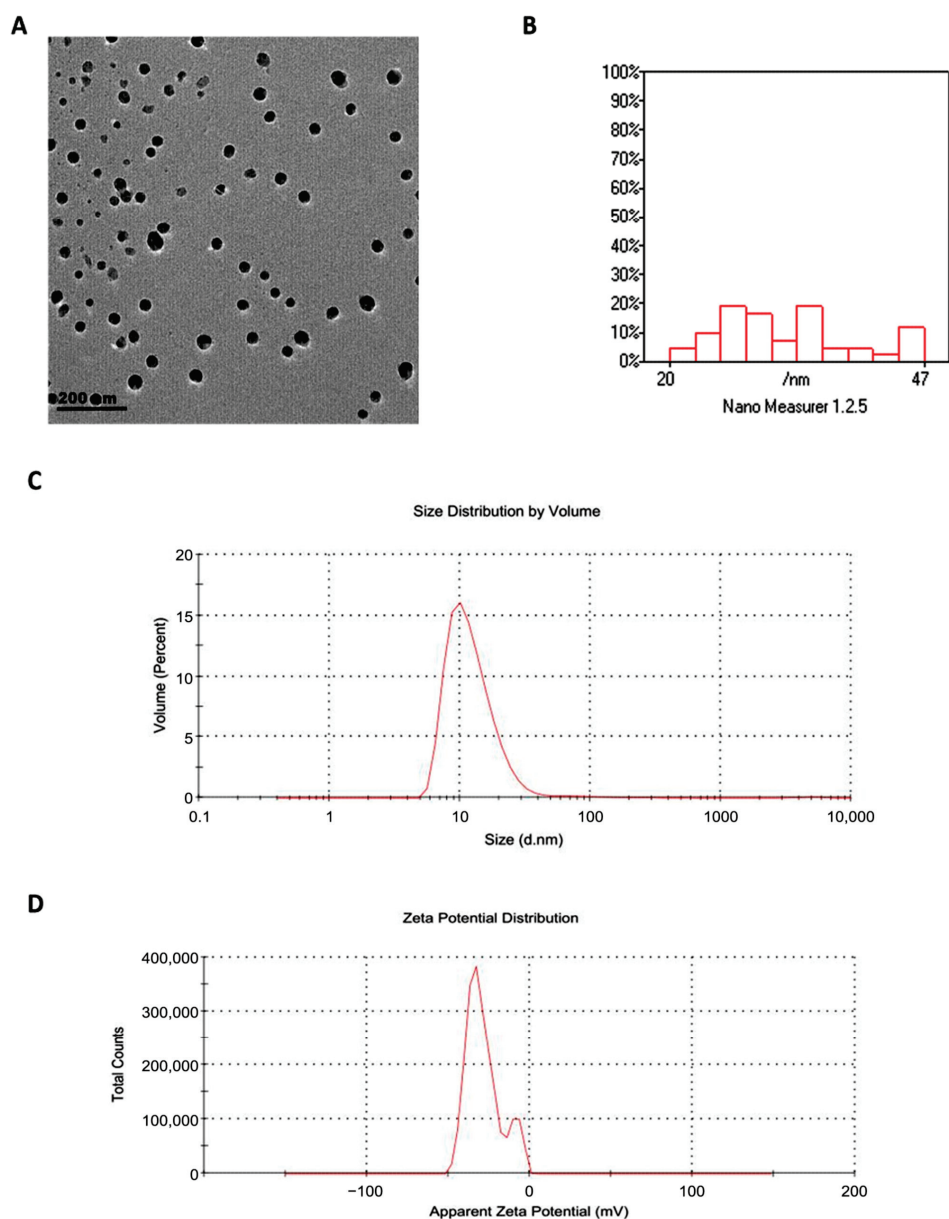
Before analysis, the normality of data distribution and homogeneity of variances were verified using the Shapiro–Wilk and Levene’s tests, respectively. One-way ANOVA (SAS Institute, 2012, Proc ANOVA) was performed to compare means among experimental groups, and Tukey’s post hoc test was employed for multiple comparisons. Results are reported as mean ± standard error (SE), with statistical significance set at  $p < 0.05$ . Data visualization was performed with GraphPad Prism 9.0, and multivariate analyses (principal component analysis (PCA) and heatmap clustering) were conducted with SRplot.

### 3. Results

#### 3.1. Characterization of Grape Seed Oil Nanoemulsion (GSONE)

Transmission electron microscopy revealed that GSONE particles were spherical and uniform in morphology (Figure 3A) and ranged in diameter from 20 to 47 nm (Figure 3B). Dynamic light scattering (DLS) analysis yielded a hydrodynamic diameter of 88 nm, which is larger than the TEM values, reflecting particle-solvent interactions and hydration shell formation. The PDI was 0.542, suggesting moderate size dispersion within the formulation (Figure 3C). The zeta potential was measured at −28 mV, confirming good electrostatic

stability of the nanoemulsion suspension (Figure 3D). All measurements were performed on freshly prepared samples and independently verified in replicate runs to ensure consistency and accuracy.



**Figure 3.** Physicochemical characterization of grape seed oil nanoemulsion (GSONE). **(A)** Transmission electron microscopy (TEM) image shows predominantly spherical and uniformly dispersed nanoparticles. **(B)** Particle size distribution histogram indicates that most particles range from 20 to 47 nm in diameter. **(C)** Dynamic light scattering (DLS) analysis displays size distribution by volume (Z-average: 88 nm; PDI: 0.542). **(D)** Zeta potential measurement reveals a value of  $-28$  mV, indicative of electrostatic stability of the nanoemulsion suspension.

### 3.2. Growth Performance and Tumor Weight/Volume Changes

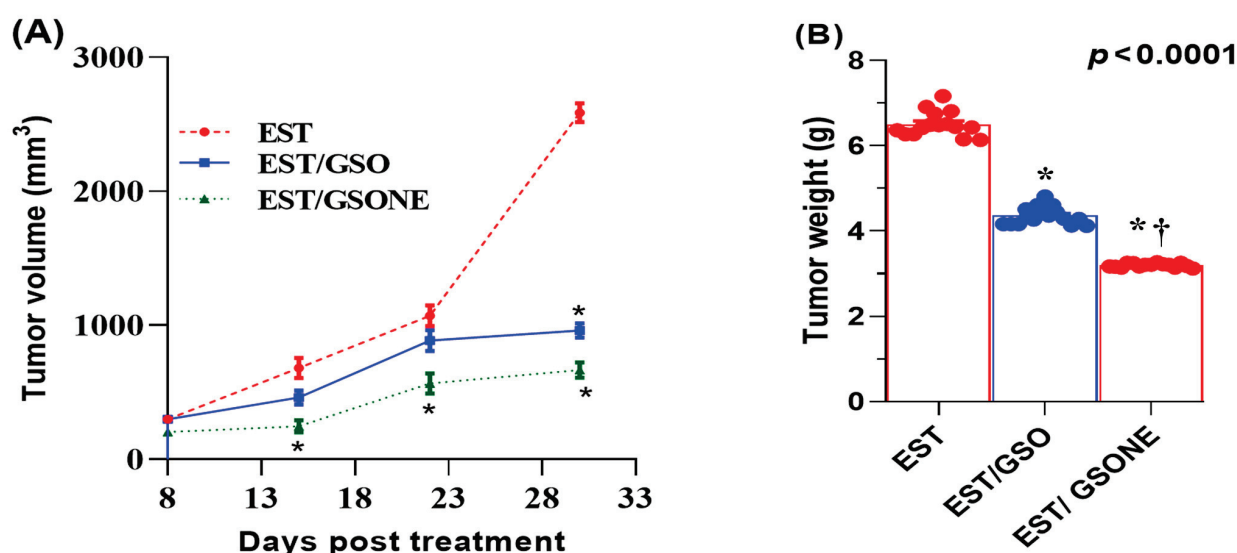
Treatment effects on growth performance and tumor burden were quantified in mice bearing EST following administration of either crude GSO or its nanoemulsion form (Table 3). The EST group displayed a significant reduction in net body weight relative to all controls. Both GSO and GSONE therapies improved body weight, with the EST/GSONE group demonstrating a greater reversal of cachexia than the EST/GSO group. Tumor weight was highest in the untreated EST group, while both treatments led to marked reductions;

notably, GSONE yielded the most significant effect, achieving a 52.89% decrease in tumor mass compared to the untreated EST group. Across all measurement time points, tumor volume was lowest in the EST/GSONE group, intermediate in the EST/GSO group, and highest in the untreated EST group, with nanoemulsion consistently outperforming crude oil (Figure 4).

**Table 3.** Growth performance and tumor weight changes induced by grape seed oil nanoemulsion compared to raw grape seed oil in mice bearing Ehrlich solid carcinoma.

| Parameters | Control      | GSO          | GSONE        | EST            | EST/GSO         | EST/GSONE        | <i>p</i> -Values |
|------------|--------------|--------------|--------------|----------------|-----------------|------------------|------------------|
| IBW (g)    | 21.86 ± 0.33 | 21.69 ± 0.29 | 21.44 ± 0.26 | 21.93 ± 0.38   | 21.56 ± 0.37    | 21.38 ± 0.48     | 0.746            |
| FBW (g)    | 27.46 ± 0.24 | 27.86 ± 0.31 | 27.93 ± 0.22 | 27.38 ± 0.19   | 26.87 ± 0.25    | 27.34 ± 0.18     | 0.041            |
| NW(g)      | 27.46 ± 0.24 | 27.86 ± 0.31 | 27.93 ± 0.22 | 20.62 ± 0.19 * | 22.43 ± 0.25 *# | 24.16 ± 0.12 *#† | 0.003            |
| BWG (g)    | 5.60 ± 0.09  | 6.17 ± 0.02  | 6.49 ± 0.04  | −1.30 ± 0.19 * | 0.87 ± 0.12 *#  | 2.79 ± 0.37 *#†  | <0.0001          |

GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: Grape seed oil (4 mg/kg body weight) + EAC cells (0.2 mL); EST/GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight) + EAC cells (0.2 mL) (each group  $n = 15$ ). IBW: Initial body weight; FBW: Final body weight; NW: Net Weight (g); body weight after subtraction of tumor mass in tumor-bearing groups; BWG: Body Weight Gain (g); calculated as NW—IBW to reflect net gain or loss excluding tumor weight; TM: Tumor. Values are presented as mean ± SE. \* Significantly different from the control group; # Significantly different from the EST group; † Significantly different from the EST/GSO group. The significance level is  $p < 0.05$ .

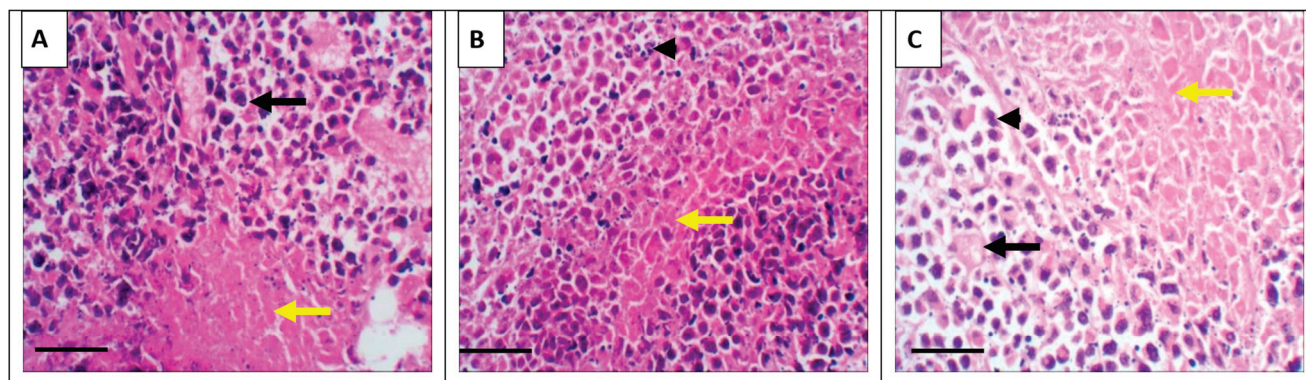


**Figure 4.** Changes in Tumor volume (A) and tumor weight (B) in mice bearing Ehrlich solid carcinoma (EST) and treated with grape seed oil nanoemulsion or raw grape seed oil. Each group ( $n = 15$ ). EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: Grape seed oil (4 mg/kg body weight) + EAC cells (0.2 mL); EST/GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight) + EAC cells (0.2 mL) (each group  $n = 15$ ). Standard bars represent the standard error of the mean (SEM). \* Significantly different from the EST group; † Significantly different from the EST/GSO group.

### 3.3. Tumor Histopathology

Representative hematoxylin and eosin (H&E)-stained sections of tumor tissues from each experimental group are shown in Figure 5. Distinct histopathological features were evident among the groups. The control group displayed uniform, densely packed tumor cells with occasional necrotic foci. In contrast, treatment with GSO (Panel B) and, particularly, GSONE (Panel C) resulted in marked changes in tumor architecture, including reduced cellular density, prominent areas of necrosis, and greater stromal separation. These morphological changes underscore the modulatory effects of GSO and GSONE on

tumor tissue structure and are consistent with reduced tumor aggressiveness and enhanced therapeutic response.



**Figure 5.** Representative photomicrograph of solid tumors from the positive control and different experimental groups. (A) The EAC group shows tumor tissue infiltrating the muscle layer and forming solid sheets of undifferentiated tumor cells (yellow arrow), with tumor cells showing high-grade anaplasia, pleomorphism, and abnormal mitosis (black arrow). (B) The treated group with grape seed oil (GSO) showed a massive reduction in tumour size and cell count (arrowhead) between the skeletal muscle bundle (yellow arrow). (C) The treated group with GSO nanoemulsion (GSONE) showed a reduction in the quantity of the active neoplastic cells, vacuolation (black arrows), and degeneration of the neoplastic cells with karyolysed nuclei (arrowhead) and a skeletal muscle bundle (yellow arrow). All images at = 400×. Scale bar = 50  $\mu$ m.

#### 3.4. Blood Biochemical Parameters

Blood biochemical profiling revealed pronounced disruptions in protein metabolism and liver function in mice bearing EST (Table 4). The EST group showed significant reductions in serum total protein, albumin, and globulin levels compared with controls. Administration of GSONE significantly improved these parameters, normalizing values to levels indistinguishable from those of the control group. Globulin concentrations remained similar between the EST/GSO and EST/GSONE treated groups. Markers of hepatic injury, including AST, ALT, ALP, and total bilirubin, were significantly elevated in the EST group compared to controls. Both GSO and GSONE treatments significantly attenuated these elevations, with the most significant restorative effect observed in the EST/GSONE cohort, whose values approached those of healthy controls.

**Table 4.** Blood biochemical changes induced by grape seed oil nanoemulsion compared to raw grape seed oil in mice bearing Ehrlich solid carcinoma.

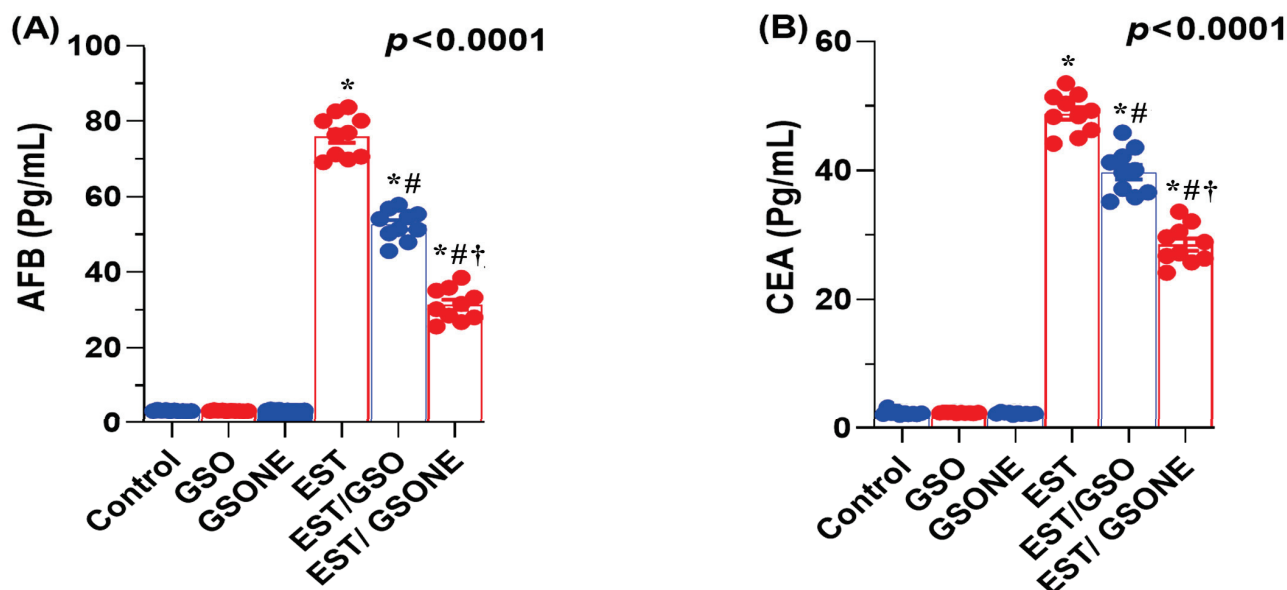
| Parameters | Control          | GSO              | GSONE            | EST                 | EST/GSO             | EST/GSONE           | <i>p</i> -Values |
|------------|------------------|------------------|------------------|---------------------|---------------------|---------------------|------------------|
| TP (g/dL)  | 6.88 $\pm$ 0.29  | 6.90 $\pm$ 0.31  | 6.92 $\pm$ 0.19  | 4.13 $\pm$ 0.13 *   | 5.38 $\pm$ 0.32 *#  | 6.19 $\pm$ 0.27 #†  | 0.0146           |
| Alb (g/dL) | 3.79 $\pm$ 0.13  | 3.93 $\pm$ 0.17  | 3.98 $\pm$ 0.21  | 2.16 $\pm$ 0.07 *   | 2.71 $\pm$ 0.12 *#  | 3.53 $\pm$ 0.18 #†  | 0.0273           |
| Glo (g/dL) | 3.09 $\pm$ 0.16  | 2.97 $\pm$ 0.14  | 2.94 $\pm$ 0.06  | 1.97 $\pm$ 0.06 *   | 2.67 $\pm$ 0.10 #   | 2.66 $\pm$ 0.09 #   | 0.0047           |
| TB (mg/dL) | 0.42 $\pm$ 0.09  | 0.38 $\pm$ 0.07  | 0.36 $\pm$ 0.06  | 1.32 $\pm$ 0.25 *   | 0.86 $\pm$ 0.03 *#  | 0.58 $\pm$ 0.10 #   | <0.0001          |
| ALP (U/L)  | 88.32 $\pm$ 4.26 | 86.25 $\pm$ 5.18 | 85.41 $\pm$ 6.22 | 128.14 $\pm$ 7.52 * | 101.21 $\pm$ 6.91 # | 95.33 $\pm$ 4.93 #  | 0.0026           |
| ALT (U/L)  | 46.26 $\pm$ 3.24 | 45.21 $\pm$ 4.35 | 45.03 $\pm$ 5.11 | 89.25 $\pm$ 4.89 *  | 78.29 $\pm$ 6.23 *  | 54.26 $\pm$ 5.39 #† | 0.0001           |
| AST (U/L)  | 76.32 $\pm$ 6.66 | 73.41 $\pm$ 4.20 | 72.27 $\pm$ 5.38 | 118.39 $\pm$ 6.15 * | 96.20 $\pm$ 4.44 *# | 84.06 $\pm$ 5.93 #  | 0.0001           |

GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: Grape seed oil (4 mg/kg body weight) + EAC cells (0.2 mL); EST/GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight) + EAC cells (0.2 mL). Values are presented as mean  $\pm$  SE, with each group ( $n = 10$ ). TP: Total protein; Alb: Albumin; Glo: Globulin; TB: Total Bilirubin; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase. \* Significantly different from the control group; # Significantly different from the EST group; † Significantly different from the EST/GSO group. The significance level is  $p < 0.05$ .



### 3.5. Tumor-Associated Biomarkers

Serum concentrations of AFP and CEA were markedly elevated in mice bearing EST compared with all control groups, indicating a higher tumor burden. Upon treatment with crude GSO or its nanoemulsion counterpart (GSONE), both AFP and CEA levels were significantly reduced. Notably, the EST/GSONE group showed a more pronounced decrease in tumor markers than the EST/GSO group, supporting the enhanced antitumor efficacy of the nanoemulsion formulation (Figure 6A,B). These findings support the value of AFP and CEA as sensitive indicators of therapeutic response and tumor suppression in this model.

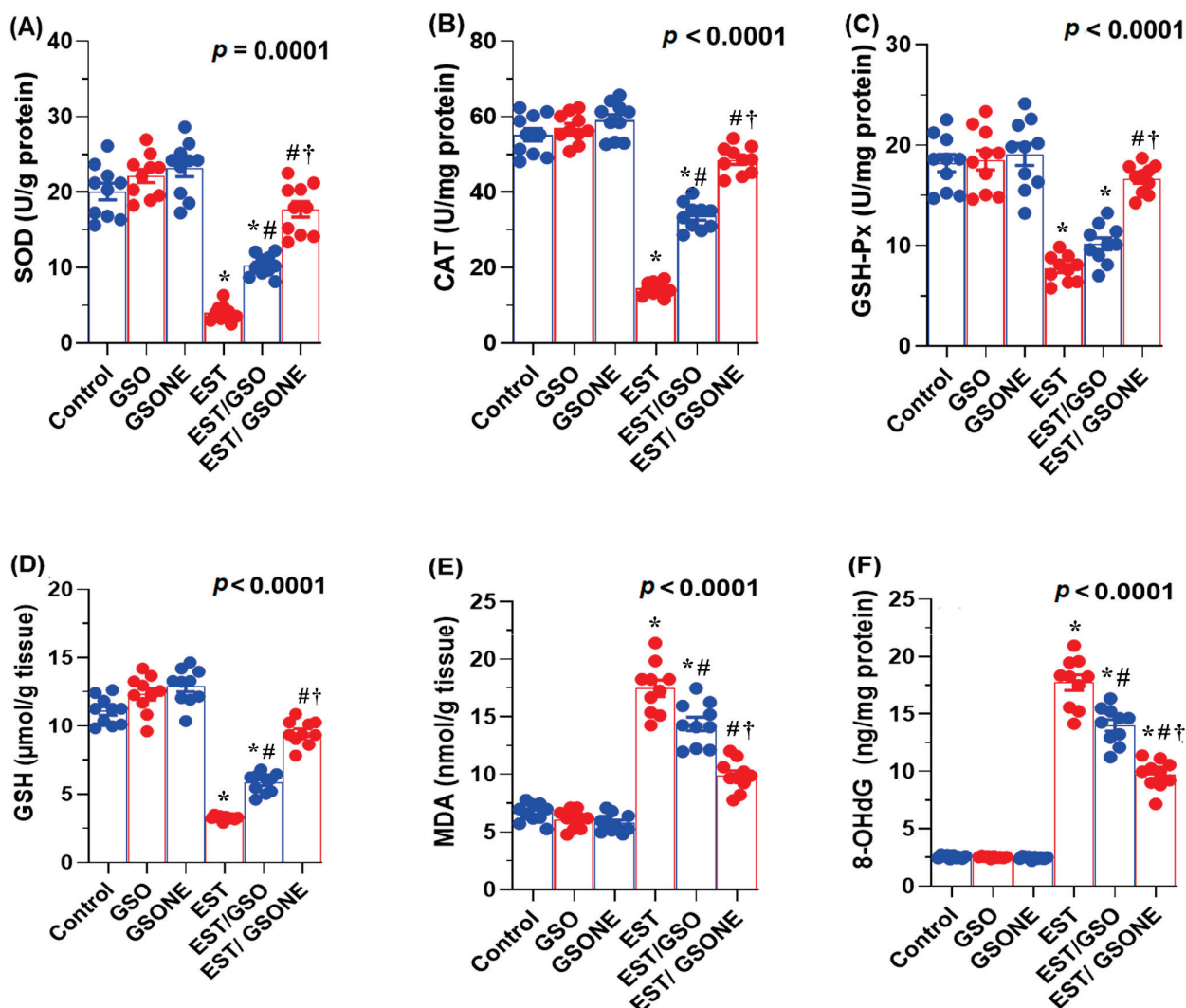


**Figure 6.** Effects of grape seed oil nanoemulsion (GSONE) and crude grape seed oil (GSO) on serum tumor-associated biomarkers in Ehrlich solid carcinoma (EST)-bearing mice. (A) Alpha-fetoprotein (AFP) and (B) carcinoembryonic antigen (CEA) serum levels in control, EST, EST/GSO, and EST/GSONE groups. GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: GSO + EAC cells; EST/GSONE: GSONE + EAC cells. Data are presented as mean ± SE (each group  $n = 10$ ). Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. \* Significantly different from the control group; # Significantly different from the EST group; † Significantly different from the EST/GSO group.

### 3.6. Redox Status and DNA Oxidative Damage

Mice bearing EST exhibited significantly decreased hepatic antioxidant defenses, as evidenced by lower levels of reduced GSH, SOD, CAT, and GSH-Px compared to healthy controls. Treatment with either crude GSO or its nanoemulsion (GSONE) markedly restored these antioxidant parameters, with the EST/GSONE group demonstrating the most robust recovery across all markers (Figure 7A–D). Tumor-induced oxidative stress, as reflected by elevated MDA concentrations, was significantly reduced after intervention, with GSONE showing superior efficacy and restoring MDA levels to those of the control group (Figure 7E). Furthermore, DNA oxidative damage, assessed by hepatic 8-hydroxy-2'-deoxyguanosine (8-OHdG), was highest in the EST group and effectively minimized following treatment, with the lowest 8-OHdG values observed in the EST/GSONE cohort (Figure 7F). These data underscore the potent antioxidant and protective effects of GSONE against tumor-induced oxidative stress and genotoxicity.

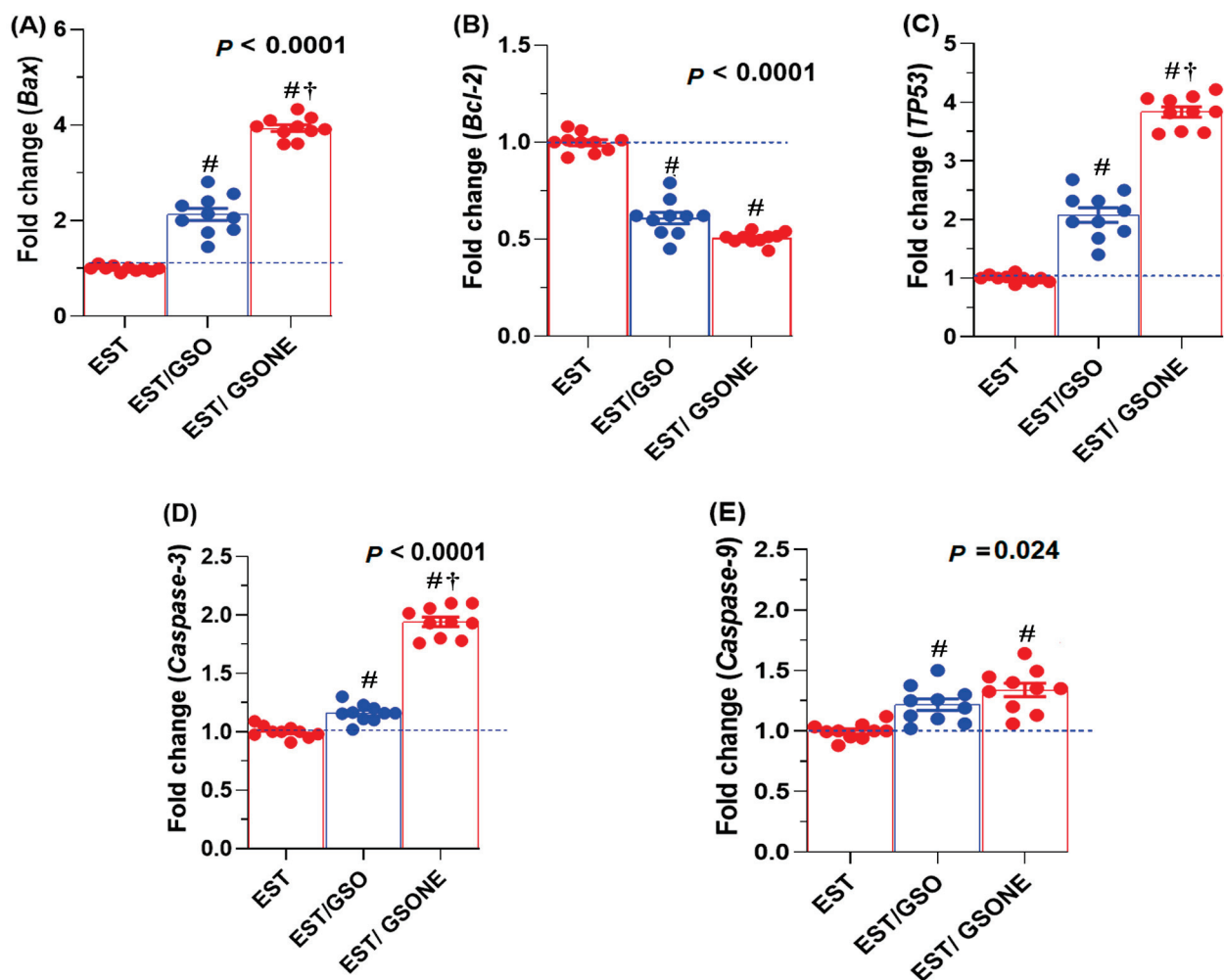




**Figure 7.** Effects of grape seed oil nanoemulsion (GSONE) and crude grape seed oil (GSO) on hepatic redox status and DNA oxidative damage in Ehrlich solid carcinoma (EST)-bearing mice. (A–D) Antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reduced glutathione (GSH) levels; (E) hepatic malondialdehyde (MDA) concentrations as a marker of lipid peroxidation; (F) 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels as an indicator of DNA oxidative damage. GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: GSO + EAC cells; EST/GSONE: GSONE + EAC cells. Each group ( $n = 10$ ). Bars represent mean  $\pm$  SE. A one-way ANOVA with Tukey's post hoc test was used. \* Significantly different from the control group; # Significantly different from the EST group; † Significantly different from the EST/GSO group.

### 3.7. Apoptotic Gene Expression Profiles

Expression analysis revealed that pro-apoptotic genes, including *Bax*, *TP53*, *caspase-3*, and *caspase-9*, were significantly affected by GSO administration (Figure 8). Treatment with either crude grape seed oil (GSO) or its nanoemulsion (GSONE) restored the expression of these markers, with *Bax*, *TP53*, and *caspase-3* levels significantly higher in the EST/GSONE group compared to EST/GSO. At the same time, *caspase-9* did not show a statistically significant difference between the two treated groups. Conversely, the anti-apoptotic gene *Bcl-2* was markedly suppressed by both treatments, with no significant difference between the EST/GSO and EST/GSONE-treated groups.

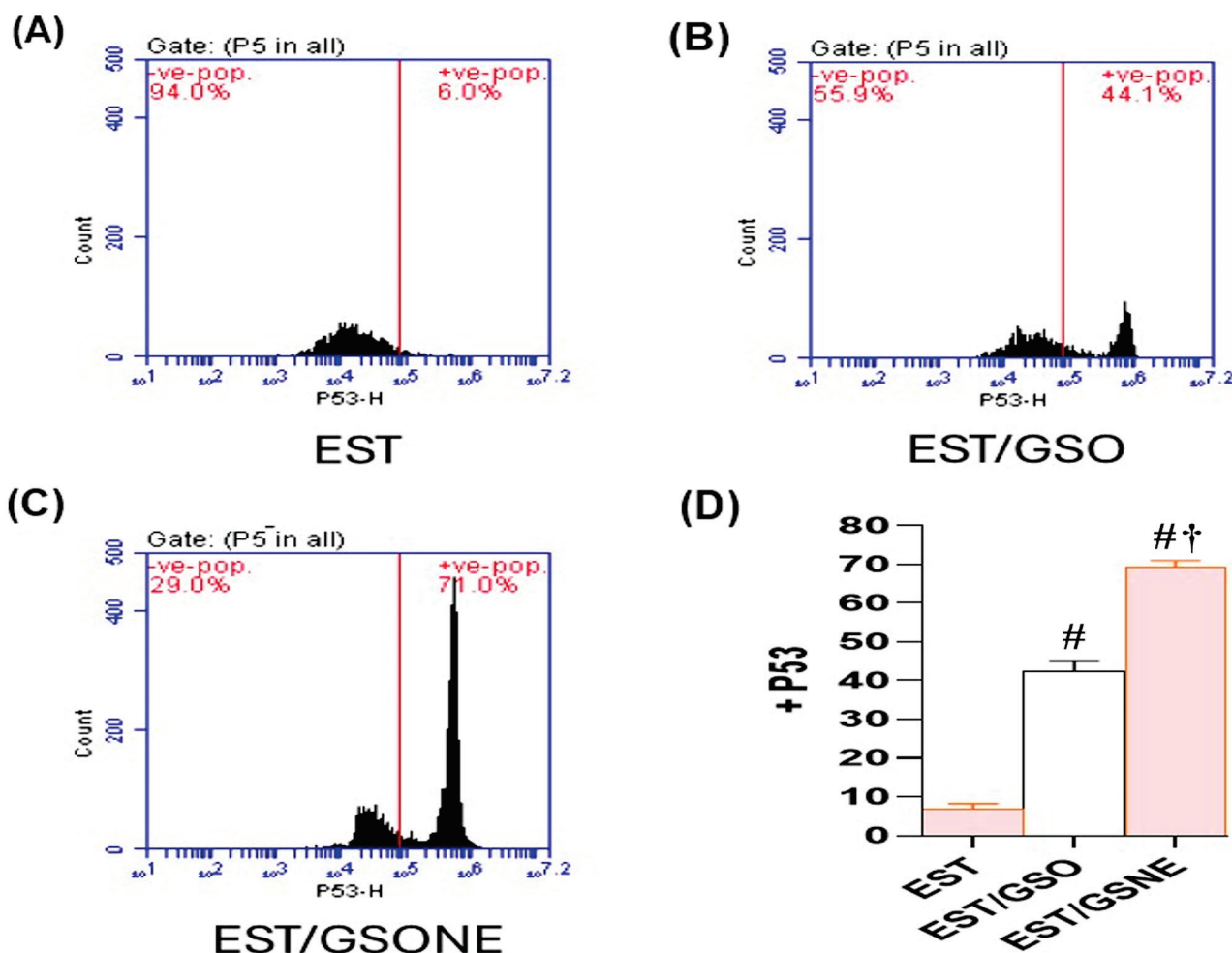


**Figure 8.** Effects of grape seed oil nanoemulsion (GSONE) and crude grape seed oil (GSO) on apoptotic gene expression profiles in Ehrlich solid carcinoma (EST)-bearing mice. Expression levels of (A) *Bax* (Bcl-2-associated X protein), (B) *Bcl-2* (B-cell lymphoma 2), (C) *TP53* (tumor protein 53), (D) *Caspase-3* (cysteine-aspartic acid protease-3), and (E) *Caspase-9* (cysteine-aspartic acid protease-9) in control, EST, EST/GSO, and EST/GSONE groups. GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: GSO + EAC cells; EST/GSONE: GSONE + EAC cells. Each group ( $n = 10$ ). Values are presented as mean  $\pm$  SE. Statistical comparisons were performed using one-way ANOVA with Tukey's post hoc test. # Significantly different from the EST group; † Significantly different from the EST/GSO group.

Flow cytometric analysis (Figure 9A–C) further confirmed these findings at the cellular level: untreated ESTs demonstrated only 6.0% p53-positive cells, indicating minimal apoptosis. Treatment with GSO increased p53 positivity to 44.1%, while GSONE elevated this fraction to 71.0%, demonstrating significantly greater apoptotic activation than in both the EST and EST/GSO groups. Quantitative assessment (Figure 9D) showed that GSONE therapy was most effective at augmenting p53 expression and apoptotic cell frequency in tumor tissues.

Flow cytometric assessment of cleaved caspase-3 in EST cells revealed a marked enhancement of apoptotic execution following treatment with grape seed oil formulations (Figure 10A–D). In the untreated EST group, only 7.7% of cells were positive for cleaved caspase-3, indicating minimal basal apoptotic activity. Administration of crude grape seed oil (EST/GSO) increased the proportion of cleaved caspase-3-positive cells to 26.6% ( $p < 0.05$  vs. EST), suggesting moderate induction of apoptosis. Treatment with grape seed oil

nanoemulsion (EST/GSONE) significantly elevated cleaved caspase-3 expression to 69.1% ( $p < 0.05$  vs. both EST and EST/GSO), reflecting robust activation of the caspase-dependent apoptotic pathway. Quantitative analysis (Figure 10D) confirmed that GSONE conferred the highest apoptotic index, followed by GSO and the untreated control, highlighting the superior pro-apoptotic efficacy of nanoemulsion formulation.

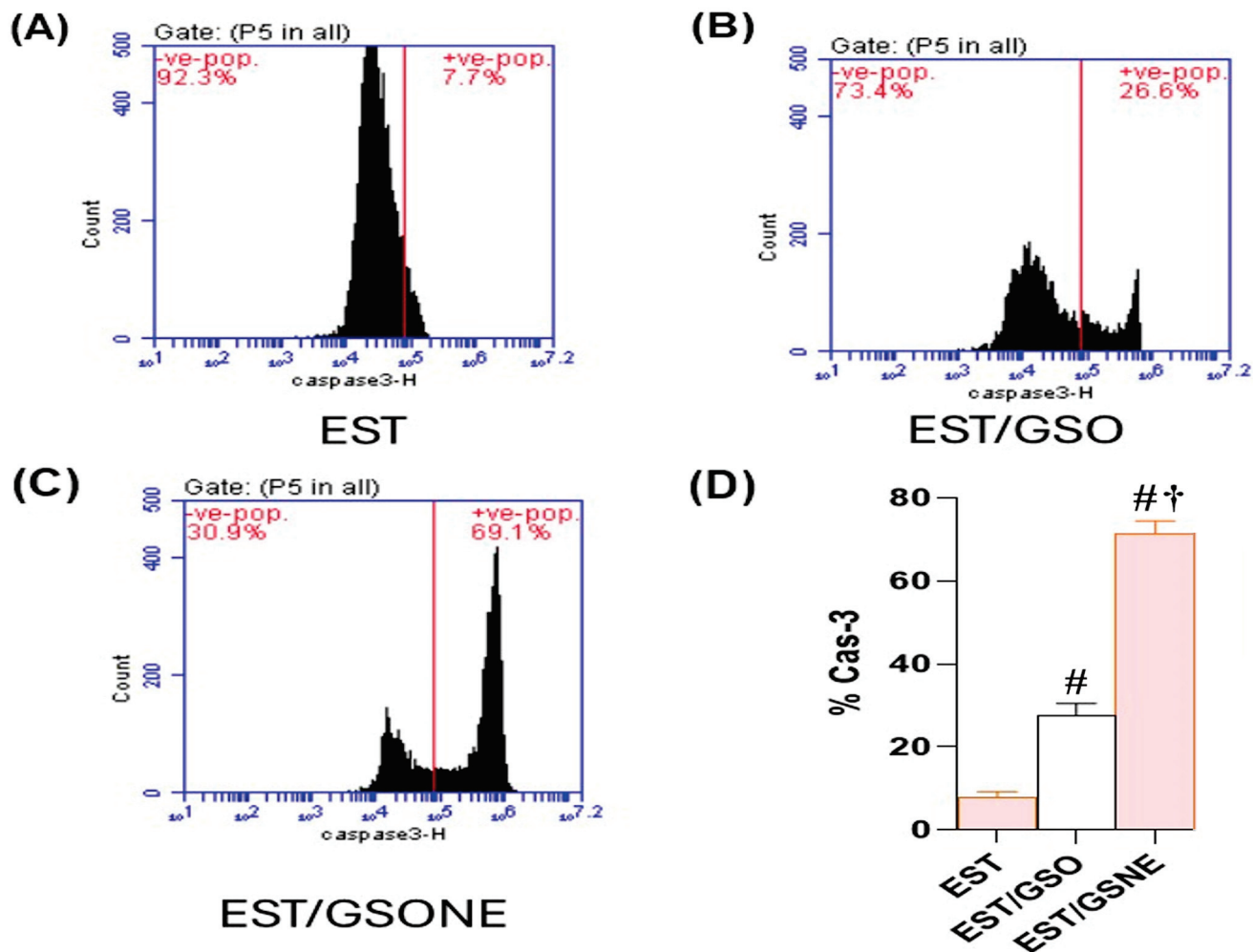


**Figure 9.** Flow cytometric analysis of p53 expression in Ehrlich solid tumor (EST) cells following treatment with grape seed oil formulations. (A–C) Representative dot plots showing p53-positive cell populations in untreated EST (A), EST treated with crude grape seed oil (GSO; B), and EST treated with grape seed oil nanoemulsion (GSONE; C). (D) Quantitative assessment demonstrates a significant, formulation-dependent increase in p53 expression, with GSONE treatment showing the greatest induction ( $p < 0.05$  vs. EST and EST/GSO) (each group  $n = 10$ ). Data are presented as mean  $\pm$  SD. # Significantly different from the EST group; † Significantly different from the EST/GSO group.

### 3.8. Liver Histopathology and Ultrastructural Changes

Histological evaluation of liver tissue showed that mice treated with either crude grape seed oil (GSO) or its nanoemulsion form (GSONE) exhibited normal hepatic architecture, including intact central veins, well-organized lobular structures, and healthy hepatocytes with centrally positioned nuclei and uniform cytoplasmic morphology (Figure 11A–C). In contrast, hepatic tissue from mice bearing Ehrlich solid tumors (EST) demonstrated pronounced pathological changes, vacuolar degeneration, central vein congestion, and severe nuclear necrosis (Figure 11D). Treatment with GSO or GSONE in EST-bearing mice restored liver architecture, evidenced by well-formed hepatic cords and lobular structures (Figure 11E,F), indicating notable protection against tumor-induced damage. Quantitative

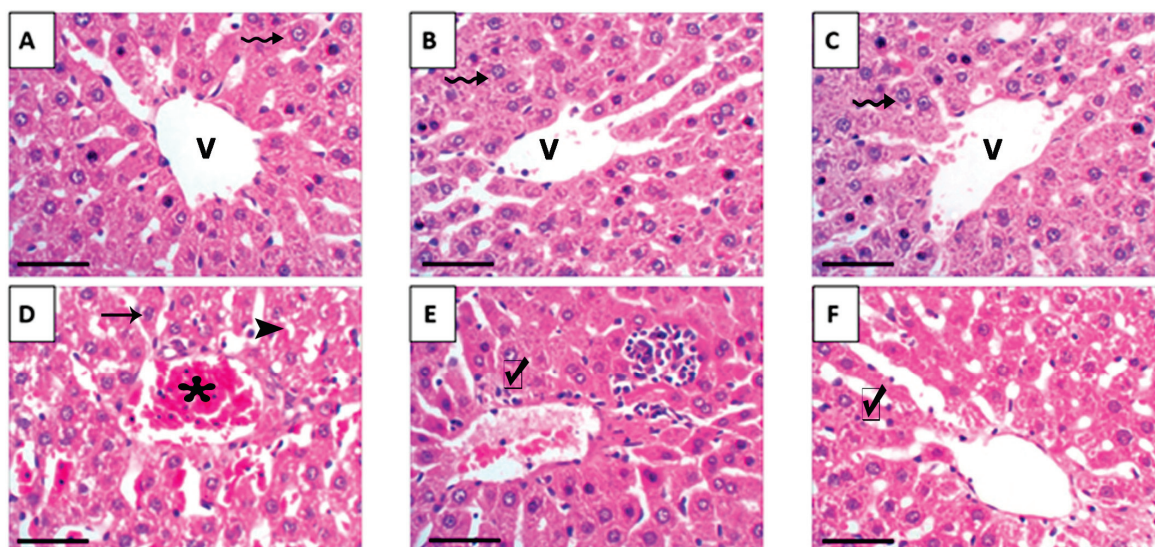
scoring of liver injury confirmed marked injury in the EST group compared with controls. At the same time, GSONE treatment was particularly effective at alleviating tissue injury severity, with no significant difference in histopathological scores between the EST/GSO and EST/GSONE groups (Figure 12).



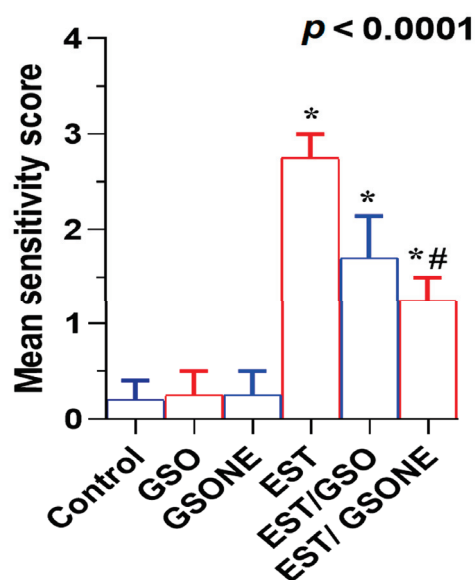
**Figure 10.** Flow cytometric assessment of cleaved caspase-3 expression in Ehrlich solid carcinoma (EST) cells following treatment with grape seed oil formulations. (A–C) Representative dot plots showing cleaved caspase-3-positive cell populations in untreated EST (A), EST treated with crude grape seed oil (GSO; (B)), and EST treated with grape seed oil nanoemulsion (GSONE; (C)). (D) Quantitative analysis demonstrates a significant, formulation-dependent increase in caspase-3 activation, with GSONE treatment yielding the highest apoptotic index ( $p < 0.05$  vs. EST and EST/GSO) (each group  $n = 10$ ). Data are presented as mean  $\pm$  SD. # Significantly different from the EST group; † Significantly different from the EST/GSO group.

Ultrastructural analysis further supported these findings. Normal hepatocyte ultrastructure, including organized mitochondria, rough endoplasmic reticulum, and nuclei with distinct nucleoli, was maintained in control, GSO, and GSONE groups (Figure 13A–C). EST-bearing mice showed classic signs of cellular necrosis, irregular nuclei, chromatin condensation, extensive vacuolation, cytoplasmic degeneration, and disrupted mitochondrial cristae (Figure 13D). Restoration of near-normal ultrastructure, characterized by limited mitochondrial degeneration and preservation of organelle integrity, was observed in mice treated with either GSO or GSONE after EST induction (Figure 13E,F), highlighting the treatments' hepatoprotective effects.



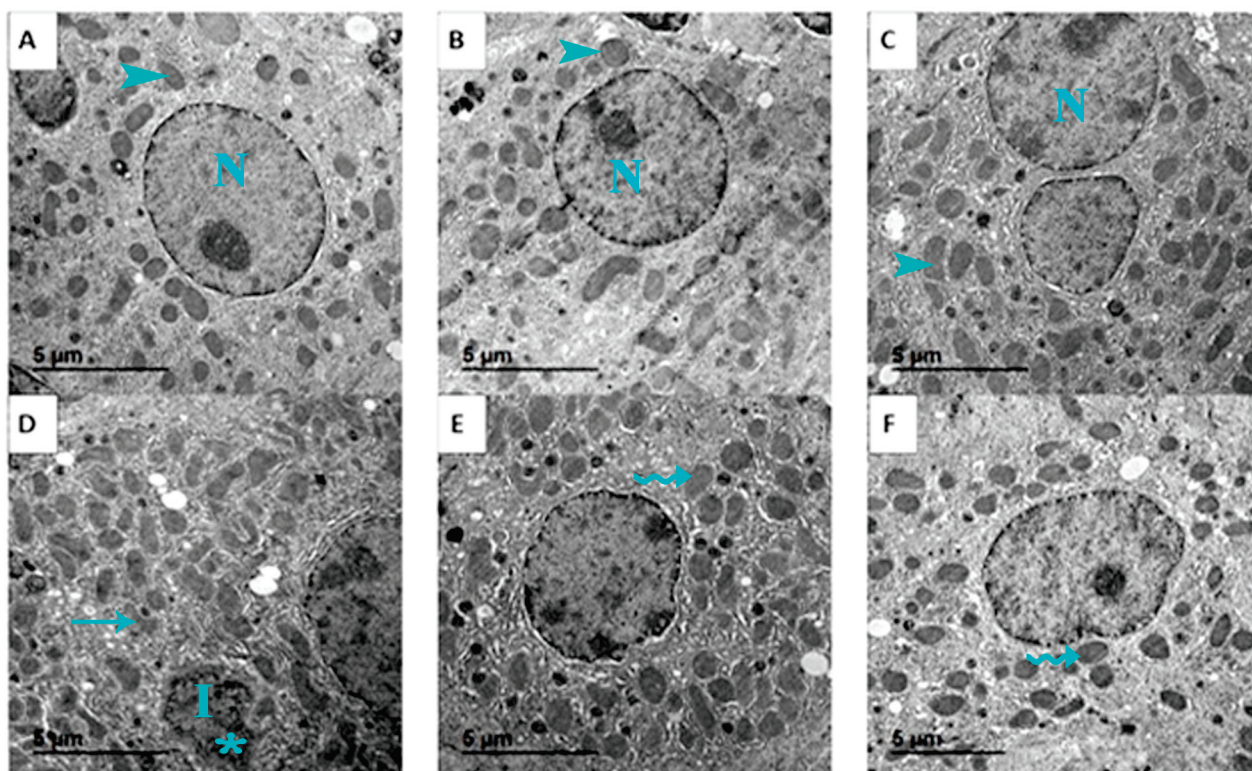


**Figure 11.** Representative photomicrographs of liver tissue from the control group and various experimental groups. (A) Control group: exhibits normal hepatic architecture that has a central vein (V) and healthy hepatocytes (wavy arrow). (B) GSO: grape seed oil (4 mg/kg body weight); shows normal hepatic architecture with central vein (V) and healthy hepatocytes (wavy arrow). (C) GSONE: grape seed oil nanoemulsion (4 mg/kg body weight); displays normal hepatic architecture with central vein (V) and healthy hepatocytes (wavy arrow). (D) EST: inoculated with Ehrlich ascites carcinoma (EAC) cells (0.2 mL); demonstrates vacuolar degeneration (arrowheads), central vein congestion (asterisk), and severe nuclear necrosis (arrow). (E) EST/GSO: grape seed oil (4 mg/kg body weight) + EAC cells (0.2 mL); shows restored liver architecture with well-formed hepatic cords (checkmark). (F) EST/GSONE: grape seed oil nanoemulsion (4 mg/kg body weight) + EAC cells (0.2 mL) shows well-formed hepatic cords (checkmark). All images were captured at 400× magnification; scale bar = 50 μm.



**Figure 12.** Mean severity scores of liver tissue damage in Ehrlich solid tumor (EST)-bearing mice treated with crude grape seed oil (GSO) or grape seed oil nanoemulsion (GSONE). Liver injury was assessed by semi-quantitative histopathological scoring in control, EST, EST/GSO, and EST/GSONE groups. GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich solid tumor cells (0.2 mL); EST/GSO: GSO + EST cells; EST/GSONE: GSONE + EST cells. Data are presented as mean ± SE (each group  $n = 10$ ). Statistical differences between groups were determined by one-way ANOVA and Tukey's post hoc test. \* Significantly different from the control group; # Significantly different from the EST group.

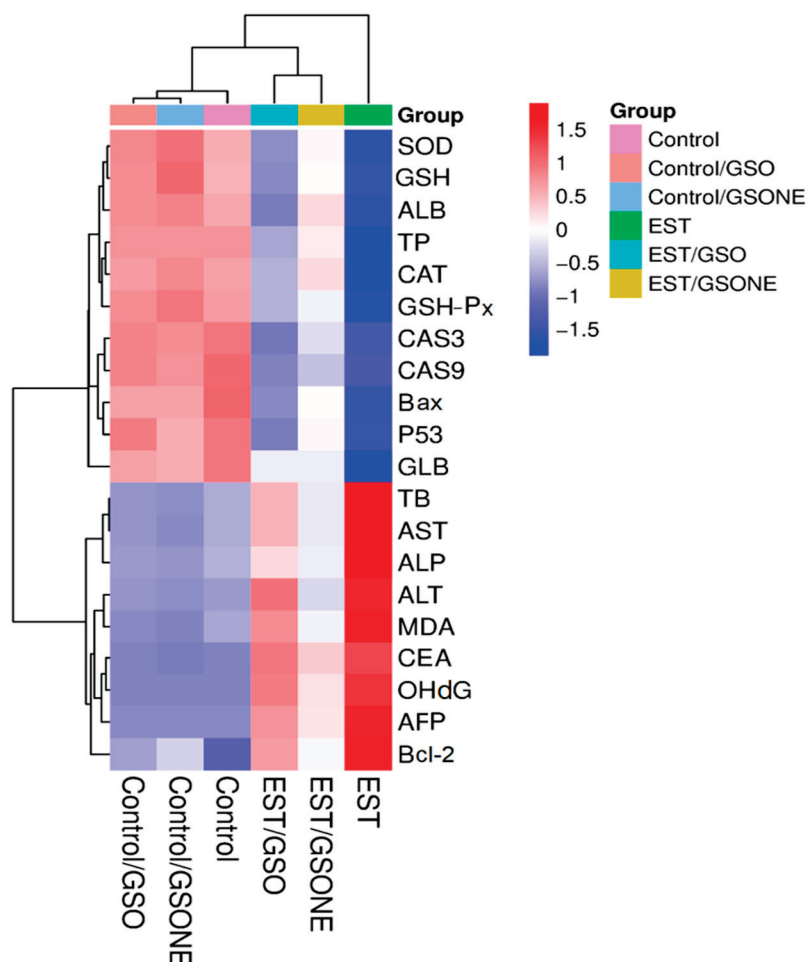




**Figure 13.** Representative transmission electron micrographs showing the liver ultrastructure from the control and experimental groups. (A) Control group: shows normal hepatocyte ultrastructure with nucleus (N) and healthy mitochondria (arrowhead). (B) GSO: grape seed oil (4 mg/kg body weight); maintains normal hepatocyte ultrastructure with nucleus (N) and healthy mitochondria (arrowhead). (C) GSONE: grape seed oil nanoemulsion (4 mg/kg body weight); maintains normal hepatocyte ultrastructure with nucleus (N) and healthy mitochondria (arrowhead). (D) EST: inoculated with Ehrlich ascites carcinoma (EAC) cells (0.2 mL); exhibits classic signs of cellular necrosis, including irregular nuclei (I), chromatin condensation (asterisk), extensive vacuolation, cytoplasmic degeneration, and disrupted mitochondrial cristae (arrow). (E) EST/GSO: grape seed oil (4 mg/kg body weight) + EAC cells (0.2 mL); shows restoration of near-normal ultrastructure with limited mitochondrial degeneration (wavy arrow). (F) EST/GSONE: grape seed oil nanoemulsion (4 mg/kg body weight) + EAC cells (0.2 mL) shows limited mitochondrial degeneration (wavy arrow), highlighting an observable hepatoprotective effect.

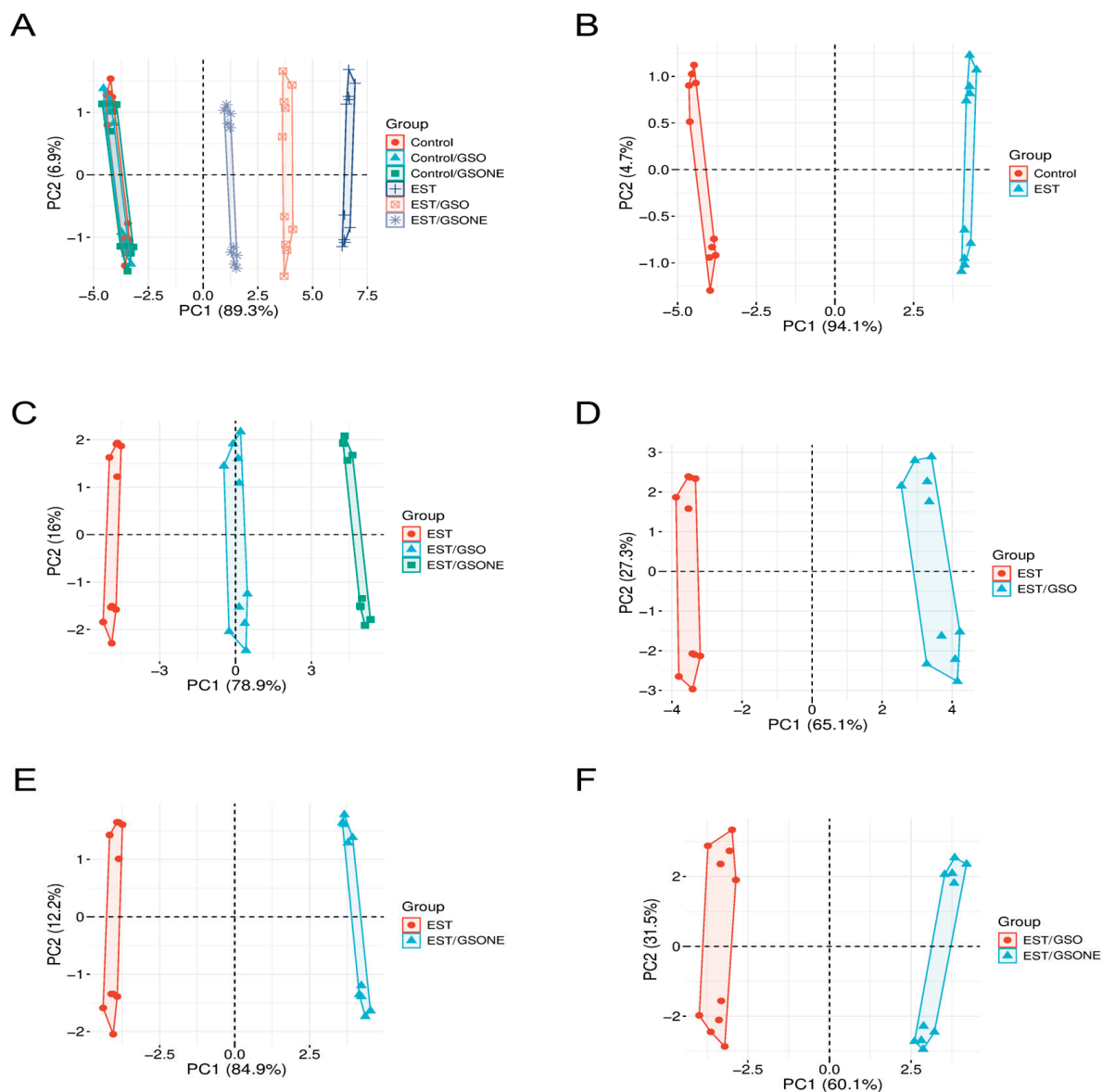
### 3.9. Multivariable Analyses

Hierarchical clustering heatmap analysis (Figure 14) delineated distinct biochemical and molecular expression profiles across experimental groups. The control and non-tumor treatment groups (Control/GSO, Control/GSONE) clustered together, characterized by elevated antioxidant enzyme activities (SOD, CAT, GSHP-x, GSH), albumin (ALB), and total protein (TP), alongside lower levels of pro-apoptotic and oxidative stress markers. In contrast, the EST group formed a separate cluster, characterized by diminished antioxidant parameters and elevated liver enzymes (AST, ALT, ALP), oxidative stress indices, and apoptotic markers, reflecting the metabolic and cellular disruption induced by tumor burden. Groups treated post-tumor induction (EST/GSO, EST/GSONE) occupied intermediate positions in the heatmap, demonstrating partial restoration of antioxidant status and suppression of tumor-related markers. Notably, the EST/GSONE group exhibited a more pronounced shift toward the healthy control profile than the EST/GSO group, highlighting the superior modulatory and therapeutic efficacy of the grape seed oil nanoemulsion formulation.



**Figure 14.** Hierarchical clustering heatmap of biochemical and molecular markers across experimental groups. Color gradients from red (upregulation) to blue (downregulation) reflect the relative abundance of markers, while clustering highlights the relationships among control, treatment-only, tumor-bearing, and combined treatment/tumor groups. GSO: Grape seed oil (4 mg/kg body weight); GSONE: Grape seed oil nanoemulsion (4 mg/kg body weight); EST: Inoculated with Ehrlich Ascites Carcinoma (EAC) cells (0.2 mL); EST/GSO: GSO + EST cells; EST/GSONE: GSONE + EST cells. Each group ( $n = 10$ ). SOD: Superoxide dismutase; GSH: Reduced glutathione; ALB: Albumin; TP: Total protein; CAT: Catalase; GSH-Px: Glutathione peroxidase; CAS3/9: Caspase 3/9; Bax: Bcl-2-associated  $\times$  protein; P53: Oncoprotein 53; GLB: Globulin; TB: Total bilirubin; AST: Aspartate transaminase; ALP: Alkaline phosphatase; ALT: Alanine transaminase; MDA: malondialdehyde; CEA: Cancer embryonic antigen; OhdG: Hydroxy-2'-deoxyguanosine; AFP: alpha feto protein; Bcl-2: B-cell lymphoma 2.

Principal component analysis (Figure 15) was performed to visualize multivariate differentiation among experimental groups based on biochemical and molecular features. The first principal component (PC1) accounted for 89.3% of the total variance (Figure 15A), clearly separating the tumor-bearing EST group from control and treatment-only cohorts, with healthy animals treated with GSO or GSONE clustering closely with controls. Pairwise PCA comparisons reinforced these distinctions: Figure 15B demonstrates near-complete segregation between the control and EST groups along PC1 (94.1%), indicating substantial tumor-induced biochemical shifts. Comparison of EST and EST/GSO (Figure 15C) revealed distinct clusters. Collectively, these multivariate results demonstrate that both GSO and GSONE confer biochemical protection in tumor-bearing mice, with the nanoemulsion formulation exhibiting superior restorative capacity.



**Figure 15.** Principal component analysis (PCA) of biochemical and molecular profiles across experimental groups. (A) PCA plot showing the distribution of all experimental groups: Control, Control/GSO, Control/GSONE, EST, EST/GSO, and EST/GSONE; (B) PCA plot comparing Control and EST groups; (C) PCA plot comparing EST, EST/GSO, and EST/GSONE groups; (D) PCA plot comparing EST and EST/GSO groups. (E) PCA plot comparing EST and EST/GSONE groups. (F) PCA plot comparing EST/GSO and EST/GSONE groups. PCA plots display the multivariate distribution of samples, with colored points representing individual animals and ellipses denoting group clustering. GSO: Grape seed oil (4 mg/kg body weight); GSONE: grape seed oil nanoemulsion (4 mg/kg body weight); EST: Ehrlich solid tumor; EST/GSO: GSO + EST cells; EST/GSONE: GSONE + EST cells.

#### 4. Discussion

Ehrlich carcinoma remains a robust and widely used experimental tumor model that effectively mimics several biological and histopathological features of aggressive human cancers, notably rapid proliferation, low differentiation, and high responsiveness to antitumor intervention [25]. The evolution of this tumor model is known to induce oxidative stress, a hallmark of many malignancies, where an imbalance between oxidant production and antioxidant defenses results in DNA damage, lipid peroxidation, and mitochondrial dysfunction [42]. Such a redox imbalance not only promotes mutagenesis but



also contributes to liver injury and systemic metabolic disruption. Therefore, counteracting oxidative stress using dietary antioxidants and phytochemicals represents a promising strategy for both cancer prevention and supportive therapy [43]. Therefore, counteracting oxidative stress using dietary antioxidants and phytochemicals represents a promising strategy for both cancer prevention and supportive therapy [44].

Extensive evidence has established the chemopreventive and therapeutic effects of grape seed-derived compounds in different cancer models [45,46]. Studies in colon, prostate, lung, and skin cancer models have demonstrated that grape seed extracts (GSEs) rich in polyphenols and proanthocyanidins inhibit proliferation, induce apoptosis, and suppress angiogenesis through pathways involving PI3K/Akt, MAPK, and NF- $\kappa$ B signaling [47–54]. GSO shares similar bioactivity, attributed to its unique composition of linoleic acid, tocopherols, phytosterols, and polyphenols, which exhibit pro-apoptotic and antioxidant mechanisms [55]. However, despite its promising biological activity, GSO's poor aqueous solubility and oxidative instability have limited its practical use in biomedical formulations [23].

The incorporation of GSO into a nanoemulsion (GSONE) provides clear advantages in improving its stability, dispersion, and bioavailability [56,57]. Nanocarrier-based systems, including oil-in-water nanoemulsions, have demonstrated enhanced anticancer efficacy by facilitating cellular uptake and controlled release of hydrophobic phytochemicals [58]. In the present study, the nanoformulation exhibited a mean particle size of ~88 nm and zeta potential of  $-28$  mV, parameters reported to favor intestinal permeability and cellular internalization [59–63]. These physicochemical characteristics are consistent with earlier nanoemulsion studies that improved the antitumor activity of other natural oils by enhancing free radical scavenging and mitochondrial uptake [64–67].

This study's findings that GSONE reduced tumor volume and weight more effectively than crude GSO are in agreement with reports demonstrating that nanoencapsulation potentiates the cytotoxic and apoptotic actions of grape polyphenols [68,69]. Zhu et al. and Al-Ashmawy et al. similarly observed that GSO and proanthocyanidin-rich extracts decreased tumor cell viability by modulating caspase-dependent apoptosis and VEGF-linked angiogenesis [46,70]. The improved therapeutic activity of GSONE may therefore result from enhanced intracellular delivery of active lipophilic constituents that upregulate pro-apoptotic signals (Bax, TP53, caspase-3, caspase-9) and downregulate anti-apoptotic markers (Bcl-2), thereby promoting the intrinsic apoptotic cascade. Comparable mitochondrial apoptotic activation has been described for GSE, where proanthocyanidins increased cytochrome c release and stimulated caspase-3 cleavage in cervical cancer and colon cancer cells [71,72].

Biochemical restoration observed in this study also supports the hepatoprotective potential of GSONE. Elevated AST, ALT, ALP, and bilirubin levels are hallmarks of hepatic stress in tumor-bearing mice. Normalization of these parameters after GSONE treatment mirrors earlier findings, demonstrating that GSO restores hepatic antioxidant capacity in hepatotoxic models [73]. Moreover, the observed reductions in tumor biomarkers AFP and CEA are consistent with previous studies showing that polyphenolic grape derivatives improve liver integrity and reduce systemic oxidative stress [36,74,75].

Reduction in oxidative stress biomarkers (MDA, 8-OHdG) and concomitant elevation of endogenous antioxidants (SOD, CAT, GPx, GSH) demonstrate the enhancement of antioxidant defense mechanisms by GSONE, reinforcing the activation of Nrf2/ARE signaling known from other grape-derived interventions [76,77]. Similar redox modulation has been observed in GSE-treated models of head/neck and colon cancer, where reduced ROS generation coincided with improved mitochondrial function and DNA repair [78,79].

These antioxidant responses not only mitigate tumor-associated metabolic disturbance but also limit damage to hepatic and systemic tissues [80].

The multivariate analysis further consolidated the superiority of the nanoemulsion system relative to crude oil, showing distinct clustering toward healthy control profiles. Such global biochemical normalization underscores the systemic benefits of improved delivery systems in functional nutrient-based chemoprevention [81,82]. Comparable behavior has been reported in studies utilizing lipid-based nanoparticles containing grape seed bioactives, which demonstrated selective cytotoxicity toward tumor cells while sparing normal cell lines [83–85].

Collectively, this work situates GSONE within the broader context of grape-derived phytochemicals and nanotechnology-based delivery systems for cancer management. Previous investigations have confirmed that grape seed components act through multiple converging mechanisms, including antioxidant, anti-inflammatory, pro-apoptotic, and antiangiogenic effects. The present findings add value by demonstrating that nanoformulation magnifies these effects *in vivo*, producing superior restoration of hepatic and systemic homeostasis.

Several limitations should be acknowledged. The EST model, while well-validated, represents a single aggressive tumor type that may not fully reflect the heterogeneity of human cancers. The present study focused on one specific nanoemulsion formulation; variations in particle size, surfactant composition, or preparation methods could yield different outcomes. The short observation period assessed acute effects but precluded evaluation of long-term safety and sustained therapeutic benefits. Additionally, a direct comparison with standard chemotherapeutic agents was not conducted, limiting the assessment of relative therapeutic potential. While enhanced bioavailability was demonstrated, the precise mechanisms underlying nanoemulsion-mediated tissue distribution and cellular uptake remain to be fully elucidated. Another important limitation is that the phytochemical composition of the grape seed oil (GSO) used in this study was not experimentally analyzed in our laboratory. The interpretation of its chemical profile and bioactive constituents was based on previously published data rather than direct analysis, which may introduce minor variations in reproducibility or compositional consistency. Also, the transcriptomic changes underlying the effects of grape seed oil and nanoemulsion were evaluated using targeted qPCR rather than RNA sequencing (RNA-seq). Although qPCR provided specific mechanistic insights into apoptosis and oxidative stress regulation, RNA-seq would offer a comprehensive, genome-wide view of the signaling pathways modulated by the treatments. Due to current budgetary and time constraints, this approach was not pursued, but it remains an essential future direction for validating and extending the present findings.

Similarly, flow cytometric analysis of apoptosis markers (p53, cleaved caspase-3) was performed on dispersed tumor cells without additional gating for epithelial markers such as EpCAM or cytokeratin, thereby restricting analysis to tumor cell populations. Moreover, while the observed loss of viable cells reflected the cytotoxic effects of GSO and GSONE, immunohistochemistry (IHC) or immunofluorescence (IF) could provide improved spatial localization and quantitative confirmation of apoptotic marker expression in tumor tissues.

Future research should aim to integrate omics-based profiling, long-term safety assessments, and direct mapping of nanocarrier-tumor interactions to fully delineate the molecular pathways engaged by grape seed oil nanoemulsions. By bridging nutrition, nanotechnology, and oncology, such approaches will advance the translational potential of dietary phytochemical nanoformulations for cancer prevention and adjunctive therapy.



## 5. Conclusions

This study demonstrates that GSONE confers greater protection against systemic oxidative stress and secondary hepatic injury induced by EST than crude GSO. The superior efficacy of GSONE can be attributed to its enhanced physicochemical properties, including improved stability, water solubility, and bioavailability, afforded by its nanoformulation. Mechanistically, GSONE acts through multiple complementary pathways, exerting potent antioxidant effects, restoring cellular redox balance, modulating oxidative stress-related biochemical parameters, and preserving liver histoarchitecture during tumor challenge. Importantly, GSONE also exhibits pronounced anti-apoptotic activity in tumor cells by downregulating some pro-apoptotic players and mitigating apoptosis-associated molecular disruptions, thereby highlighting its therapeutic promise for the management of malignancies driven by oxidative and metabolic stress.

These findings underscore the potential of nanoemulsion-based delivery systems to enhance the clinical utility of natural bioactives, such as grape seed oil, and support further translational investigation of GSONE as a novel adjunct in cancer therapy.

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## Abbreviations

The following abbreviations are used in this manuscript:

|       |                            |
|-------|----------------------------|
| ALT   | Alanine aminotransferase   |
| ALP   | Alkaline phosphatase       |
| AFP   | Alpha fetoprotein          |
| AST   | Aspartate aminotransferase |
| Bax   | Bcl-2-associated x protein |
| Bcl-2 | B-cell lymphoma 2          |
| CEA   | Carcinoembryonic antigen   |
| CAT   | Catalase                   |
| cDNA  | Complementary DNA          |
| DLS   | Dynamic light scattering   |
| EAC   | Ehrlich Ascites Carcinoma  |

|        |                                         |
|--------|-----------------------------------------|
| ELFA   | Enzyme-linked fluorescent assay         |
| EST    | Ehrlich solid Tumor                     |
| GAPDH  | Glyceraldehyde phosphate dehydrogenase  |
| GSH    | Glutathione (reduced)                   |
| GSO    | Grape seed oil                          |
| GSONE  | Grape seed oil nanoemulsion             |
| GSH-Px | Glutathione peroxidase                  |
| H&E    | Hematoxylin and eosin                   |
| MDA    | Malondialdehyde                         |
| NCI    | National Cancer Institute               |
| p53    | Oncoprotein 53                          |
| PBS    | Phosphate-buffered saline               |
| PCA    | Principal component analysis            |
| PDI    | Polydispersity index                    |
| PE     | Phycoerythrin                           |
| SE     | Standard error                          |
| SOD    | Superoxide dismutase                    |
| TBARS  | Thiobarbituric acid reactive substances |
| TEM    | Transmission electron microscopy        |
| TP53   | Tumor protein 53 gene                   |
| TP     | Total protein                           |

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## Article

# Cucurbitacin B from Cucurbitaceae Plants: Treating Pancreatic Cancer via Inducing Mitophagy, Inhibiting Glycolysis, and Enhancing Immune Function

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**Abstract:** Background: Cucurbitacin B (CuB) is a relatively unique and valuable component in plants of the Cucurbitaceae family due to its diverse and remarkable physiological activities, but its specific mechanisms in regulating tumor metabolism and immune response remain unclear. The hypoxic tumor microenvironment (TME) of pancreatic cancer induces metabolic reprogramming in cancer cells, causing them to rely on glycolysis for energy. LDHA, a key enzyme in glycolysis, can suppress glycolysis and tumor growth when inhibited. Objective: The objective of this study was to investigate the mechanism of CuB against pancreatic cancer and its effect on the immune system. Methods: In this study, cell migration/invasion assays, immunofluorescence, ELISA, Western blot, CETSA, flow cytometry, mouse models, and metabolomic and transcriptomic analyses were utilized to systematically elucidate the mechanism by which CuB inhibits pancreatic cancer and activates the immune system. Results: This study confirms that CuB inhibits pancreatic cancer by suppressing the PI3K/Akt/mTOR pathway and activating PINK1/Parkin to induce mitophagy, thereby inhibiting cell migration, invasion, and proliferation. It down-regulates the expression of LDHA to block glycolysis, reduce lactate production and efflux, and improve the acidic TME. CuB also induces ICD to activate dendritic cells, promote CD8<sup>+</sup> T-cell and M1 macrophage infiltration, and reduce the levels of regulatory T cells. Metabolomic and transcriptomic analyses validate CuB's dual effects on metabolic reprogramming and immune activation. Conclusions: This study, for the first time, reveals that CuB induces mitophagy via the PI3K/Akt/mTOR and PINK1/Parkin pathways to selectively eliminate damaged mitochondria and suppress tumor energy metabolism. CuB inhibits pancreatic cancer through a triple mechanism—inducing mitophagy, inhibiting glycolysis, and activating immunity—which provides innovative insights for pancreatic cancer therapy.

**Keywords:** Cucurbitacin B; pancreatic cancer; mitophagy; glycolysis; immunity

## 1. Introduction

Cucurbitacin B (CuB) is a unique and physiologically active component found in cucurbitaceous plants, primarily present in their fruits, seeds, or vines. CuB has demonstrated multifaceted effects, including antitumor, anti-inflammatory, antibacterial, and antiviral activities. Additionally, it exhibits antifeedant and repellent properties against certain

insects, making it a natural potential plant protectant for crop pest control. Due to its remarkable therapeutic efficacy against various cancers [1,2], several drugs with CuB as the main ingredient or lead compound are currently in clinical trials or have already been applied in clinical treatments.

In the 2022 global cancer statistical analysis, pancreatic cancer ranked as one of the most lethal malignancies worldwide, accounting for 4.8% of all cancer fatalities [3]. Pancreatic ductal adenocarcinoma (PDAC), accounting for 95% of pancreatic cancers, is one of the most invasive malignant tumors [4]. PDAC has numerous fibrogenic mechanism-related cells but poor vascularization, leading to continuous intratumoral hypoxia. Therefore, to ensure energy supply, PDAC cells change from oxidative phosphorylation (OXPHOS) to glycolysis. As a result, most pyruvic acid (PA) is converted to lactic acid (LA) instead of entering the tricarboxylic acid (TCA) cycle [5]. Such glycolytic reprogramming, classically known as the Warburg effect, is pathognomonic of cancer metabolism and prominently observed in PDAC cells [6]. On the one hand, lactate inhibits the functions and viability of T cells and NK cells, aiding immune evasion [7,8]. On the other hand, the enhanced energy metabolism hinders the growth of immune cells by competing with T cells for energy and nutrients within the tumor microenvironment (TME) [9,10]. Lactate dehydrogenase A (LDHA), a key enzyme in aerobic glycolysis, catalyzes the conversion of PA to LA. Thus, it serves as a viable target for tumor therapy, primarily by inhibiting tumor metabolic reprogramming, decreasing lactate secretion, and consequently ameliorating the TME [11]. The special tumor immune microenvironment (TIME) of “cold” tumors severely impairs the efficacy of immunotherapy, characterized by T-cell deficiency or exclusion, accompanied by abundant tumor-promoting macrophages, anti-inflammatory cytokines, and immunosuppressive cells [12,13]. Pancreatic cancer is a typical “cold” tumor, with scarce adaptive immune cells but a significant enrichment of innate immune cells. Therefore, improving the tumor-suppressive immune microenvironment and activating antitumor immune cells are also key to the treatment of pancreatic cancer.

Studies indicate that the antitumor activity of CuB is linked to autophagy [14,15], as it induces DNA damage by elevating ROS levels and activating the PI3K/Akt/mTOR pathway [16]. Research has revealed that the PI3K/Akt/mTOR pathway facilitates the transition to aerobic glycolysis and reprogramming of glucose metabolism [17]. By inhibiting mTORC1, the expression of LDHA and other metabolic effectors can be downregulated, and when this is combined with a PI3K inhibitor, the inhibitory effect on cancer cells is enhanced [18]. Furthermore, CuB has been shown to modulate immune responses, specifically by reducing the expression of cytokines such as NOS, COX-2, and MHC-II in macrophages [19]. Therefore, given these pleiotropic effects and the urgent need for novel therapeutics in pancreatic cancer, CuB is an intriguing candidate for targeting both tumor metabolism and immune dysregulation. However, the exact mechanisms by which CuB modulates tumor metabolism and immune responses in pancreatic cancer are not well understood.

Here, we report the CuB-induced mitophagy of pancreatic cancer via the PI3K/Akt/mTOR and PINK1/Parkin pathways, subsequently modulating the key enzyme levels in the glycolysis pathway and reducing the energy supply for pancreatic cancer cells. In particular, the downregulation of LDHA expression decreases the conversion of PA to LA, and the TME is also improved, with an increase in the infiltration of immune cells. Therefore, we reveal that CuB can induce cellular mitophagy, inhibit glycolysis in pancreatic cancer cells, and improve the TME in terms of metabolic balance through the PI3K/Akt/mTOR and PINK1/Parkin pathways and LDHA downregulation. Significantly, LDHA serves as a promising target for CuB to exert its effects in inhibiting glycolysis and enhancing the TME.

## 2. Materials and Methods

### 2.1. Cell Culture

Human pancreatic cancer cells (PANC-1) (MeilunBio, Dalian, China) and mouse PANC-02 cells (Procell, Wuhan, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA, C11995500BT) supplemented with 10% fetal bovine serum (FBS; Corning, MA, USA, 35-081-CV) and 1% penicillin–streptomycin–amphotericin B (Biorigin, Beijing, China, BN28053) and incubated in an incubator set at 37 °C with a carbon dioxide concentration of 5%.

### 2.2. Cell Viability Determination

PANC-1 cells were seeded in 96-well plates and treated with DMEM or CuB for 24, 48, or 72 h. The optical density at 450 nm was measured following the guidelines of Cell Counting Kit-8 (cck-8 kit) (NCM Biotech, Suzhou, China, C6005).

In the colony formation experiment, cells were seeded in 6-well plates and treated with DMEM or CuB (Shanghai YuanYe Bio-Technology Co., Ltd., Shanghai, China, ≥98%, LOT: J16IB220291). After 24 h, cells in different groups were transferred into new 6-well plates (1000 cells/well). After being washed twice with cold PBS, the colonies were stored in fixative and stained with 0.1% crystal violet. Then, these colonies were counted, and images were taken of them.

In the wound healing experiment, a 96-well plate was used. A scratch was created in each well when its cell density was about 90%. Before applying the DMEM or CuB treatment at different concentrations, we washed the cells twice with  $1 \times$  PBS to reduce the impact of cell proliferation on the results. The wound healing process in the same area was observed using an inverted light microscope at 0 and 24 h.

The invasion ability of PANC-1 cells was assessed using a Matrigel® Invasion Chamber (Corning, Inc., Lowell, MA, USA). The upper chamber was filled with 200 µL of DMEM or varying CuB concentrations, while the lower chamber contained 500 µL of medium supplemented with 10% FBS. Following a 24-h incubation period, cells from both chambers were fixed with 4% paraformaldehyde for 15 min. After two washes with PBS, staining was performed using 0.1% crystal violet. Excess dye was removed by means of repeated PBS washes until the background became clear. Cells remaining in the upper chamber were gently wiped away with a cotton swab. Images were captured using an inverted fluorescence microscope.

### 2.3. DAPI, Calcein AM/PI, and Monodansyl Cadaverine (MDC) Staining

For cellular experiments, adherent cultures in 6-well plates were maintained in DMEM supplemented with various CuB concentrations for 24 h. Following fixation (4% paraformaldehyde, 10 min), nuclei were counterstained with DAPI (Biorigin, Beijing, China, BN20923) and visualized using a Nikon Eclipse Ti Inverted Microscope (Nikon, Japan) after PBS washes. Parallel samples were processed for the viability assessment using the Calcein AM/PI dual-staining kit per the manufacturer's protocol (Lablead, Beijing, China, C30002), with live/dead cell quantification performed on three independent fields per well.

To evaluate autophagy, an MDC stain kit (Beyotime, Shanghai, China, C3018S) was used to detect acidic vesicle organelles such as autophagosomes. After being treated using the same method as above, cells were stained with MDC for 40 min in the dark at 37 °C. Finally, the cells were washed with a  $1 \times$  Buffer and examined using Confocal Laser Scanning Microscopy (CLSM) (Leica SP8, Wetzlar, Germany). The cells were cultured in a 96-well plate using the same treatment method as above, and their fluorescence (Eex/Eem = 335 nm/512 nm) was detected using a fluorescence microplate reader.

#### 2.4. Flow Cytometry

Cells were processed using the same method as above and collected. Cell apoptosis rates were quantified by means of flow cytometry using an Annexin V-FITC detection kit (Beyotime, Shanghai, China, C1062L).

#### 2.5. Measurement of Cellular ATP Content

ATP levels were detected using the ATP Content Assay Kit (SolarBio, Beijing, China, BC0305). PANC-1 cells were processed using the same method as above. Then, the cell lysate or supernatant was collected. The working reagent was mixed with the cell lysate or supernatant and Reagent 1. Absorption was immediately (within 10 s) measured at 340 nm. The mixed solution was incubated at 37 °C for 3 min before retesting. The ATP content was calculated according to the instruction manual.

#### 2.6. Calreticulin (CRT) and High Mobility Group Box 1 (HMGB1) Measurement

After being treated using the same method as above, cells were fixed and then permeabilized with 0.5% Triton. The cells were blocked with 5% BSA, then incubated with anti-HMGB1 (A19529, 1:500) or anti-CRT (ab92516, 1:800) overnight at 4 °C. After washing with cold 1 × PBS, fluorescent secondary antibody was added to the culture plate, and DAPI staining was performed simultaneously. Finally, the fluorescence was observed via CLSM.

The PANC-1 cells were plated on the indicated day of treatment. Then, the cell supernatant or cell lysate was obtained, and its content was measured according to the instructions of the CRT (FineTest, Wuhan, China, EH1659) or HMGB1 (FineTest, Wuhan, China, EH0884) ELISA kit.

#### 2.7. Glucose Consumption Assay

After processing using the same method as above, a Glucose Content Assay Kit (SolarBio, Beijing, China, BC2505) was used to treat the cells. The cell sediment was resuspended in distilled water and boiled for 10 min. The supernatant was collected by means of centrifugation and incubated with the working reagent for 15 min. The absorbance was detected using a fluorescence microplate reader at 505 nm.

#### 2.8. Lactate Secretion Assay

Cells or supernatants were processed and collected as described above. After ultrasonic lysis, they were treated according to the instructions of the CheKine<sup>TM</sup> Micro Lactate Assay Kit (Abbkine, Wuhan, China, KTB1100). Their absorbance was detected using a fluorescence microplate reader at 450 nm.

#### 2.9. PA Secretion Assay

A pyruvic acid production assay was performed by using the Pyruvate Content Assay Kit (SolarBio, Beijing, China, BC2200). Cells or supernatants were processed and collected as described above and lysed by means of ultrasonication. After the cells were mixed with Reagents 1 and 2 in sequence, their absorbance was measured at 520 nm.

#### 2.10. Enzyme Activity Related to Glycolysis

PANC-1 cells were processed and collected as described above. Then, the enzymatic activities of LDHA (FineTest, Wuhan, China, EH2155), PC (Elabscience, Wuhan, China, E-BC-K608-M), and G6PD (FineTest, Wuhan, China, EH1022) in the supernatant were detected according to the instructions of the corresponding reagent kits.



### 2.11. NAD<sup>+</sup>/NADH Ratio Assay

PANC-1 cells were inoculated into a 96-well plate containing 50 µL of culture medium after different treatments according to the instructions of the NAD/NADH-Glo™ Assay (Promega, Madison, MI, USA, G9071). Subsequently, each well was treated according to the kit's instructions, and the signals were measured using a luminometer.

### 2.12. Extracellular Acidification Rate (ECAR) Detection

PANC-1 cells were processed and collected as described above. Following the instructions for the Extracellular Acidification Rate (ECAR) Fluorometric Assay Kit (Elabscience, Wuhan, China, E-BC-F069), the supernatants were resuspended in distilled water. The cells were seeded in a new 96-well plate and incubated in the dark at 37 °C for 30 min. Immediately after the working solution was added, the fluorescence value was detected with a fluorescence microplate reader (Ex/Em = 490 nm/535 nm). After continued incubation for another 30 min, the fluorescence value was detected once more.

### 2.13. Extracellular Oxygen Consumption Rate (ORC) Detection

The cellular oxygen consumption rate was measured using the Extracellular OCR Plate Assay Kit (Dojindo, Kumamoto, Japan, E297). The cells were seeded in a 96-well plate with a black transparent background. After 12 h, the culture medium or working solution was added and incubated at 37 °C for 30 min in a microplate reader. Mineral oil was immediately added and incubated for 5 min after the drug or culture medium was added. The fluorescence intensity was detected every 10 min for 200 min in continuous reading mode using a microplate reader.

### 2.14. RT-qPCR

RNA extraction was carried out with the SteadyPure Universal RNA Extraction Kit (Accurate, Changsha, China, AG21017). Complementary DNA was created using an Evo M-MLV reverse transcription kit (Accurate, Changsha, China, AG11706). Quantitative PCR analysis was conducted on an iQ5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) employing SYBR Green Pro Taq HS Premixed qPCR Kit III (Accurate, Changsha, China, AG11739). All gene expression data were normalized to those for GAPDH, with the primer sequences provided in Supplementary Table S1. All the reagent kits were purchased from Accurate.

LDHA mRNA (transcript number NM\_001165414.2) was used as an overexpression vector. The eukaryotic transient vector pRP [Exp]-Neo CMV > hLDHA [NM\_001165414.2]: IRES: EGFP (Vector ID: DS10038) and pRP [Exp]-NeoR CMV > EGFP control vector (Vector ID: MJ-282) were constructed by Beijing MIJIA Scientific Co., Ltd. (Beijing, China). After Sanger sequencing, the endotoxin-free plasmid was extracted and prepared for transfection into host cells.

The Lipofect5000 transfection reagent (Lablead, Beijing, China, T2123) was used to transfect LDHA overexpression plasmids into cells. First, according to the instructions from the reagent supplier, the cells were cultured in a complete medium without antibiotics until the cell density reached 80%. The Lipofect5000 and plasmid mixture prepared in fresh medium were then replaced in the wells and cultured for another 36 h.

### 2.15. Cellular Thermal Shift Assay (CETSA)

Logarithmically growing PANC-1 cells were passaged into T75 flasks. When the cell confluency reached 90%, the cells were divided into two groups, treated with either 0.1% (*v/v*) DMSO or 50 µg/mL CuB, respectively. After incubation for 60 min following the administration of the treatments, the cells were collected by means of centrifugation and

washed with PBS (0.01 M, pH 7.4). To the cell pellet, 1 mL of pre-chilled PBS (0.01 M, pH 7.4) containing 1% PMSF (Solarbio, Beijing, China, P0100) was added, and the mixture was left to stand on ice for 30 min. The cells were distributed into 6 centrifuge tubes (approximately 150 µL per tube) and heated in a metal bath at 42, 47, 52, 57, 62, or 67 °C for 3 min, followed by equilibration at room temperature (RT) for 3 min. Cells were lysed using a repeated freeze–thaw method (vortex for 30 s → freeze in liquid nitrogen for 1 min → thaw in a 37 °C water bath for 3 min → vortex for 30 s; repeated 3 times to ensure complete lysis). After centrifugation at  $12,000\times g$  at 4 °C for 20 min, the supernatant was transferred to a new EP tube. The supernatant was mixed with 30 µL of loading buffer (Servicebio, Wuhan, China, G3011), vortexed thoroughly, and heated at 99 °C for 5 min for protein denaturation. Finally, the samples were subsequently analyzed via Western blotting (WB).

#### 2.16. Animals and Tumor Models

Male C57BL/6N mice (20–22 g) were purchased from Beijing SiPeiFu Laboratory Animal Technology Co. (Beijing, China). The experiment was performed under the guideline of the Laboratory Animal Ethics Committee of Beijing University of Traditional Chinese Medicine (BUCM-2024050702-2069). To establish a mouse model of pancreatic cancer, a 100 µL cell suspension ( $5 \times 10^6$  cells) was subcutaneously injected into the right upper limb of each mouse. When the tumor size reached about 100 mm<sup>3</sup>, the mice were randomly divided into three groups, with 6 mice in each group. The CuB groups were injected with CuB at a concentration of 0.5 mg/mL in 1% DMSO diluted with physiological saline (0.9% NaCl), while the control group was injected with physiological saline (0.9% NaCl) and the DMSO group received 0.1% DMSO as vehicles. Throughout the entire process, the size of the tumors was measured using a vernier caliper, and the tumor volume was calculated using the following formula:  $\text{volume} = 0.5 \times L \times W \times W$ , where L and W represent the tumor length and width. We also performed HE staining on major organs (heart, liver, spleen, and kidneys) to determine *in vivo* biosafety; the levels of liver (ALT, AST, and ALP) and kidney (GFR, UREA, and CREA) function indicators were also evaluated.

#### 2.17. Immunofluorescence (IF)

Fixed tumor tissues (5 µm sections) were blocked with 5% BSA, then co-incubated with antibodies and DAPI. TUNEL (Servicebio, Wuhan, China, G1504) and ROS (Beyotime, Shanghai, China, S0033S) staining was performed. Antifluorescence quenching mounting medium (Lablead, Beijing, China, D0120) was used. Sections were observed and imaged via CLSM; the fluorescence intensity was quantified using ImageJ (ImageJ 1.54f). Image quantification was performed blindly. Antibody details are presented in Table S2.

#### 2.18. Expression of Lactic Acid, Pyruvate, and Cytokines in Tumors

Tumor tissues from the control and CuB groups were harvested and homogenized, and the supernatant was collected by means of centrifugation. The LA and PA concentrations in the supernatant were detected using a reagent kit. In addition, fresh tumor tissues were mixed with 0.9 mL pre-cooled PBS at a ratio of 1:9 (*w/v*) and homogenized on ice. The homogenate was centrifuged at 2000 rpm at 4 °C for 20 min, and the supernatant was placed on ice for subsequent detection. The pH values of tumor suspensions from mice in different treatment groups were measured using a precision pH meter (Hangzhou Qiwei Instrument Co., Ltd., Hangzhou, China), which was calibrated with standard buffer solutions (pHs 4.00, 6.86, 9.18) according to the instruction manual before detection. The levels of cytokines (i.e., iNOS, TNF-α, INF-γ, Arg1, IL-4, and IL-10) in mouse tumor lysate were detected using ELISA kits.

### 2.19. Metabolomic Analysis

For the metabolomic analysis, 30 mg of sample was mixed with 600  $\mu$ L of pre-cooled 80% (*v/v*) methanol containing two steel balls. The mixture was pre-cooled at  $-40^{\circ}\text{C}$ , ground for 2 min, sonicated in an ice-water bath, and then incubated at  $-40^{\circ}\text{C}$  overnight. After centrifugation (12,000 rpm,  $4^{\circ}\text{C}$ , 20 min), 150  $\mu$ L of the supernatant was stored at  $-80^{\circ}\text{C}$  for subsequent LC-MS analysis. Another 150  $\mu$ L of the supernatant was dried and derivatized with 80  $\mu$ L of methoxyamine hydrochloride (15 mg/mL in pyridine) at  $37^{\circ}\text{C}$  for 60 min, followed by the addition of 50  $\mu$ L of BSTFA, 20  $\mu$ L of *n*-hexane, and 10  $\mu$ L of internal standards (C8-C24 prepared in chloroform); the mixture was reacted at  $70^{\circ}\text{C}$  for 60 min and then left at room temperature for 30 min. GC-MS was used for the metabolic profiling of energy metabolites (including those related to glycolysis, the tricarboxylic acid cycle, and amino acids). Quality control (QC) samples were prepared by mixing equal volumes of extracts from all samples. Raw data were processed using XCMS v4.5.1 (for baseline filtering, peak detection, etc.) and MS-DIAL for the deconvolution analysis. Compounds were identified based on their retention time, mass, fragments, and isotopes, combined with the HMDB, Lipidmaps v2.3, METLIN, and LuMet-Animal3.0 databases. Information on the internal standards and experimental reagents is provided in Table S3.

### 2.20. Western Blotting (WB)

Protein samples underwent separation via SDS-PAGE (Lablead, P0085) and were then transferred using a transfer buffer (servicebio, G2145). After being blocked for 2 h, the membrane was incubated with the primary antibody overnight at  $4^{\circ}\text{C}$ . Before incubation with the secondary antibody, the membrane was washed with PBS. Finally, an ultrasensitive ECL chemiluminescent solution (Biorigin, BN16009) was used, and images were taken using the chemiluminescence image analysis system.  $\beta$ -actin was used as the internal reference, and specific information on the antibodies used is shown in Table S2.

### 2.21. In Vivo Immunotherapeutic Mechanism

On the 11th day, the mice in each group were humanely sacrificed. Tumor tissues and spleens were then collected and subjected to flow cytometry analysis. Minced tissue was ground with PBS and sieved. For lymph node dendritic cells, sieved cells were centrifuged at 1000 rpm for 10 min; the pellet was washed with PBS, resuspended in 200  $\mu$ L PBS, incubated with antibodies at room temperature for 30 min, then detected on a BD LSRFortessa Cell Analyzer. For the spleen and tumor, sieved cells were centrifuged at 1500 rpm for 10 min; the pellet was resuspended in 2 mL DMEM, layered over 1 mL room-temperature Lymphoprep, and centrifuged at  $800\times g$  for 25 min at room temperature. The buffy coat was collected, washed with PBS, centrifuged at 1000 rpm for 10 min, incubated with antibodies at room temperature for 30 min, then detected on a BD LSRFortessa Cell Analyzer. Antibodies were diluted to half the maximum recommended concentration, as per the product instructions.

Moreover, immunofluorescence staining was carried out on the tumor tissues to determine the expression levels of CRT and HMGB1. The antibodies used are listed in Table S2.

### 2.22. Transcriptomic Analysis

Libraries were sequenced on Illumina Novaseq 6000, yielding 150 bp paired-end reads. RNA integrity (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA) was ensured with an RIN of  $\geq 7.0$ . The libraries were then constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit following the manufacturer's instructions. The sequencing depth was

approximately 40 million reads per sample to ensure sufficient sequencing coverage. Raw reads were processed with Fastp 1 to remove low-quality ones. After StringTie2 assembly, Cuffcompare enabled gene structure extension and novel transcript identification. ASprofile analyzed alternative splicing of differential transcripts/exons. SNPs/InDels were identified using SAMtools/BCFtools (details: <http://samtools.sourceforge.net/mpileup.shtml> (accessed on 15 March 2025)), with SnpEff annotating variant effects (e.g., amino acid changes). Subsequently, a bioinformatics analysis of the RNA-Seq data was performed using R software (v 3.2.0), in which the DESeq2 package in R was used to identify differentially expressed genes (DEGs). Hypergeometric-based enrichment analyses (GO, KEGG, Reactome, WikiPathways) in R screened significant terms, which were visualized as bar charts, chord diagrams, and bubble charts via R.

### 2.23. Statistical Analysis

The data are presented in the form of the mean  $\pm$  the standard error of the mean (SEM), analyzed using GraphPad Prism 10.2.3. For comparisons of two groups, unpaired *t*-tests were used; for comparisons of multiple groups, one-way ANOVAs with Tukey's post hoc test were used. Significance was inferred at  $p < 0.05$ . Normality (Shapiro–Wilk) and variance homogeneity (Levene) were tested; parametric methods were applied only when assumptions were met. Details of the specific statistical methods, *p*-values, and sample sizes (*n*) for each experiment are provided in the figures and their respective legends.

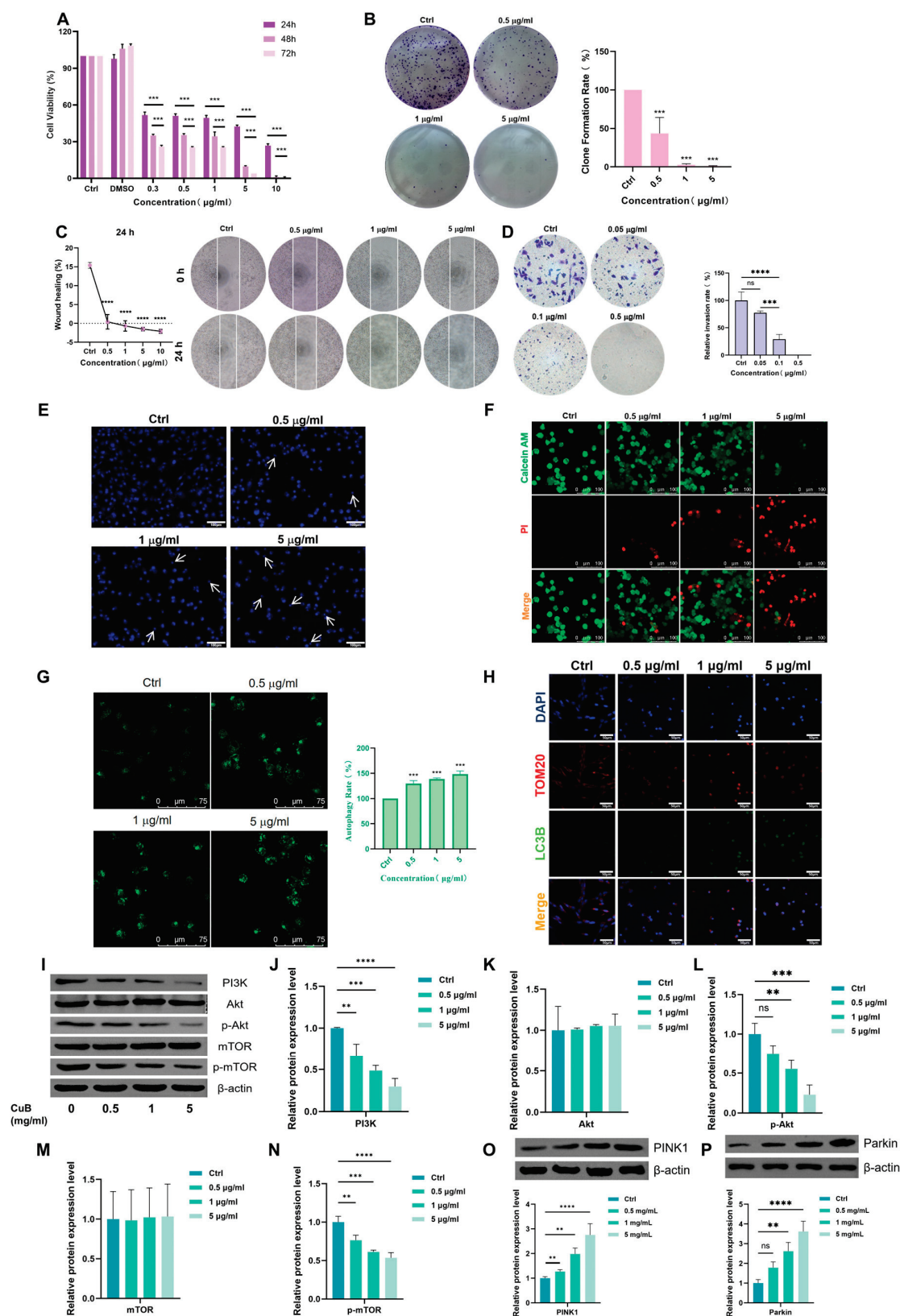
## 3. Results

### 3.1. Anti-Pancreatic Cancer Activity of CuB

We explored CuB's antitumor activities through in vitro cell experiments. To determine the effect of CuB on the proliferation of PANC-1 cells, the cells were incubated with CuB for 24 or 48 h. Our results showed that CuB significantly inhibited the proliferation of PANC-1 cells (Figure 1A) in a time- and dose-dependent manner, and the IC<sub>50</sub> values were 1.0, 0.11, and 0.022  $\mu\text{g/mL}$  for 24, 48, and 96 h. Even at low concentrations, CuB significantly inhibited the colony formation and migration abilities of PANC-1 cells (Figure 1B,C). A Transwell assay with Matrigel showed that CuB reduced the invasion ability of PANC-1 cells (Figure 1D). As shown in Figure 1E,F, CuB also induced cell death in a concentration-dependent manner, causing the cell nuclei to shrink and become brighter.

To further study the mechanism of cell death, we also examined the autophagic effect of CuB on PANC-1 cells. The number of autophagic vesicles increased significantly in cells treated with CuB (Figure 1G). Meanwhile, as the concentration of CuB increased, the intensity of the fluorescence co-localization of the autophagy protein LC3B and mitochondria (labeled by TOM20) was enhanced (Figure 1H), indicating that the ability of autophagosomes to recognize and clear damaged mitochondria improved, and the level of mitophagy increased. The results (Figure 1I–P) of WB in vitro showed that after drug administration, PI3K, *p*-mTOR, and *p*-Akt expression decreased, total mTOR and total Akt remained unchanged, and PINK1 and Parkin expression increased. Therefore, CuB may induce mitophagy through the PI3K/Akt/mTOR and PINK1/Parkin pathways.



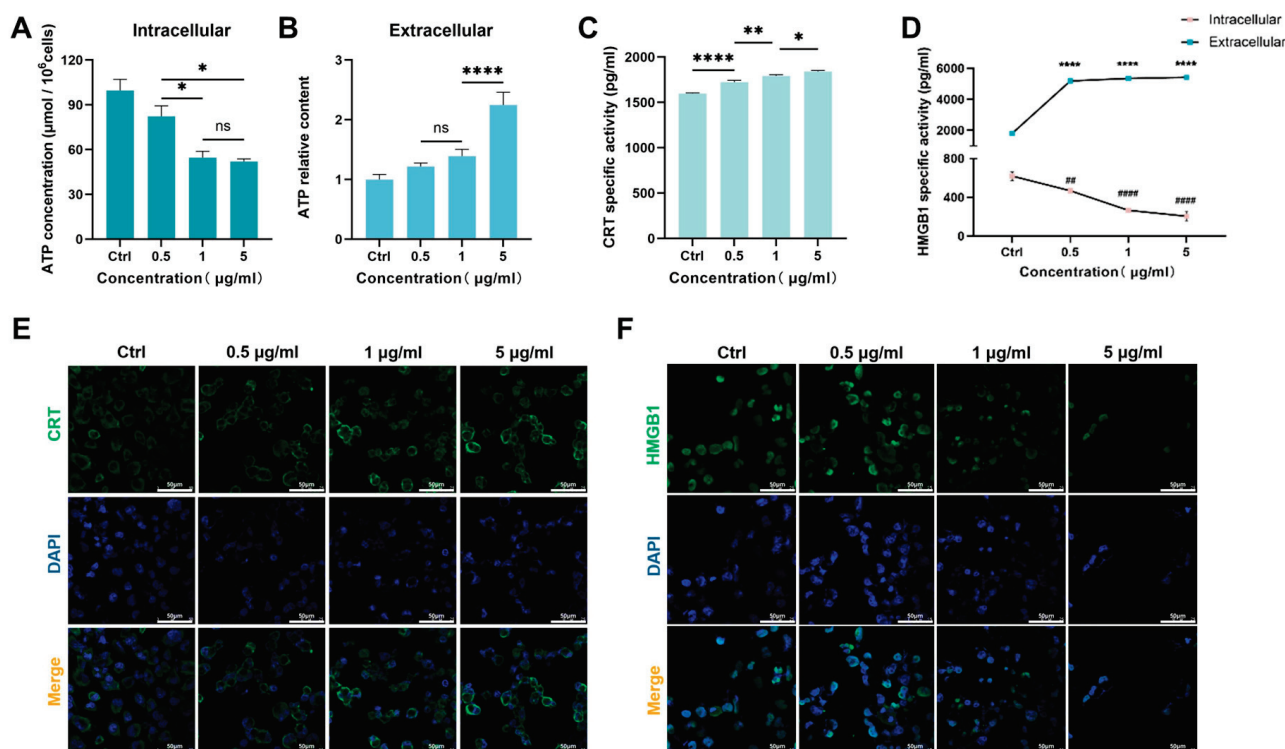


**Figure 1.** Anti-pancreatic cancer activity of CuB. (A) CuB has potent growth inhibitory effects on PANC-1 cells. Bars,  $\pm$ standard error (SE). (B) Effect of CuB on colony formation. (C,D) Effects of CuB on migration and invasion. (E,F) DAPI and Calcein AM/PI staining. (G) Effect of CuB on autophagy. (H) Immunofluorescence staining of DAPI, LC3B, and TOM20. (I–P) Effects of CuB on proteins related to the PI3K/Akt/mTOR and PINK1/Parkin pathways. Bars,  $\pm$ SE; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .



### 3.2. CuB Induces Immunogenic Cell Death (ICD)

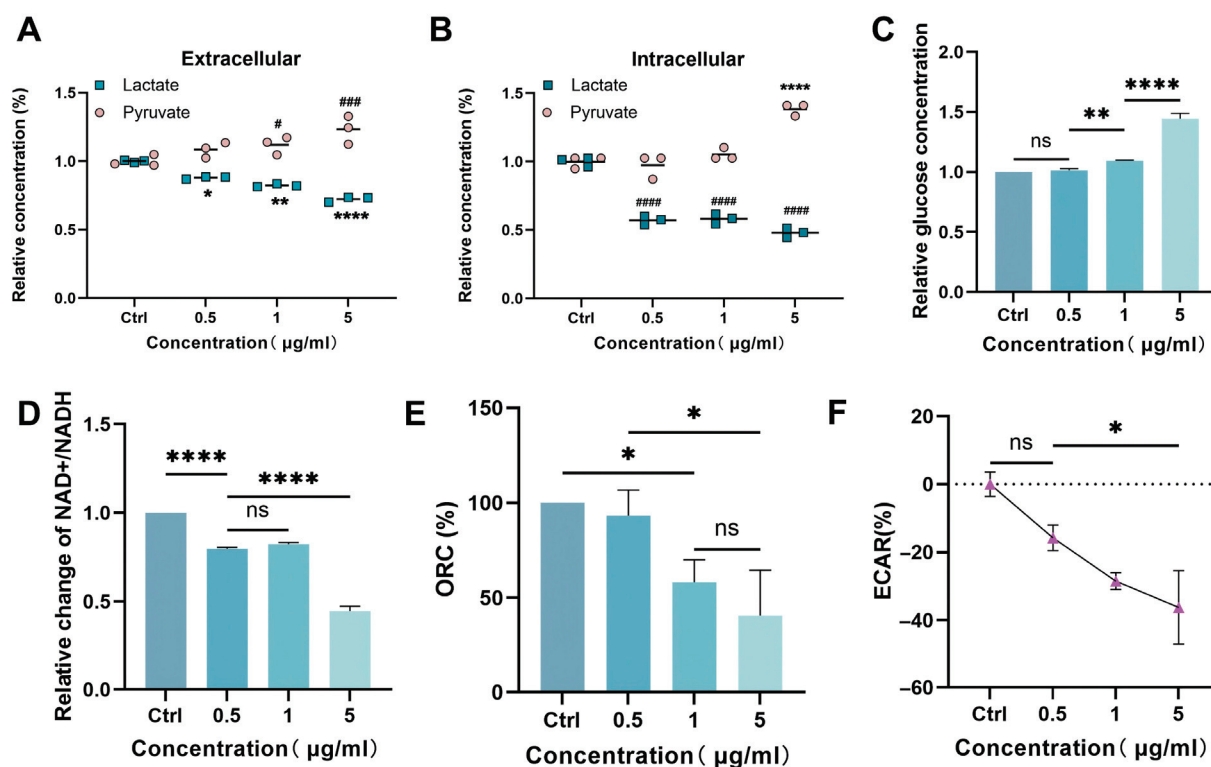
ICD can directly cause cell death and trigger immune responses to combat cancer. This process is marked by the presentation of CRT on the cell surface. Additionally, it involves the secretion of mediators, including the non-histone chromatin protein HMGB1 and ATP [20,21]. We also investigated the effect of CuB on ICD. ATP is released in an autophagy-dependent manner from cells during the ICD process [22]; therefore, we detected the ATP content. Figure 2A,B shows that CuB significantly reduced intracellular ATP levels but increased ATP release. Additionally, CuB promoted the release of HMGB1 and the exposure of CRT, while the intracellular expression of HMGB1 was decreased (Figure 2C–F). This suggests that during the action of CuB, an ICD response occurred in the tumor cells, which enhanced the antitumor immune response.



**Figure 2.** CuB induces immunogenic cell death. Effects of CuB on the intracellular (A) and (B) extracellular ATP contents in PANC-1 cells. The levels of (C) CRT and (D) HMGB1 in PANC-1 cells. (E) CRT and (F) HMGB1 immunofluorescence staining. Bars,  $\pm$ SE; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ , ##  $p < 0.01$ , #####  $p < 0.0001$ .

### 3.3. Cucurbitacin B-Mediated Inhibition of Glycolysis

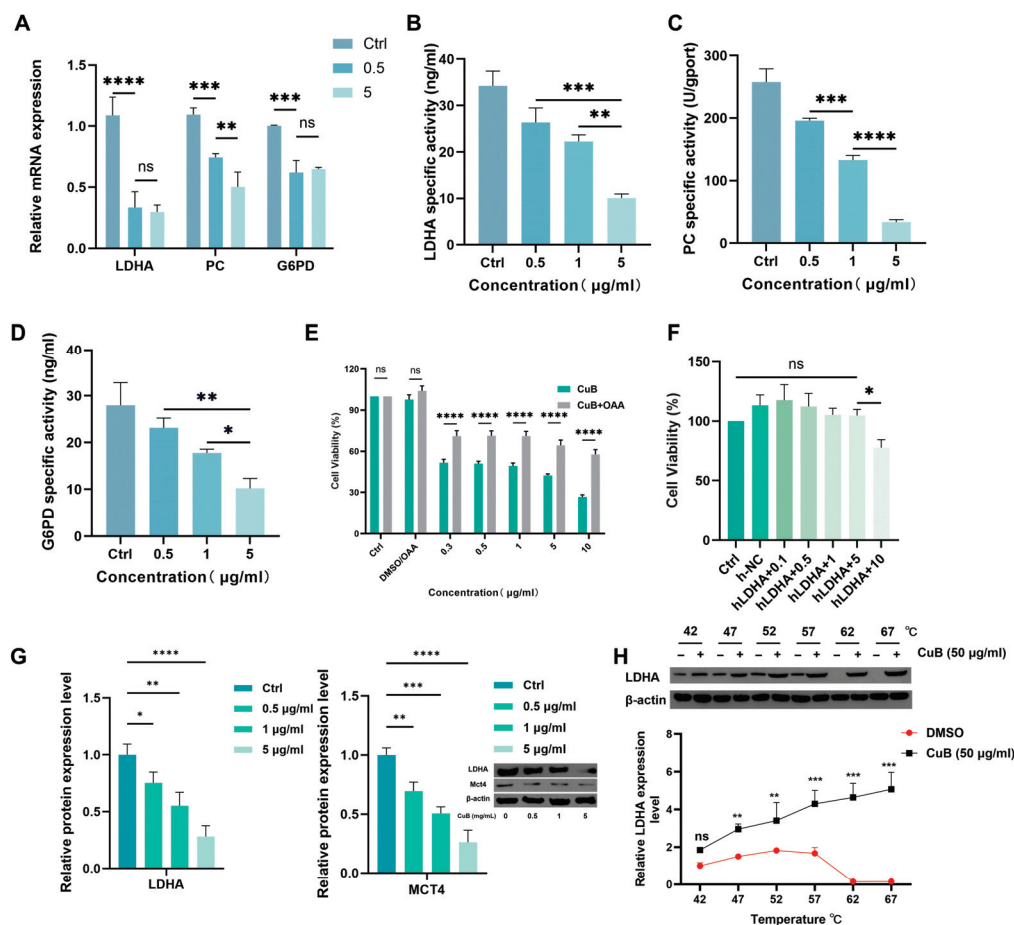
To explore the impacts of CuB on cellular glucose metabolism, we initially measured the levels of both intracellular and extracellular LA and PA. The findings indicated that following drug administration, the concentrations of both intracellular and extracellular PA rose, whereas those of LA declined (Figure 3A,B), indicating that the glycolytic pathway might have been inhibited. The accumulation of PA led to the inhibition of glucose metabolism, resulting in a decrease in glucose consumption (Figure 3C). Additionally, we found that CuB decreased the  $\text{NAD}^+/\text{NADH}$  ratio, along with both the OCR and ECAR (Figure 3D–F), indicating that the cellular oxidative phosphorylation metabolic pathway was also inhibited.



**Figure 3.** Cucurbitacin B-mediated inhibition of glycolysis. (A) Extracellular and (B) intracellular LA and PA contents in PANC-1 cells. (C) Intracellular glucose concentration in PANC-1 cells. NAD<sup>+</sup>/NADH ratio (D), ORC (E), and ECAR (F) in PANC-1 cells under different concentrations of CuB. Bars,  $\pm$ SE; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ , #  $p < 0.05$ , ###  $p < 0.001$ , ####  $p < 0.0001$ .

To further clarify the mechanism of CuB's effect on glucose metabolism, we detected the transcription of LDHA (a key enzyme in glycolysis), PC (a key enzyme in the TAC), and G6PD (a key enzyme in the PPP). The transcription levels of all three enzymes were downregulated after drug administration (Figure 4A), which was also confirmed by the ELISA results (Figure 4B–D). Among them, the activities of PC and LDHA were more significantly affected by CuB than were those of G6PD. Pyruvate reacts with CO<sub>2</sub> in a carboxylation reaction catalyzed by PC to generate oxaloacetate (OAA), which then enters the TCA cycle pathway. Our experiments demonstrated that CuB significantly inhibited PC activity (Figure 4C). Thus, we examined the effect on PANC-1 cells with added OAA (2 mM). The results indicated that the addition of OAA (2 mM) only slightly rescued the growth of PANC-1 cells treated with CuB (Figure 4E). Moreover, the inhibitory effect of CuB on PANC-1 cells with LDHA overexpression (Figure S1) was significantly attenuated (Figure 4F). This indicated that CuB mainly restrained the expression of LDHA enzyme, suppressing the glycolytic pathway.

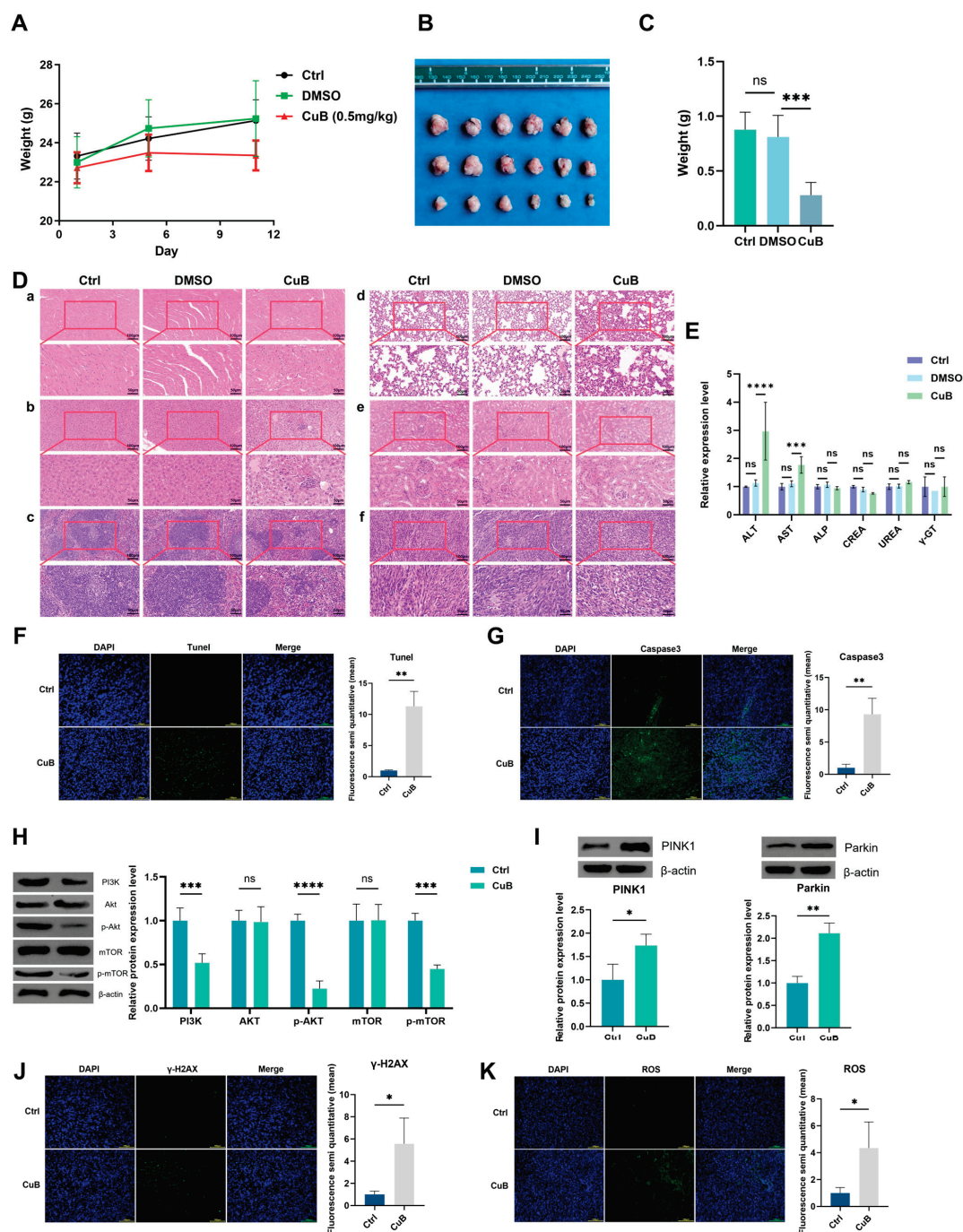
The WB experiment also confirmed that the LDHA level decreased in the CuB-treated group (Figure 4G). In addition, we investigated MCT4 expression by means of WB. The downregulation of MCT4 expression (Figure 4G) indicated that CuB also reduced lactate efflux. To further determine whether LDHA is the target of CuB, we performed a CETSA (Figure 4H). The LDHA activity in the DMSO group decreased with an increase in temperature but was retained after treatment with CuB. These results indicate that CuB may influence the glucose metabolism reprogramming of cells by inhibiting the activities of enzymes related to glucose metabolism in PANC-1 cells—particularly the activity of LDHA, a key enzyme in glycolysis—thereby reducing lactate production and ATP generation and consequently suppressing aerobic glycolysis and growth in PANC-1 cells.



**Figure 4.** The mechanism by which CuB inhibits glycolysis. (A) The transcription levels and (B–D) ELISA quantitative analysis results of three enzymes in PANC-1 cells treated with CuB. (E) The viability of CuB-treated PANC-1 cells with/without OAA. (F) The cell viability of CuB-treated PANC-1 cells after LDHA overexpression. (G) Protein expression of LDHA and MCT4 in PANC-1 cells under CuB. (H) CETSA. Bars,  $\pm$ SE; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### 3.4. In Vivo Antitumor Effect of CuB

To explore the in vivo anticancer effect of CuB, we established a PANC02 tumor-bearing mouse model and examined the changes in tumor volume and weight in three different treatment groups (saline, 0.1% DMSO, and CuB). First, the in vivo safety of the CuB treatment was affirmed by tracking body weight, assessing blood biochemical indices, and conducting HE staining. The experimental data indicated that CuB did not cause a significant reduction in the body weight of the mice (Figure 5A). However, compared with those for the other groups, the rate of weight gain was decreased, which might be related to the regulatory effect of CuB on mouse metabolism. It also caused no evident inflammation or damage in the major organs of the mice (including heart, spleen, lung, and kidneys), but some inflammation occurred in the liver (Figure 5E). Furthermore, there was a significant difference in the growth of tumors (Figure 5B,C) among the CuB-treated group, the saline group, and the 0.1% DMSO group. H&E staining (Figure 5D) showed that tumors in CuB-treated mice exhibited pronounced cellular damage, including widespread structural disintegration and nuclear loss, underscoring the remarkable antitumor potency of CuB. To further assess tumor cell apoptosis, TUNEL and caspase-3 assays were performed. Figure 5F,G demonstrate marked increases in TUNEL-positive cells and caspase-3 expression in the CuB group compared with the controls, confirming CuB's capacity to trigger apoptotic pathways in tumor cells.



**Figure 5.** In vivo antitumor effect of CuB. (A) Weights of mice ( $n = 6$ ). Volumes (B) and weights (C) of tumors at the end of treatment ( $n = 6$ ). (D) HE staining images of the heart (a), liver (b), spleen (c), lung (d), kidney (e), and tumors (f) of mice at the end of different treatments. (E) Blood biochemical index analysis;  $n = 6$ . IF staining of TUNEL (F) and caspase-3 (G) in tumor tissue. (H,I) WB analysis of proteins related to the PI3K/Akt/mTOR and PINK1/Parkin signaling pathways.  $\gamma$ -H2AX staining (J) and ROS staining (K) in tumor tissue.  $n = 3$ ; bars,  $\pm$ SE; statistical significance determined via unpaired two-tailed Student's  $t$ -test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

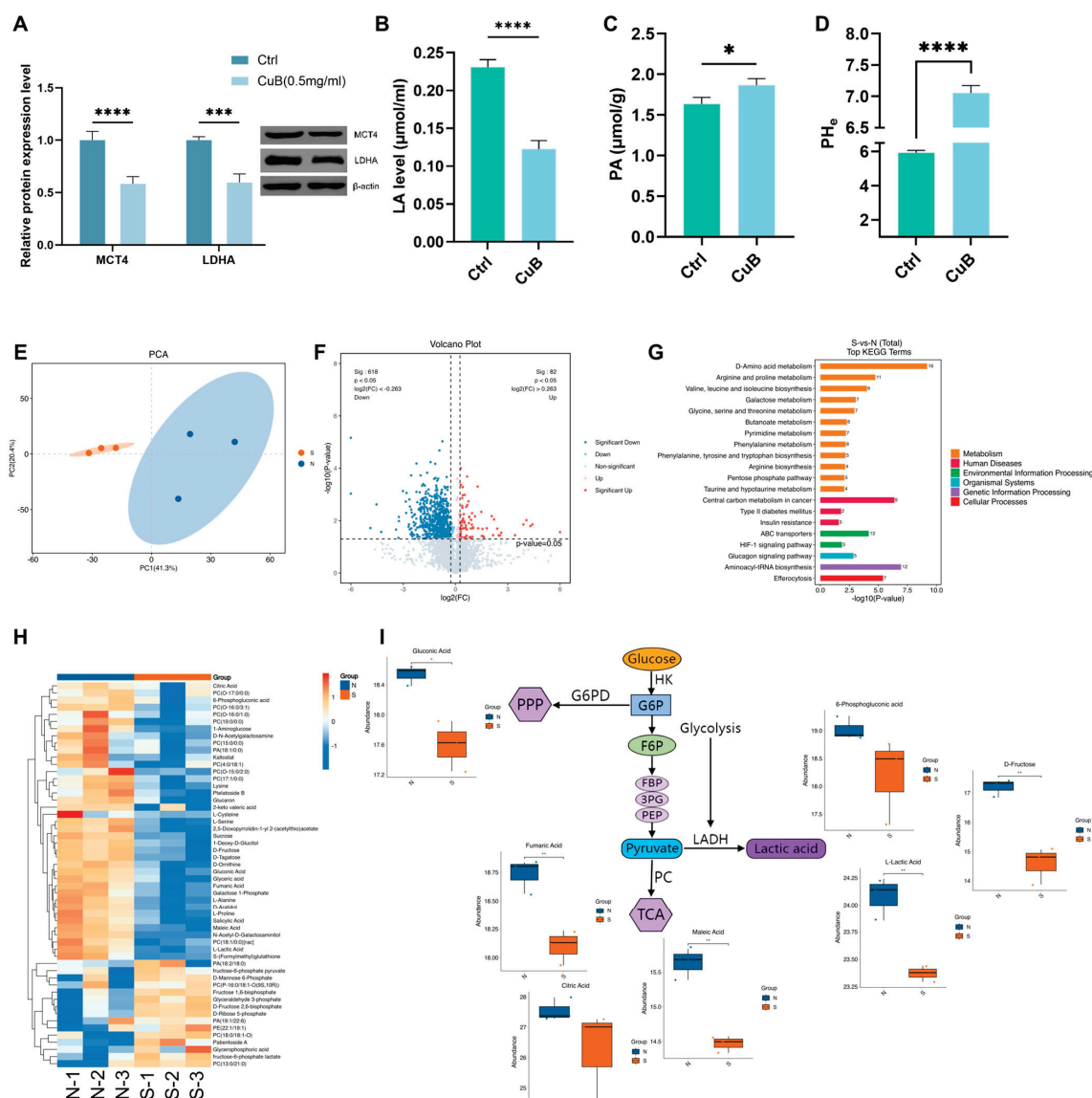
We further detected the expression of mTOR,  $p$ -mTOR, PI3K, Akt,  $p$ -Akt, PINK1, and Parkin in tumor tissues via WB. The results (Figure 5H,I) were consistent with those of the in vitro experiments. CuB autophagy induction is associated with the induction of ROS and the promotion of DNA damage [23–25]. Therefore, we also detected the levels of  $\gamma$ -H2AX and ROS in tumor tissues by means of immunofluorescence. In this study, as



shown in Figure 5J,K, the fluorescence of both  $\gamma$ -H2AX and ROS in the CuB treatment group was enhanced.

### 3.5. In Vivo Effect of CuB on Tumor Metabolism

In pancreatic cancer, high glycolytic rates and the accumulation of LA contribute to the creation of the acidic pericellular TME and activate immunosuppressive pathways [26]. To elucidate the potential mechanism of CuB in regulating glycolytic metabolism and reversing an immunosuppressive TME, we first investigated the glycolysis-related enzyme levels in vivo after CuB treatment. Consistent with the in vitro results, in tumor tissues, the expression levels of LDHA and MCT4 in the CuB treatment group were decreased (Figure 6A), the content of LA was reduced, and the content of PA was increased (Figure 6B,C). Studies have shown that MCTs, especially MCT4, which transports LA out of the cell, are important for pH regulation [27]. In this study, we found that the pH value of tumor tissues in mice significantly increased to 7.05 (Figure 6D), indicating that the acidic microenvironment was improved.



**Figure 6.** In vivo metabolism effect of CuB. (A) Protein expressions of LDHA and MCT4 in tumor tissue ( $n = 3$ ). Concentrations of LA ( $n = 6$ ) (B) and PA ( $n = 3$ ) (C). (D) pH values in the supernatant of mouse tumor tissue ( $n = 3$ ). (E) PCA of metabolites in tumors from mice ( $n = 3$ ). Volcano plot (F) and



KEGG enrichment analysis (G) of all DEMs between N (control group) and S (CuB-treated group). (H) Clustering heatmap of differentially expressed metabolites related to glucose metabolism. (I) Schematic of CuB-induced changes in metabolic pathways. Bars,  $\pm$ SE; statistical significance determined via unpaired two-tailed Student's *t*-test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

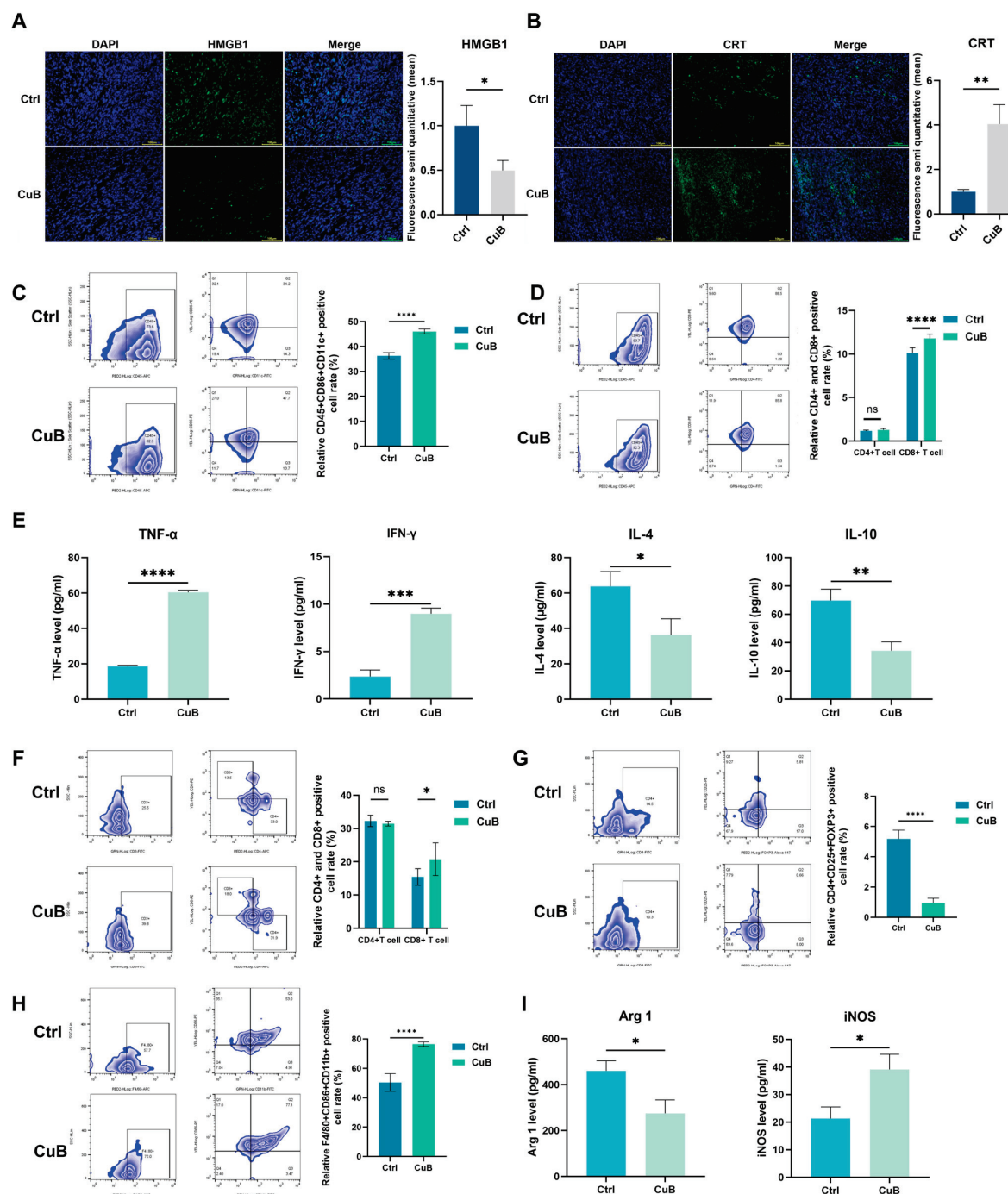
To further elucidate the anticancer effects of CuB, we conducted a metabolomics analysis. The principal component analysis (PCA) results indicated marked disparities between the normal saline group and the CuB-treated group (Figure 6E). Among all the detected differential metabolites, 214 were downregulated, while 51 were upregulated (Figure 6F). The expression of the metabolites after CuB treatment is shown in a clustered heatmap. The metabolomics analysis combined with KEGG pathway annotation revealed that the differentially expressed metabolites (DEMs) were predominantly enriched in three major metabolic pathways: amino acid metabolism, carbohydrate metabolism, and lipid metabolism (Figure 6G). Building upon our metabolomic investigation, we systematically characterized the DEMs associated with glycolytic pathways and glucose homeostasis. The clustered heatmap (Figure 6H) demonstrates that glucose metabolites like L-LA were substantially diminished in the CuB-treated group. The levels of relevant metabolites of the PPP pathway, TCA cycle, and glycolysis pathway were notably decreased (Figure 6I), suggesting significant suppression of glucose metabolism.

### 3.6. *In Vivo* Effect of CuB on Tumor Immune System

In vitro studies have shown that CuB can induce ICD. In vivo, immunofluorescence staining (Figure 7A,B) showed that treatment with CuB led to significantly higher exposure of CRT and lower exposure of HMGB1 than those in the saline group, which means that it had excellent ICD induction performance. We further examined the maturation of DCs. The maturity of DCs in the CuB-treated group was significantly increased, accounting for 46.04% (Figure 7C). Moreover, we noted that after CuB treatment, the proportion of CD8<sup>+</sup> T cells among T lymphocytes infiltrating the mouse spleen increased by 1.23-fold. Meanwhile, the CD4<sup>+</sup> T-cell proportion showed no marked disparity (Figure 7D).

Our results show that CuB significantly inhibits the secretion of LA. A reduction in LA secretion could decrease the levels of anti-inflammatory cytokines while increasing the levels of pro-inflammatory cytokines [28]. Therefore, we tested the relevant factors in the supernatant; the ELISA results (Figure 7E) showed increased expression levels of TNF- $\alpha$  and INF- $\gamma$  (3.20- and 3.79-fold, respectively) and decreased levels of IL-4 and IL-10. This indicates that CuB exerts an in vivo effect of inducing inflammatory factors that are closely related to the activation of systemic immunity [29]. Among them, cytokines such as IFN- $\gamma$  and TNF- $\alpha$  are key biomarkers produced by tumor-infiltrating T cells [30]. Then, we examined the proportions of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells among the tumor-infiltrating T cells. As illustrated in Figure 7F, the infiltration of CD8<sup>+</sup> T lymphocytes increased 1.35-fold after treatment with CuB, while CD4<sup>+</sup> T-cell populations remained statistically unchanged. Additionally, Tregs play a key role in mediating immunosuppression within the TME by inhibiting both T-cell expansion and effector cytokine secretion. In our results, the number of Treg cells in the CuB treatment group was significantly reduced (Figure 7G). Notably, CuB treatment triggered macrophage polarization toward the M1 phenotype (Figure 7H), as evidenced by upregulated M1-specific markers and concomitant downregulation of M2-associated markers (Figure 7I).

The above results demonstrate that CuB improves tumor glycolytic metabolism by inhibiting the expression of LDHA, thereby reducing LA production, remodeling systemic immunity, and increasing the infiltration of antitumor immune cells.

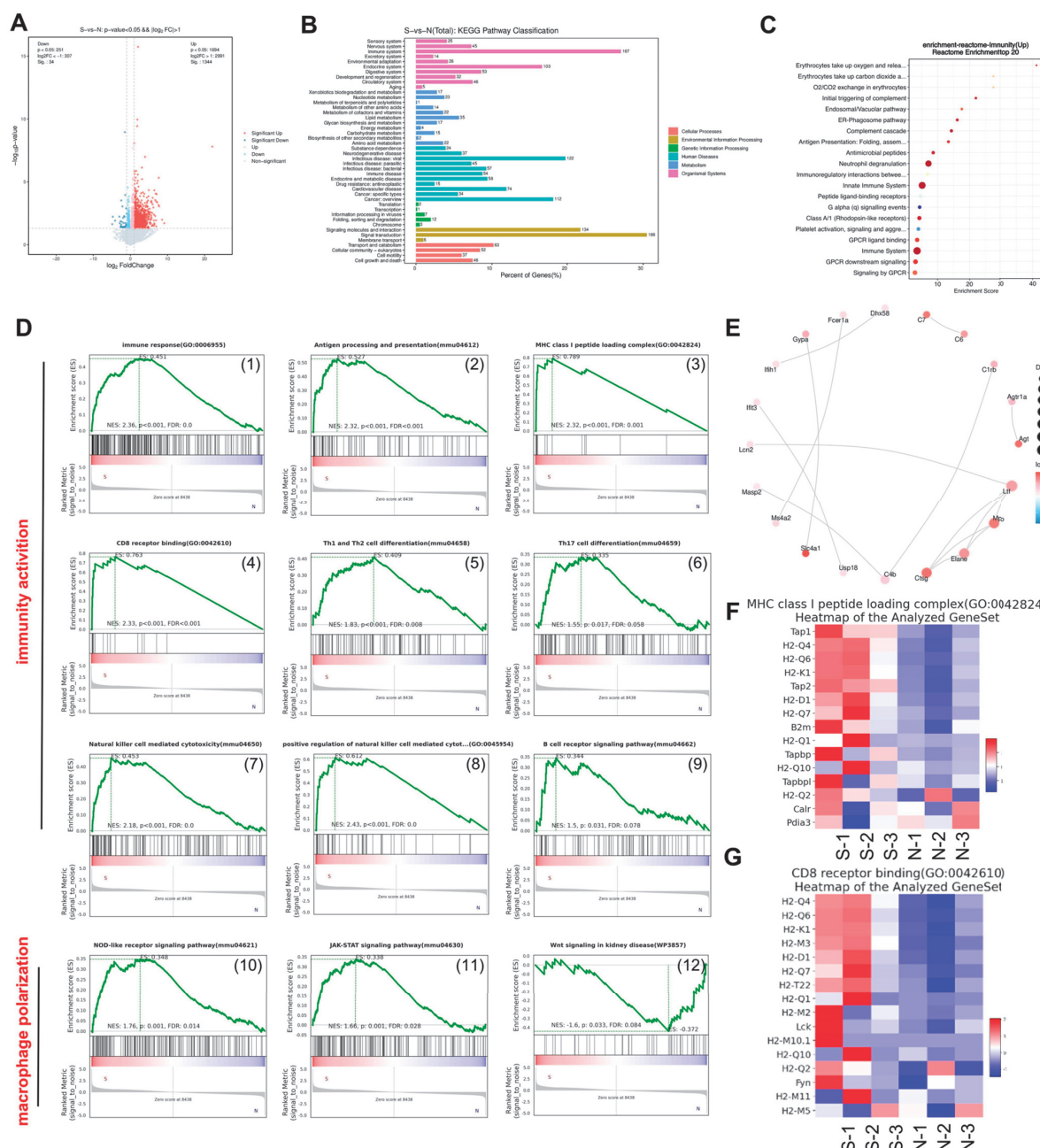


**Figure 7.** In vivo effect of CuB on tumor immune system. Immunofluorescence staining and quantitative fluorescence analysis of HMGB1 (A) and CRT (B). DC maturation in tumor tissues (C) and CD8<sup>+</sup> T cells and CD4<sup>+</sup> cells in the spleen (D). (E) Expression levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-4, and IL-10. CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells (F), Tregs (G), and M1-polarization macrophages (H) in tumor tissues. (I) Expression of M1 and M2 polarization markers.  $n = 3$ ; bars,  $\pm$ SE; statistical significance determined via unpaired two-tailed Student's  $t$ -test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### 3.7. Transcriptome Analysis

Abnormal tumor metabolism—such as glycolysis—and the accumulation of LA in tumors play important roles in establishing an immunosuppressive TME [30]. Therefore, we used RNA-Seq to explore the mechanisms by which CuB regulates glycolysis and activates the immune response. As shown in the volcano plot in Figure 8A, we detected 1378 DEGs. Upregulated genes (1344) are indicated in red, while downregulated genes (34)

are shown in blue. A KEGG pathway enrichment analysis was conducted on the DEGs. As shown in Figure 8B, the DEGs in organismal systems were mainly enriched in the immune system. The subsequent Reactome pathway analysis of immunomodulatory pathways is presented in Figure 8C. Furthermore, large immunity-associated pathways, including antigen presentation, activation of multiple immune cells, and macrophage polarization, were found via GSEA to be enriched in the CuB group (Figure 8D). The top 30 genes in the PPI network showed that most hub genes were related to immunity (Figure 8E). In particular, CuB treatment significantly activated the “MHC class I peptide loading complex” and “CD8 receptor binding pathway” (Figure 8F,G) and increased the number of CD8<sup>+</sup> T cells (Figure 7F). In summary, immune function was significantly activated after CuB treatment, including antigen presentation and the activation of immune cells to enhance antitumor immune responses.

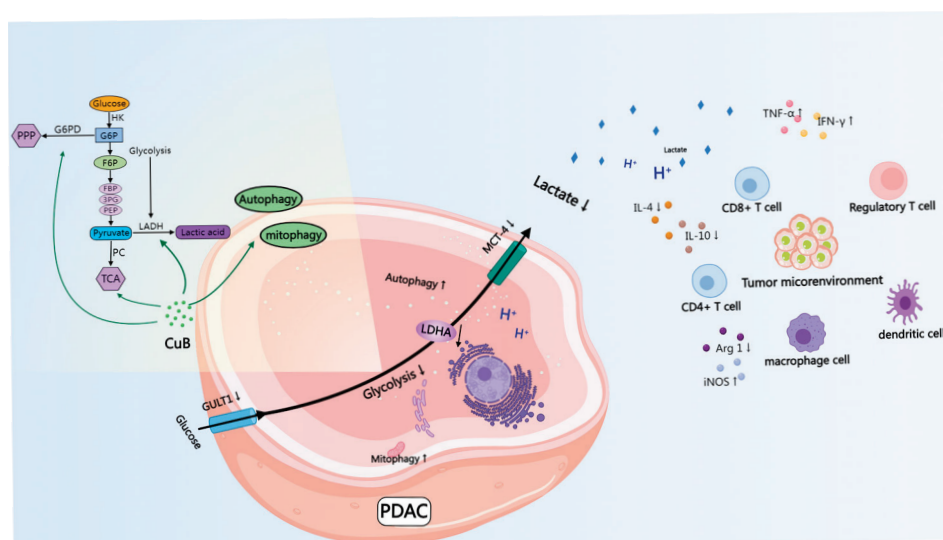


**Figure 8.** RNA-Seq analysis of tumor tissues receiving CuB. A volcano plot (A) and KEGG enrichment analysis (B) of all DEGs between N (control group) and S (CuB-treated group). (C) The results of the

Reactome enrichment analysis based on the immunomodulatory pathways. (D) The enrichment of differentially expressed genes was investigated by means of GSEA ((1)–(9) are immune activation-related pathways, and (10)–(12) are macrophage polarization-related pathways.). (E) The PPI network of differentially expressed genes. Clustering heatmaps for “MHC class I peptide loading complex” (F) and “CD8 receptor binding pathway” (G).

#### 4. Discussion

CuB exhibits broad-spectrum antiproliferative activity against multiple cancer cell types. Its mechanisms include inducing cell cycle arrest [31], triggering autophagy [23], hindering cell migration and invasion [32], and suppressing angiogenesis [33]. Moreover, CuB exhibits immune-enhancing properties by promoting the M1 polarization of macrophages [34] and enhancing dendritic cell differentiation [35]. Despite its promising effects, the full mechanism of CuB's action remains elusive. Our study revealed that CuB initiates mitophagy in PDAC cells via the PI3K/Akt/mTOR and PINK1/Parkin pathways. Additionally, it reduces lactate secretion by downregulating LDHA expression, which hinders tumor glycolysis and boosts the antitumor immune response. This multifaceted approach underscores CuB's potential as a potent anticancer agent (Figure 9).



**Figure 9.** An illustration of CuB's mechanism of action. (Created with MedPeer (medpeer.cn).)

This study revealed that CuB exerts a multifaceted influence, significantly inhibiting not only the proliferative abilities of PANC-1 cells but also their abilities to form colonies, invade, and migrate. Our finding that CuB significantly inhibits the PI3K/Akt/mTOR pathway, a well-known negative regulator of autophagy, is consistent with the findings of prior research [36,37]. The PI3K/Akt/mTOR pathway is activated after administration of the drug. Furthermore, TFAM, a key regulatory factor of mitochondrial function, shows a reduction in mitochondria that reflects mitochondrial structural damage and degradation, consistent with the occurrence of mitophagy [38]. We demonstrated that TFAM expression decreases with increasing CuB concentration (Figure S3A), while the ratio of intracellular to cytoplasmic mtDNA increases, indicating enhanced mitophagy and the release of mtDNA into the cytoplasm (Figure S3B). CuB activates the PINK1/Parkin pathway, recognized as the core machinery for mitophagy [39]. For the first time, we demonstrated that CuB induces selective autophagy, specifically mitophagy, through the PINK1/Parkin pathway. This dual regulation not only promotes the clearance of damaged mitochondria and the release of mtDNA, but also disrupts the energy metabolism balance of tumor cells, contributing to the inhibition of tumor growth.



CuB also shows a significant inhibitory effect on glycolysis in PDAC cells, crucial for breaking the “Warburg effect” on which tumor cells depend. In the CuB-treated group, indicators related to glucose metabolism—glucose consumption, the NAD<sup>+</sup>/NADH ratio, the OCR, and the ECAR—were reduced (Figure 3D–F). Simultaneously, the LA content decreased, while PA accumulated. Further study revealed that this phenomenon is mainly due to CuB suppressing the expression of LDHA, a key enzyme in glycolysis that catalyzes the conversion of PA to LA. A CETSA experiment confirmed LDHA as a direct target of CuB. Reduced LDHA activity not only impairs glycolytic energy supply but also decreases extracellular LA export through MCT4, alleviating tumor microenvironment (TME) acidosis (Figure 6D). These results form an extension of research by Li et al. [40], who linked CuB’s inhibition of glycolytic enzymes (HK, PK) to Akt/mTOR regulation, by identifying LDHA as a critical downstream effector.

In addition, the CuB-treated group exhibited an increase in extracellular ATP content, CRT exposure, and HMGB1 production inhibition—hallmarks of ICD. The DAMPs released during ICD can be phagocytosed by antigen-presenting cells (APCs), such as dendritic cells (DCs). The maturation of DCs has been demonstrated as a crucial process in initiating immune responses through facilitating antigen presentation and the infiltration of cytotoxic T lymphocytes [41]. In this study, we examined immune cells and cytokines in CuB-treated mice. The results showed that CuB promoted DC maturation, increased the number of CD8<sup>+</sup> T cells, reduced the number of Tregs, and increased the proportion of M1 macrophages. Additionally, the increase in inflammatory cytokine secretion, decrease in anti-inflammatory cytokine secretion, increase in M1 polarization markers, and decrease in M2 polarization markers suggest that CuB had a certain improvement effect on the immunosuppressive TME. Notably, the level of LA—a known suppressor of T-cell function and inducer of M2 polarization [42,43]—is reduced by CuB, further amplifying immune activation.

RNA sequencing results showed that the upregulated genes were enriched in immune-related pathways, indicating significant activation of immune function after CuB treatment. In particular, pathways related to antigen presentation were significantly activated. Enhanced IFN- $\gamma$  secretion likely drives increased MHC-I expression, facilitating antigen presentation to CD8<sup>+</sup> T cells [44] and mitigating immune escape. IF and WB results also confirmed that MHC-I expression increased after CuB treatment (Figure S4). These transcriptomic findings align with phenotypic changes in the TME (Figure 7D–F), reinforcing CuB’s ability to coordinate metabolic disruption with immune activation.

Compared with standard PDAC therapies, CuB exhibits distinct mechanisms of action. Gemcitabine (a nucleoside analog) and FOLFIRINOX (a combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) primarily target DNA synthesis or cell division but are limited by drug resistance and systemic toxicity [45]. In contrast, CuB acts through unique pathways: it directly targets glycolysis via LDHA, exploiting PDAC’s metabolic dependency, whereas gemcitabine inhibits DNA replication; it reshapes the TME to enhance antitumor immunity, a feature not prominent in FOLFIRINOX, potentially synergizing with immunotherapies. It is worth noting that CuB-induced PINK1/Parkin-mediated mitophagy accelerates mitochondrial clearance and disrupts the energy metabolism balance, as evidenced by the reduced level of oxidative phosphorylation (Figure 3E). Moreover, abnormal mitophagy may lead to the release into the cytoplasm or extracellular space of mtDNA or mitochondrial proteins, which can activate the cGAS-STING pathway or be recognized by immune cells as damage-associated molecular patterns (DAMPs). This process promotes antigen presentation and antitumor immune responses, suggesting that CuB could serve as an adjuvant for PDAC immunotherapy—for instance, by enhancing the efficacy of PD-1/PD-L1 inhibitors and improving the “immune desert” TME of PDAC. This also represents a direction for our further research.



It is worth noting that in our experiments, we found that even a low concentration of CuB can cause liver damage in mice, limiting its clinical application. Currently, a growing number of nanomaterials, such as Metal–Organic Frameworks (MOFs), exosomes, liposomes, etc., are being utilized as carriers for natural drugs. These carriers, when modified, exhibit targeting and controlled release capabilities. Among them, MOFs, composed of metal ions and organic ligands, boast a high drug loading capacity. When targeted modifications are applied, they can enhance efficacy and reduce toxicity. Hence, utilizing MOFs as carriers for the targeted delivery of CuB represents a direction for our future research.

## 5. Conclusions

In conclusion, CuB inhibits PDAC through a coordinated interplay of autophagy induction, metabolic disruption, and TME remodeling. Its unique mechanism of action, distinct from those of standard therapies, highlights its potential as a multi-target antitumor agent, with further research into delivery systems poised to address its clinical limitations. Certainly, there is still room for improvement in this article. For instance, the PANC-02 cell line model was used to establish tumor-bearing mice. Although this model can better simulate the tumor-immune interactions in immunocompetent mice, due to differences in the gene mutation spectrum compared with human PDAC, it cannot be used as a direct basis for translational therapy in human PDAC, but only provides a foundation for subsequent verification in more precise models (such as mouse models with driver gene mutations like KRAS and TP53).

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu17172809/s1>, Figure S1: The transcription levels of LDHA in PANC-1 cells; Figure S2: The nuclear magnetic resonance (NMR) results of CuB; Figure S3: (A) Immunofluorescence staining of DAPI, TFAM, and TOM20. (B) The transcription levels of mtDNA in PANC-02 cells treated with CuB (1 µg/mL); Figure S4: The IF staining (A) and protein expression of MHC-I in tumor tissue ( $n = 3$ ); Table S1: Primers used in this study; Table S2: Antibodies used in this study.(IF, WB, Flow); Table S3: Reagent Information.

**Author Contributions:** D.Y.: Writing—review & editing, Writing—original draft, Methodology, Investigation, Conceptualization. H.C.: Validation, Software, Methodology, Investigation. X.J.: Validation, Software, Formal analysis. S.L.: Validation, Software, Formal analysis. Y.S.: Software, Formal analysis. R.C.: Software, Formal analysis. Y.F.: Software, Formal analysis. X.D.: Software. C.Q.: Validation. J.N.: Writing—review & editing, Funding acquisition. X.Y.: Writing—review & editing, Writing—original draft, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The experiment was performed under the guideline of the Laboratory Animal Ethics Committee of Beijing University of Traditional Chinese Medicine (Approval code: BUCM-2024050702-2069, approval date: 13 May 2024).

**Data Availability Statement:** Data will be made available on request.

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

## Abbreviations

CuB, Cucurbitacin B; DAMPs, damage-associated molecular patterns; ECAR, Extracellular Acidification Rate; GSEA, gene set enrichment analysis; GT, Gamma-Glutamyl Transferase; ICD, Immunogenic Cell Death; LA, lactic acid; OAA, oxaloacetate; OCR, Oxygen Consumption Rate; OXPHOS, oxidative phosphorylation; PA, pyruvic acid; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; TIME, tumor immunosuppressive microenvironment; TME, tumor microenvironment; TNF- $\alpha$ , Tumor Necrosis Factor-alpha.

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## Article

# Growth of Renal Cancer Cell Lines Is Strongly Inhibited by Synergistic Activity of Low-Dosed Amygdalin and Sulforaphane

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**Abstract: Background:** Plant derived isolated compounds or extracts enjoy great popularity among cancer patients, although knowledge about their mode of action is unclear. The present study investigated whether the combination of two herbal drugs, the cyanogenic diglucoside amygdalin and the isothiocyanate sulforaphane (SFN), influences growth and proliferation of renal cell carcinoma (RCC) cell lines. **Methods:** A498, Caki-1, and KTCTL-26 cells were exposed to low-dosed amygdalin (1 or 5 mg/mL), or SFN (5  $\mu$ M) or to combined SFN-amygdalin. Tumor growth and proliferation were analyzed by MTT, BrdU incorporation, and clone formation assays. Cell cycle phases and cell cycle-regulating proteins were analyzed by flow cytometry and Western blotting, respectively. The effectiveness of the amygdalin–SFN combination was determined using the Bliss independence model. **Results:** 1 mg/mL amygdalin or 5  $\mu$ M SFN, given separately, did not suppress RCC cell growth, and 5 mg/mL amygdalin only slightly diminished A498 (but not Caki-1 and KTCTL-26) cell growth. However, already 1 mg/mL amygdalin potently inhibited growth of all tumor cell lines when combined with SFN. Accordingly, 1 mg/mL amygdalin suppressed BrdU incorporation only when given together with SFN. Clonogenic growth was also drastically reduced by the drug combination, whereas only minor effects were seen under single drug treatment. Superior efficacy of co-treatment, compared to monodrug exposure, was also seen for cell cycling, with an enhanced G0/G1 and diminished G2/M phase in A498 cells. Cell cycle regulating proteins were altered differently, depending on the applied drug schedule (single versus dual application) and the RCC cell line, excepting phosphorylated Akt which was considerably diminished in all three cell lines with maximum effects induced by the drug combination. The Bliss independence analysis verified synergistic interactions between amygdalin and SFN. **Conclusions:** These results point to synergistic effects of amygdalin and SFN on RCC cell growth and clone formation and Akt might be a relevant target protein. The combined use of low-dosed amygdalin and SFN could, therefore, be beneficial as a complementary option to treat RCC. To evaluate clinical feasibility, the in vitro protocol must be applied to an in vivo model.

**Keywords:** amygdalin; sulforaphane; renal cell carcinoma; tumor growth; cell cycling

## 1. Introduction

The use of complementary and alternative medicine (CAM) has significantly increased among cancer patients in the last decades. A survey on cancer patients in France and



Poland indicated that 85% are CAM users [1,2]. The number of cancer patients in Sweden and Germany integrating CAM into their treatment schedules has been estimated to be between 50 and 70% [3,4]. Overall, in Europe, more than 50% of cancer patients utilize CAM [5]. CAM encompasses non-conventional therapies such as phytomedicine and probiotics, and mind-body practices such as acupuncture and hypnosis. Among these options, plant-derived compounds are most frequently used, based on evidence that a balanced diet high in fruits and vegetables reduces cancer risk [6,7].

The integration of plant-derived natural products, either as isolated bioactive compounds or as plant extracts, into conventional cancer treatment is associated with the hope of alleviating disease symptoms, mitigating side effects, boosting the immune system, and preventing disease recurrence. Of the many available herbal drugs, amygdalin and sulforaphane (SFN) are among the ones purported to exert distinct anti-tumor properties.

The natural compound amygdalin, a cyanogenic glycoside found in high concentrations in bitter almonds and fruit kernels such as peaches and apricots, metabolizes into hydrogen cyanide (HCN) in the body through  $\beta$ -glucosidase activity [8]. The proponents of amygdalin therapy assume that HCN selectively accumulates in tumor cells due to their enrichment with  $\beta$ -glucosidase, forcing selective tumor cell destruction [8]. In vitro data point to amygdalin triggered apoptosis induction with an increase of Bax, a decrease of Bcl-2 proteins and alterations of caspase-3 activity, cell growth blockade by acting on the cyclin-cdk axis and akt-mTOR signaling, and invasion suppression via altered integrin  $\alpha$  and  $\beta$  receptor expression [9–11]. Animal studies show that amygdalin may also down-regulate cancer growth in vivo [12,13]. Nevertheless, its anti-tumor efficacy remains contentious. An old study conducted in 1982, involving 175 cancer patients, did not report a clinical benefit of amygdalin [14]. No high-standard clinical studies have been initiated since then, leaving its anti-tumor potential unresolved. Rather, warnings have been issued that excessive amygdalin consumption may lead to severe cyanide poisoning [15]. During recent years, amygdalin has regained high regard as a cytotoxic and chemopreventive remedy [16,17].

SFN, an isothiocyanate abundant in green vegetables such as broccoli, kale, cabbage and cauliflower, is highly popular among cancer patients, since it is said to prevent oncogenesis and tumor progression [18]. Its anti-cancer effects have been demonstrated on a panel of human tumor cell lines, including bladder, lung, breast, and colon cancer cells, by modulating epigenetic as well as non-epigenetic pathways involving interactions with the Akt/MAPK and the Nrf2/ARE signaling pathways [19]. Detailed information on the molecular mode of action of SFN is provided in [20]. There are further reports pointing to preventive and therapeutic effects of SFN in in vivo tumor models [21,22]. Attenuation of oncogenic pathways along with an increase in tumor suppressor proteins under SFN have been shown in clinical trials [23,24]. SFN has recently been declared one of the “Big Five” phytochemicals targeting cancer stem cells [25].

Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for approximately 90% of cases [26]. Once metastasized, RCC is difficult to treat. It does not respond to chemoradiotherapy, and cytokine-based therapy has failed due to insufficient efficacy. The concept of drug targeting with receptor tyrosine kinase inhibitors (TKIs) is also not as efficient as initially thought, due to resistance development [25]. The introduction of immune checkpoint inhibitors (ICIs), often combined with TKIs or other ICIs, has significantly improved the prognosis for patients with metastatic disease. However, this approach is associated with severe immune-related adverse effects [27], and only a minority of patients profit from this type of therapy [28].

Thus, integrating amygdalin and SFN into current treatment could offer potential benefits to RCC patients. The present study was conducted to elucidate growth suppressive properties and the underlying molecular mechanisms of both compounds on RCC cells in vitro, with particular emphasis on low-dosed amygdalin.

## 2. Materials and Methods

### 2.1. Cell Cultures

RCC cell lines Caki-1 and KTCTL-26 were purchased from LGC Promochem (Wesel, Germany). A498 cells were provided by Cell Lines Service (Heidelberg, Germany). All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2% HEPES (2-(4-(2-Hydroxyethyl)-1-piperazine)-ethanesulfonic acid) buffer, 1% Glutamax (all Gibco/Invitrogen, Karlsruhe, Germany), and 1% penicillin/streptomycin (both Sigma-Aldrich, München, Germany) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### 2.2. Drugs

Amygdalin from apricot kernels (Sigma-Aldrich, Taufkirchen, Germany) was freshly dissolved in cell culture medium at low concentrations of 1 and 5 mg/mL [10,29]. SFN was provided by Biomol, Hamburg, Germany. Based on earlier dose-response analyses, SFN was applied at 5 µM [30,31]. The tumor cells were treated with amygdalin alone, with SFN alone or with both amygdalin and SFN. Controls remained untreated.

### 2.3. Cell Growth and Apoptosis

Cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction test (MTT-assay). Drug treated versus untreated carcinoma cell lines were plated out into 96-multiwell plates (50 µL/well,  $1 \times 10^5$  cells/mL) and incubated for 24, 48, and 72 h. Then, 10 µL of MTT/well (Roche Diagnostics, Penzberg, Germany) was added for 4 h. Cells were then lysed using sodium dodecyl sulfate (10% SDS in 0.01 M HCl) and incubated overnight at 37 °C. A multi-well ELISA reader determined the absorbance at 570 nm (Tecan Infinite M200, Männedorf, Switzerland). After subtracting background absorbance and matching with a standard curve, the results were expressed as mean cell number. The mean cell number was set at 100% after 24 h of incubation.

Apoptosis was evaluated by the annexin V-FITC Apoptosis Detection kit (BD Pharmingen, Heidelberg, Germany). Tumor cell lines were washed twice with PBS, and then incubated with 5 µL of annexin V-FITC and 5 µL of propidium iodide in the dark for 15 min. Cells were then analyzed using the FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany). A total of 10,000 events were collected for each sample. CellQuest software version 6.0 (BD Biosciences) served to quantify the percentage of apoptotic (early and late), necrotic, and vital cells.

### 2.4. Bliss Independence Model

Synergistic, additive, or antagonistic effects between SFN and amygdalin were determined using the Bliss independence model [32]. The calculation was based on the MTT data with the assumption that each of the drugs applied, amygdalin and SFN, induces its anti-cancer effect independently by targeting different pathways. The tumor cells were treated with either SFN [5 µM] or amygdalin [1 or 5 µM] or the two combined for 24, 48, and 72 h. The response, in terms of growth inhibition, was normalized to the untreated control at the respective time point (set to 100%). The Bliss predicted inhibition rate I<sub>Bliss</sub> was then calculated by the formula

$$I_{\text{Bliss}} = I_{\text{amyg}} + I_{\text{SFN}} - (I_{\text{amyg}} \times I_{\text{SFN}})$$

Synergism is defined by  $I_{\text{Bliss}} > \text{observed inhibition}$  by the drug combination.  $I_{\text{Bliss}} < \text{observed inhibition}$  by the drug combination reflects antagonism, and  $I_{\text{Bliss}} = \text{observed inhibition}$  by the drug combination defines an additive effect.

### 2.5. Clonogenic Growth and Cell Proliferation

To evaluate clonogenic tumor growth, 500 cells/well were transferred to 6-well-plates. A 2 mL cell culture medium was additionally added (drug-containing versus drug-free medium). Following 5–10 days of incubation without medium change during this time,

cell colonies were fixed and counted. Clones  $\geq 50$  cells were counted as one colony using a Zeiss ID 03 light microscope (Zeiss AG, Oberkochen, Germany). Tumor cell proliferation was measured using a BrdU (5-bromo-2'-deoxyuridine) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem/Merck Biosciences, Darmstadt, Germany). Drug treated versus non-treated tumor cells were seeded onto 96-well microtiter plates (50  $\mu$ L/well,  $1 \times 10^5$  cells/mL) and incubated with BrdU labeling solution (20  $\mu$ L/well). After 24 or 48 h, cells were fixed and detected using anti-BrdU mAb, according to the manufacturer's instructions. Absorbance was measured at 450 nm by a microplate ELISA reader (Tecan Infinite M200, Männedorf, Switzerland). Values were expressed as a percentage compared to untreated controls, set to 100%.

## 2.6. Cell Cycle Analysis

A cell cycle analysis was performed with RCC cell lines grown to subconfluency. To carry out cell cycle analysis, treated and non-treated tumor cell populations were stained with the dye propidium iodide, using the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, Heidelberg, Germany), and then subjected to flow cytometry with a FACSCalibur (BD Biosciences). A total of 10,000 events per sample were analyzed. Data acquisition was conducted with Cell-Quest software, version 5.1. The cell cycle distribution was calculated using ModFit software, version 3.3 (BD Biosciences). The number of gated cells in the G1, G2/M, or S phase was finally depicted as the percentage of the total number of cells in all phases. Experiments were conducted with both asynchronous and synchronous tumor cells. Tumor cells were synchronized at the G1-S boundary with aphidicolin (1  $\mu$ g/mL; Sigma-Aldrich) 24 h before starting cell cycle analysis and subsequently resuspended in fresh (aphidicolin-free) medium for 2 h.

## 2.7. Western Blot Analysis

The expression of proteins involved in cell cycle regulation was analyzed in Caki1, A498, and KTCTL-26 cells. The tumor cell lysates were applied to a 7–12% polyacrylamide gel (depending on the protein size to be detected) and electrophoresed for 90 min at 100 V. Proteins were then transferred to nitrocellulose membranes (1 h, 100 V), blocked with nonfat dry milk for 1 h, and incubated overnight with the following monoclonal antibodies: Anti-CDK1/Cdc2 (IgG1, clone 1), anti-pCDK1/Cdc2 (IgG1, clone 44/CDK1/Cdc2 (pY15)), anti-CDK2 (IgG2a, clone 55), anti-Cyclin A (IgG1, clone 25), anti-Cyclin B (IgG1, clone 18), anti-PKB $\alpha$ /AKT (IgG1 clone 55), anti-pAKT (IgG1, Ser472/Ser473, clone 104A282), p27 (IgG1, clone G173-524), p19 (IgG1, clone 52/p19 Skp1; all: BD Pharmingen). Anti-PTEN (clone 26H9) was from Cell Signaling (Leiden, Netherlands, anti-pCDK2 (Thr160) from Thermo Fisher Scientific, Schwerte, Germany, and anti-histone H3 (IgG, clone 3H1), anti-acetylated H3 (aH3; IgG, Lys9, clone C5B11), and anti-histone H4 (IgG, clone L64C1,) were all from Cell Signaling (Leiden, The Netherlands). Anti-acetylated H4 (aH4; Lys8, polyclonal, IgG). Anti-Bax (B-9:sc-7480) and anti-Bcl-2 (N-19:sc-492) were obtained from Santa Cruz (Heidelberg, Germany). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (Upstate Biotechnology, Lake Placid, NY, USA) served as the secondary antibodies. For protein visualization, the membranes were incubated with ECL detection reagent (ECL; Amersham/GE Healthcare, München, Germany). Then, protein bands were analyzed using the Fusion FX7 system (Peqlab, Erlangen, Germany).  $\beta$ -Actin (1:1000; clone AC-15; Sigma-Aldrich) served as the internal control (see also Supplement S1). To quantify the intensity of the protein bands, the protein/ $\beta$ -actin intensity ratio was quantified using GIMP 2.8 software.

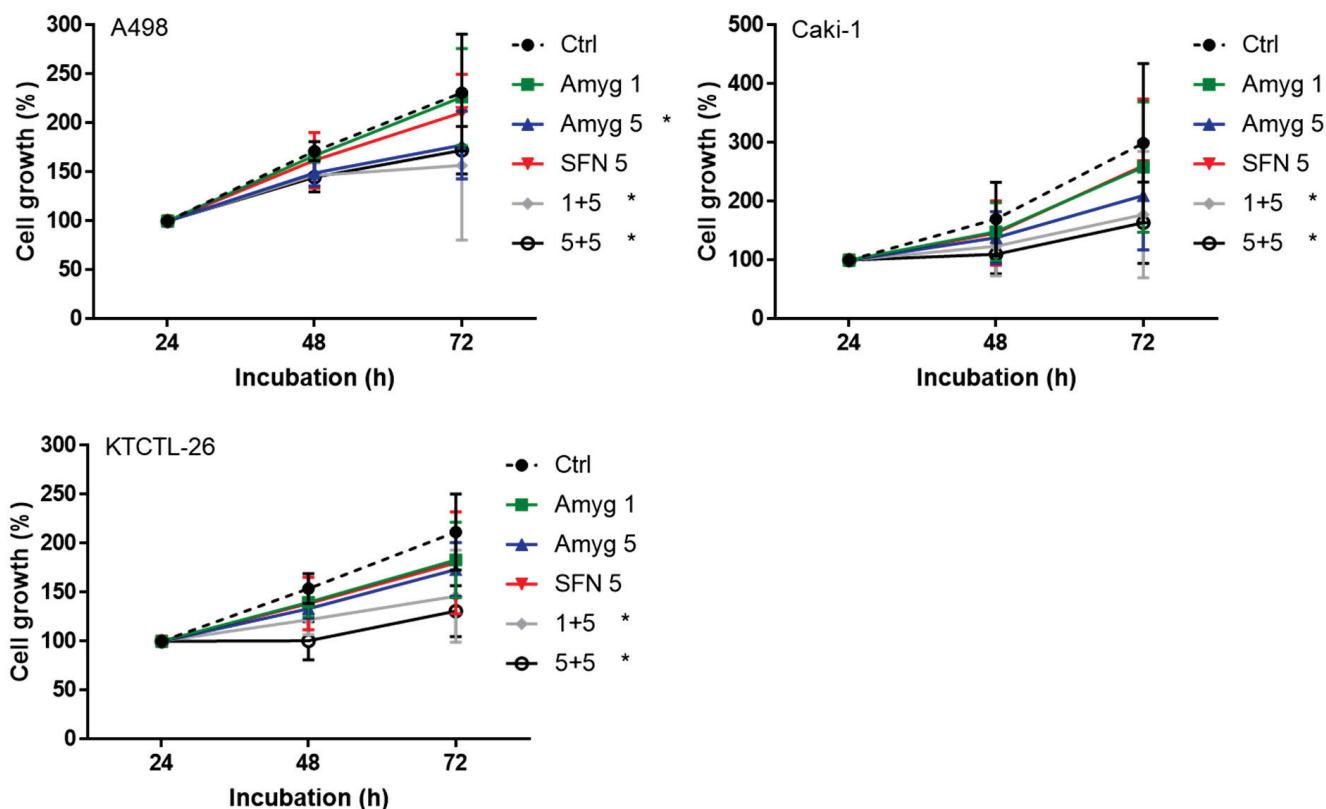
## 2.8. Statistics

All experiments were carried out three to six times. Statistical significance was calculated using the independent one-way or two-way ANOVA. Differences were considered statistically significant at a *p* value less than 0.05.

### 3. Results

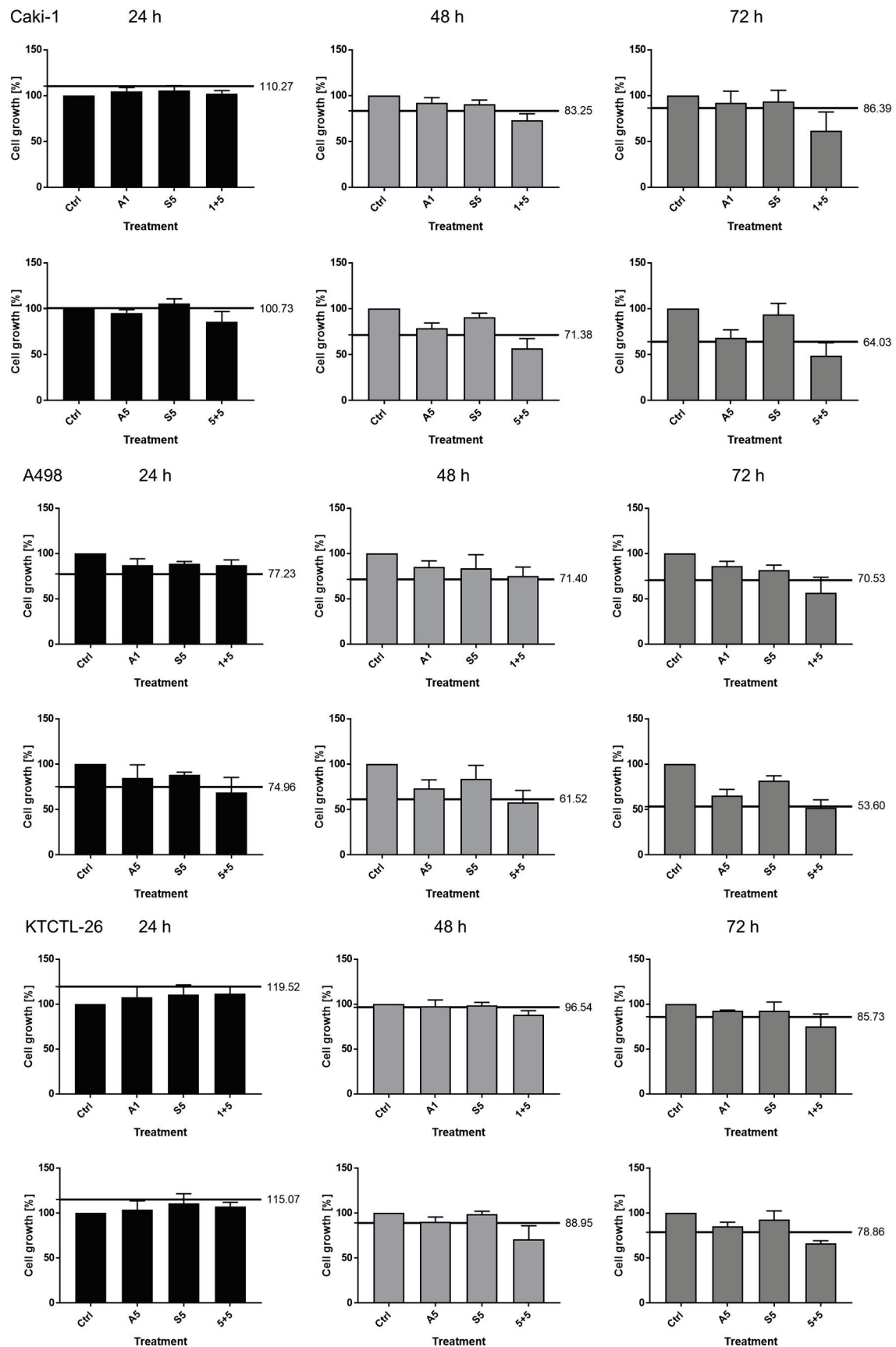
#### 3.1. Synergism of Amygdalin–SFN Combination on Tumor Growth

Amygdalin, 1 mg/mL, did not influence tumor growth in all three investigated cell lines, and 5 mg/mL amygdalin only suppressed growth in A498 cells but not in Caki-1 or KTCTL-26 cells (Figure 1). SFN, applied at 5  $\mu$ M was also only marginally effective. However, when combined with 1 or 5 mg amygdalin the RCC cell number was significantly diminished, compared to untreated controls (Figure 1). Apoptotic events were not induced by either single or dual drug treatment.



**Figure 1.** Influence of 5  $\mu$ M sulforaphane (SFN 5), 1 mg/mL or 5 mg/mL amygdalin (Amyg 1, Amyg 5) or the drug combination (5  $\mu$ M SFN + 1 mg/mL amygdalin, 1 + 5, 5  $\mu$ M SFN + 5 mg/mL amygdalin, 5 + 5) on cell growth in Caki1, A498, and KTCTL-26 cells. Cells were exposed to the drugs and evaluated after 24 (set to 100%), 48, and 72 h by the MTT assay. Controls (Ctrl) remained untreated. \* indicates significant difference to the controls (One-way ANOVA).  $n = 4$ .

Based on the Bliss independence model, synergistic inhibitory effects were already achieved after 24 h in all cell lines in the presence of SFN plus 5 mg/mL amygdalin (Figure 2). Effects on Caki-1 cells were strongest. Following 72 h treatment, synergism was seen even when SFN was combined with the lower amygdalin concentration of 1 mg/mL. Caki-1 and A-498 cells were more sensitive to the drug combination, compared to KTCTL-26 cells.



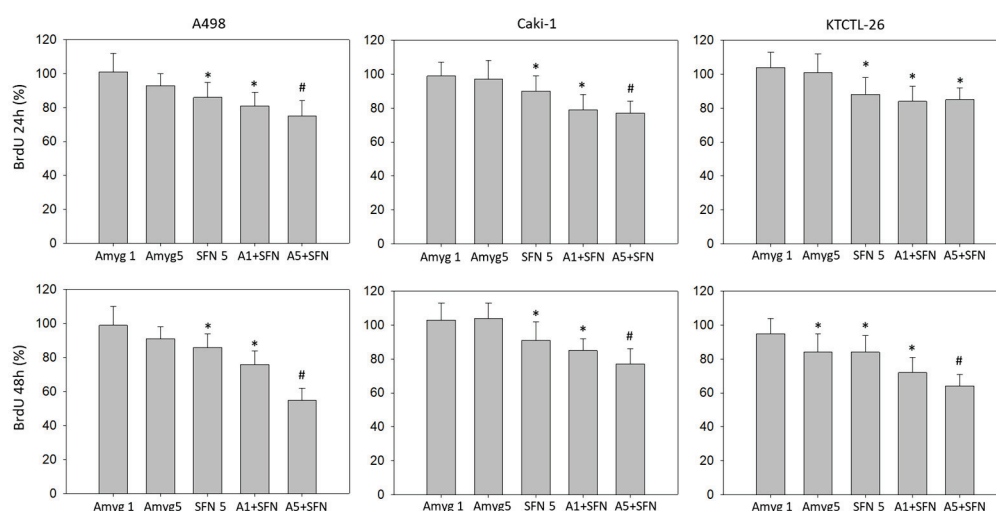
**Figure 2.** Calculation of synergism (Two-way ANOVA). A498, Caki1 and KTCTL-26 cells were treated with 1 or 5 mg/mL amygdalin (Amyg1, Amyg5) or SFN [5  $\mu$ M] or with the combination of 1 mg/mL



amygdalin + 5  $\mu$ M SFN (1 + 5) or 5 mg/mL amygdalin + 5  $\mu$ M SFN (5 + 5) for 24, 48, and 72 h. Growth characteristics were analyzed by the MTT assay and expressed as percentage inhibition, compared to the untreated control (100%). Error bars indicate standard deviation. The calculated effect from the Bliss independence model is displayed as a black line. Effect of the combination above the line = antagonistic effect, on the line = additive effect, below the line = synergistic effect.  $n = 4$ .

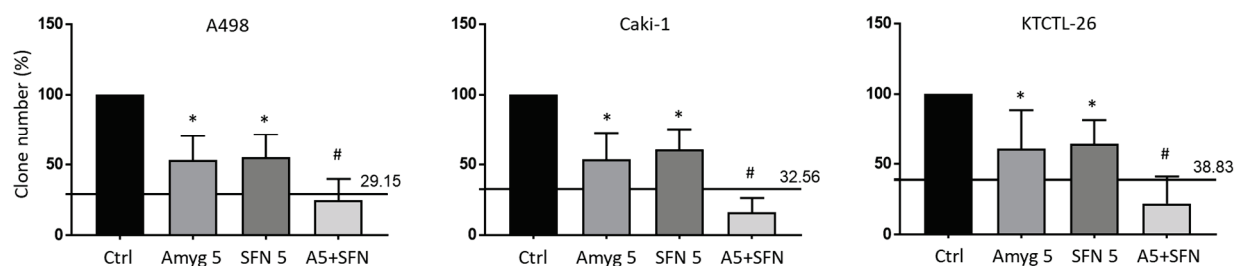
### 3.2. Suppression of Tumor Proliferation and Clone Development

Tumor proliferation, analyzed by BrdU uptake, was slightly suppressed by 5 mg/mL amygdalin after 48 h (KTCTL-26), whereas SFN diminished the proliferation of all cell lines after 24 h as well as after 48 h (Figure 3). Stronger anti-proliferation effects were induced when the SFN–amygdalin combination was applied. Although 5 mg/mL amygdalin did not alter the cells' proliferative activity (excepting KTCTL-26, 48 h), it significantly elevated the effects of SFN when the drugs were combined.



**Figure 3.** Evaluation of cell proliferation with the BrdU incorporation test. The RCC cell lines A498, Caki-1, and KTCTL-26 were exposed to 1 mg/mL or 5 mg/mL amygdalin (Amyg 1, Amyg 5), to 5  $\mu$ M sulforaphane (SFN 5) or to the amygdalin-SFN drug combination (A1 + SFN, A5 + SFN) for 24 or 48 h and then subjected to assay. Error bars indicate standard deviation. \* indicates significant down-regulation, compared to untreated controls set to 100%. # indicates significant down-regulation of the drug combination, compared to single drug use (Two-way ANOVA).  $n = 3$ .

Clonogenic growth is shown in Figure 4. Control values were set to 100%, corresponding to 27.0  $\pm$  9.2 (A498), 69.0  $\pm$  13.7 (Caki-1) or 16.7  $\pm$  7.6 (KTCTL-26) clones per well. Amygdalin (5 mg/mL) or SFN significantly reduced the number of tumor clones, independent of the cell line. Combined application was associated with a synergistic effect as calculated by the Bliss independence model.

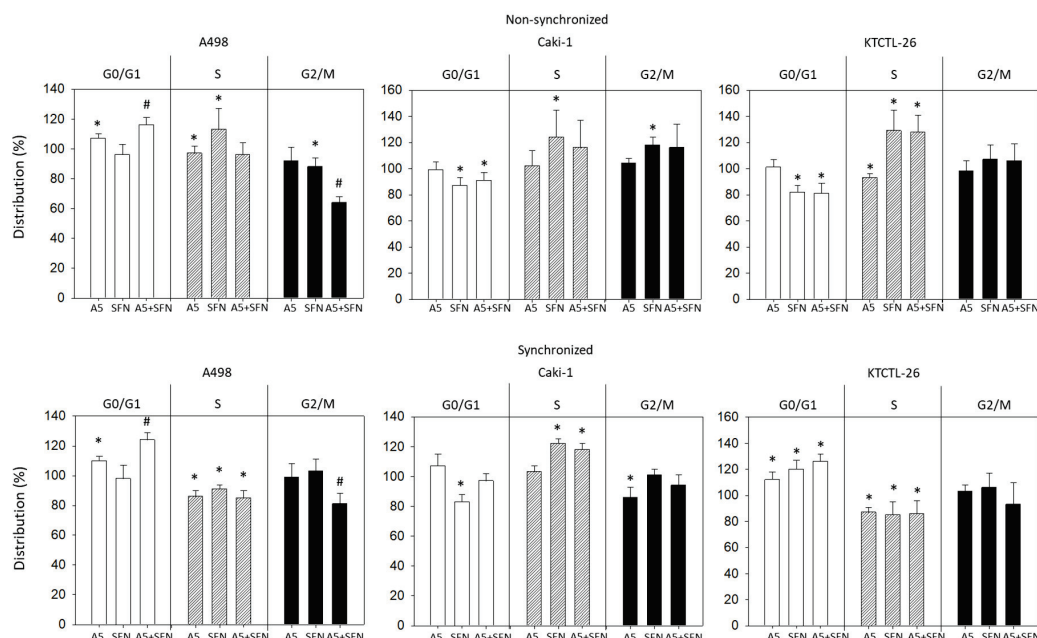


**Figure 4.** Clonogenic growth assay. Number of A498, Caki-1, and KTCTL-26 cell clones exposed to 5 mg/mL amygdalin (Amyg 5), 5  $\mu$ M sulforaphane (SFN 5), or both (A5 + SFN). Controls (Ctrl) were

incubated in cell culture medium alone. Error bars indicate standard deviation. \* indicates significant difference to untreated controls, set to 100%. # indicates significant difference of the drug combination, compared to single drug use (Two-way ANOVA). The calculated effect from the Bliss independence model is displayed as a black line. Effect of the combination above the line = antagonistic effect, on the line = additive effect, below the line = synergistic effect.  $n = 3$ .

### 3.3. Influence of Amygdalin and SFN on Cell Cycling

Cell cycle progression was followed on synchronized and non-synchronized cell populations. In the non-synchronized cell model, progression was not congruent. SFN enhanced the number of S-phase cells in all cell lines. The number of G0/G1 Caki-1 and KTCTL-26 cells was reduced, whereas the number of G0/G1 A498 cells remained unchanged. In addition, Caki-1 cells undergoing G2/M were enhanced by SFN, the percentage of A498 in G2/M, however, was lowered (Figure 5, up). Amygdalin (5 mg/mL) slightly elevated G0/G1 phase A498 cells and lowered the number of A498 and KTCTL-26 cells in the S-phase. Simultaneous drug use also altered cell cycle progression. However, superior effects, compared to the monodrug treatment, were only reflected in a G0/G1 increase and loss of G2/M in A498 cells.

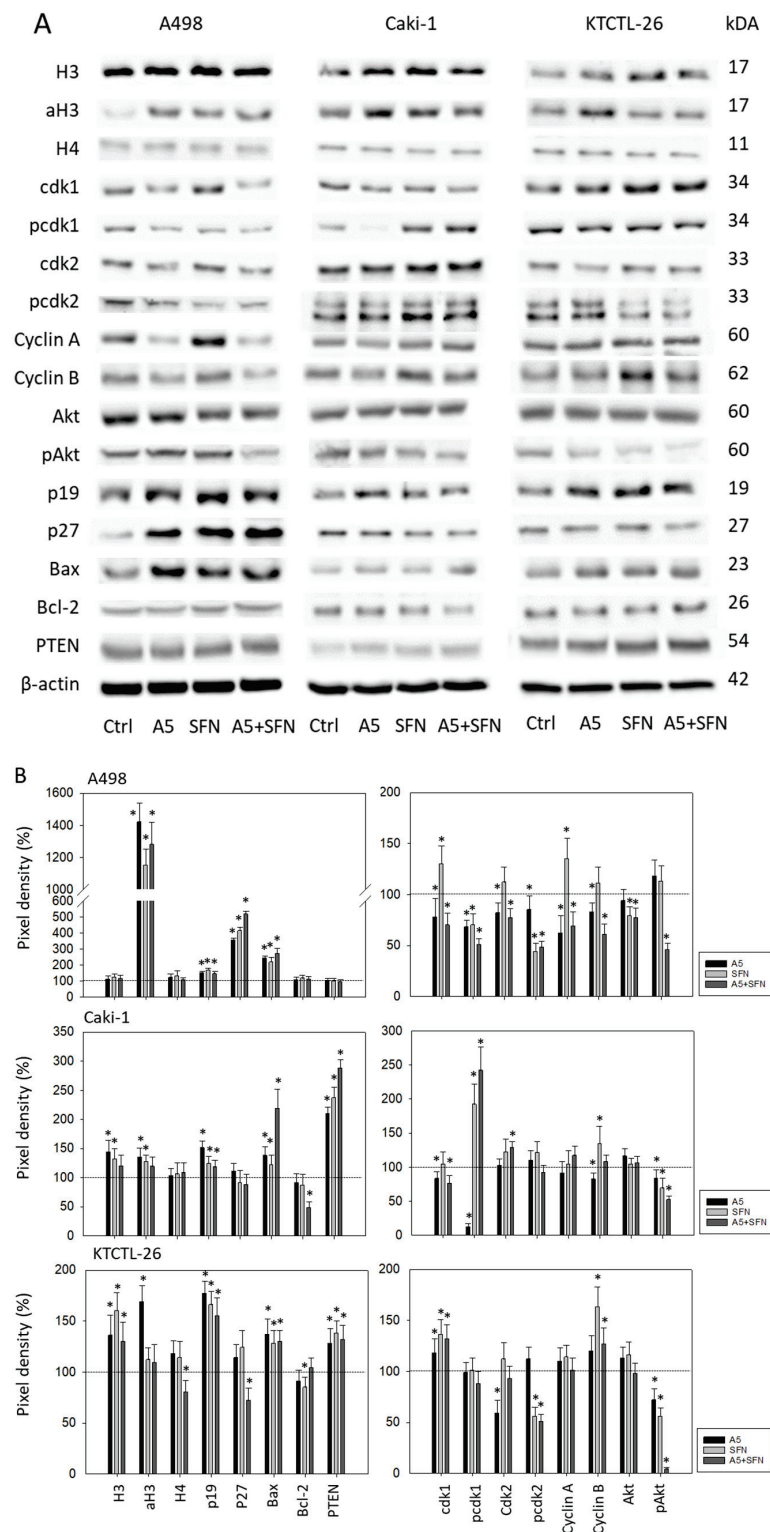


**Figure 5.** Influence of 5 mg/mL amygdalin (A5), 5  $\mu$ M sulforaphane (SFN), or both (A5 + SFN) on proportionate G0/G1, S, and G2/M-phases in A498, Caki-1, and KTCTL-26 cell lines. The upper part of the figure shows data from non-synchronized cells, the lower part from synchronized cells. Mean of three experiments. \* indicates significant difference to untreated controls (set to 100%). # indicates significant difference of the drug combination, compared to single drug use (Two-way ANOVA).

In the synchronized cell model, superior effects of the dual drug treatment were apparent in the A498 cells (Figure 5, lower). Differences to the non-synchronized cell model, however, were apparent. SFN reduced the number of S-phase A498 and KTCTL-26 cells but upregulated KTCTL-26 cells in G0/G1. Elevation of G0/G1 phase KTCTL-26 and loss of G2/M phase Caki-1 cells was observed following amygdalin exposure. The amygdalin–SFN combination diminished S-phase A498 and KTCTL-26 cells similarly to amygdalin or SFN alone. In contrast, S-phase Caki-1 cells were significantly elevated under dual treatment.

Cell cycle and apoptosis-regulating proteins were analyzed in non-synchronized cell populations. According to cell cycle progression, SFN and amygdalin did not alter protein expression in all cell lines homogeneously (Figure 6, Supplement S2, Western blots).

Amygdalin reduced cdk1 (A498, Caki-1), pcdk1 (all cell lines), cdk2 (A498, KTCTL-26), pcdk2 (A498), Cyclin A (all cell lines), Cyclin B (A498, Caki-1), pAkt (KTCTL-26), and Bcl-2 (Caki-1). However, it elevated cdk1 (moderately) in KTCTL-26, p19 (all cell lines), and Bax (all cell lines). The tumor suppressor p27 was only upregulated in A498 cells.



**Figure 6.** (A): Western blot of cell cycle and apoptosis-related proteins from A498, Caki-1, and KTCTL-26 cell lysates. Tumor cells were pretreated with 5 mg/mL amygdalin (A5), 5  $\mu$ M sulforaphane (SFN), or both compounds (A5 + SFN) for 24 h. Controls (Ctrl) remained untreated.  $\beta$ -actin served as the

internal control. One representative from three separate experiments is shown. (B): Pixel density. Values are depicted as a percentage, related to the untreated control cells (set to 100% and indicated by a horizontal line). \* indicates significant difference to controls.

SFN diminished only a few proteins: pcdk2 (A498), pAkt (Caki-1, KTCTL-26), and Bcl-2 (KTCTL-26). It, however, enhanced the following proteins: cdk1 (A498, KTCTL-26), pcdk1 (all cell lines), pcdk2 (KTCTL-26), Cyclin A (A498), Cyclin B (KTCTL-26), p19 (all cell lines), p27 (A498), Bax (all cell lines).

Amygdalin or SFN increased the histone, H3, in Caki-1 and KTCTL-26 cells. Acetylated histone, aH3, was increased in A498 (both drugs), Caki-1 (amygdalin only), and KTCTL-26 (amygdalin only). No distinct effects of amygdalin or SFN were exerted on H4, and aH4 was not detectable.

The combined use of both drugs diminished pAkt in all cell lines, much more strongly than each drug alone. An additive effect of the combined drugs was also seen with respect to p27 elevation (A498), Bax elevation and Bcl-2 reduction (both Caki-1) (see also Supplement S2).

#### 4. Discussion

The MTT assay reflected no growth inhibitory effect on RCC cells when 5  $\mu$ M SFN was applied alone. This accords with data from Gokay et al. reporting an  $IC_{50}$  of 15–20  $\mu$ M SFN being necessary to significantly block RCC cell growth [33]. Still, the MTT data should not be generalized to the effect that 5  $\mu$ M SFN exerts no action on RCC cells. BrdU incorporation as well as the clonogenic growth assay documented a significant impact of this dosage on the tumor cells' proliferative capacity. It is, therefore, conceivable that 5  $\mu$ M SFN does induce growth suppression. This has been demonstrated by others in a panel of RCC cell lines [30,34].

RCC cell growth was not altered by either 1 or 5 mg/mL amygdalin applied separately, as evidenced by the MTT test. This concurs with an earlier investigation where amygdalin concentrations of 10 mg/mL were required to significantly suppress RCC cell growth in vitro [35]. The efficacy of amygdalin may not only depend on the dosage but on the tumor entity. The  $IC_{50}$  value of amygdalin for breast cancer cells has been estimated at approximately 10 mg/mL, 25% growth reduction with 5 mg/mL [36]. Prostate and bladder cancer cell lines were already sensitive to 2.5 mg/mL amygdalin [10,28], whereas a minimum dosage of 30 mg/mL amygdalin was necessary to block pancreatic cancer cell growth [37].

Interpretation of the current data should, therefore, be restricted to the RCC cell model. Here, the SFN-amygdalin drug combination induced a substantial decrease in tumor growth, although monotherapies were without effect. Synergism was apparent even when the lowest amygdalin concentration of 1 mg/mL was used. Clonogenic growth with 5 mg/mL amygdalin was also more effectively inhibited with dual drug application, compared to single drug application. It is of particular interest that the strongest effect of the amygdalin–SFN combination was seen after 5–10 days, indicating that the therapeutic potential of this approach might be maintained for an extended time period.

Low dosing over a longer time period is important since excessive and/or high-dosed consumption of amygdalin can lead to severe cyanide intoxication. In fact, a maximum of 180 mg of HCN can be released from 500 mg of completely hydrolyzed amygdalin [38] and possibly be lethal for humans. A toxic or lethal HCN-concentration following amygdalin consumption has not been established. One patient developed severe metabolic acidosis after ingesting amygdalin with a serum HCN level of 385  $\mu$ g/dL [39]. However, it has been reported that a patient who had taken twice his usual oral amygdalin dose, and had a blood level of 600  $\mu$ g/dL cyanide showed no signs of toxicity [40]. Although a critical HCN-serum level following amygdalin metabolism has not been determined, and although our in vitro data cannot be transferred to an in vivo situation, combining amygdalin with SFN allows a considerable reduction in amygdalin dosage, thus limiting the risk of toxicity. Dichotomic effects of amygdalin have recently been reported in a rabbit

model: administration of amygdalin at high concentrations was associated with negative effects, whereas low-dosed amygdalin was beneficial [41].

This *in vitro* study shows that combining SFN with amygdalin exerts a synergistic suppression of tumor cell growth and permits a distinct reduction in amygdalin dosage. How far the amygdalin-SFN combination represents a complementary option to support conventional RCC cancer therapy requires further investigation. Amygdalin has been shown to potentiate the anti-cancer effect of sorafenib on Ehrlich ascites carcinoma *in vivo* [42] and to act synergistically with cisplatin in a breast cancer cell model [43]. Another investigation has revealed a synergistic effect of an amygdalin-vinblastine mixture on the proliferation of cervical cancer cell lines. Very strong synergism has been observed even at amygdalin concentrations of 1 and 10  $\mu\text{g/mL}$  [44]. Amygdalin has caused potent synergistic effects on osteosarcoma proliferation when combined with chemotherapeutics. Potent synergism between amygdalin and triple drug combinations has also been reported [45].

Sparse data regarding SFN's influence on RCC cells is available. In our study, 5  $\mu\text{M}$  SFN did not alter tumor cell growth, but did significantly diminish tumor cell clones. Tsaour et al. observed a decrease in RCC cell growth activity, already in the presence of 5  $\mu\text{M}$  SFN [30], whereas others have reported an  $\text{IC}_{50}$  for SFN between 6 and 20  $\mu\text{M}$ , depending on the cell line used and drug incubation time [33]. In another study, the treatment of RCC cells with 20  $\mu\text{M}$  SFN was necessary to induce a decrease in cell number [46]. These differences might be due to different RCC cell lines used in the respective studies. Varying SFN preparation techniques may also influence anti-tumor potential. A clinical trial recently investigated the impact of 500  $\mu\text{M}$  (90 mg) SFN given daily to patients with advanced pancreatic cancer. This schedule reduced tumor progression during the first six months after intake. However, some patients suffered from digestive problems, nausea, and emesis [47]. Hence, dual treatment with both SFN and amygdalin may allow dose reduction, limiting unwanted side effects.

How amygdalin and SFN interact is not clear. The Western blot data did not show homogenous additive or synergistic effects in regard to nearly all investigated cell cycle and apoptosis related proteins. Rather, cell signaling was differentially modulated, depending on the cell line and applied drug. It is, therefore, not surprising that cell cycle progression was also altered inconsistently in the presence of amygdalin or SFN alone. A superior effect of the drug combination, compared to mono-drug treatment, was only seen with A498 cells, demonstrating a significant increase of G0/G1 and decrease of G2/M-phase cells in the presence of amygdalin and SFN. In this particular case, cdk1 and 2 along with Cyclin A and B were distinctly blocked under dual drug exposure and might explain the diminished cell growth. Indeed, functional blocking of cdk1 and Cyclin B in A498 cells has been demonstrated to significantly diminish cell growth [34], and Cyclin A expression has been correlated to the G2/M phase in A498 cells [48]. Whether different characteristics of the RCC cell lines used here are responsible for the different cell cycle changes remains unresolved. Caki-1 cells express the wildtype von Hippel-Lindau (VHL) tumor-suppressor protein, and mutations of the *VHL* gene have been detected in KTCTL-26 cells, whereas A498 cells are completely VHL deficient. Therefore, VHL involvement may not explain why synergism was seen in all cell lines in the clonogenic growth assay. Another mode of action may be relevant here. It should be noted that the clones were counted after 5–10 days drug incubation, whereas cell cycling was evaluated 24 h following drug exposure. Therefore, clone data cannot strictly be associated with cell cycle progression.

Only a single protein, pAkt, was more strongly diminished in all cell lines under dual drug treatment, compared to single drug application. Hyperactivation of Akt has been demonstrated in several tumor entities, including RCC, making this protein a relevant target for cancer therapy [49]. RCC resistance development towards TKIs and ICIs involves Akt activation, as recently reported by Aweys et al. [50]. Hence, blocking Akt may optimize treatment and improve the prognosis of RCC patients. The present study shows that the herbal drugs amygdalin and SFN potently suppress Akt, making them valuable tools to fight cancer. In good accordance with this, synergistic action of SFN combined with



the natural compound formononetin on cervical cancer cell growth and apoptosis has recently been reported to be triggered by coupled deactivation of Akt [51]. The authors speculated that synergistic blockade of pAkt boosts apoptosis via cdk-cyclin suppression and G0/G1 cell cycle arrest. We did not investigate apoptosis; however, Bax has been shown to be elevated under SFN/amygdalin co-treatment in all cell lines, and Bcl-2 was distinctly reduced in Caki-1 following combined amygdalin-SFN exposure. Therefore, it cannot be excluded that synergistic deactivation of Akt may also induce apoptotic progression in a synergistic manner. In fact, SFN has already been documented to force apoptosis in pancreatic cancer cells via caspase-3 cleavage [52], and Habib et al. recently documented synergistic effects of the chemotherapeutic agent paclitaxel and SFN on prostate cancer cell apoptosis, triggered by altering Bax and Bcl-2 protein expression and caspase-3 cleavage [53]. Synergistic action on caspase 3 has also been observed when SFN is combined with gemcitabine [54], opening the question on the role of caspase-3 in our cell culture system and treatment protocol.

Respective information on amygdalin is sparse. However, simultaneous application of amygdalin and the anti-diabetic drug metformin induced stronger effects on caspase-3 and apoptosis in hepatocellular carcinoma cells, compared to single drug treatment [55]. This is important, since metformin inhibits Akt as well [56]. Finally, blocking Akt in esophageal squamous cell carcinoma cells by SFN and a further Akt-inhibitor (PP242) synergistically suppressed tumor proliferation and activated apoptotic pathways in vitro. Low-dosed SFN was only moderately effective, whereas the combined application of SFN with PP242 strongly suppressed the growth of tumor xenografts and induced cell apoptosis also in vivo [57].

The exact role of Akt, however, remains unclear. Amygdalin suppressed pAkt in Caki-1 and KTCTL-26 cells when given alone. Therefore, stronger Akt suppression seen in these cells after dual drug treatment might be an additive effect of the two drugs. However, this seems not to be the case with A498 cells, where amygdalin alone had no effect on pAkt expression. This is similar to another publication, where other combined drugs, such as SFN and the tyrosine kinase inhibitor lapatinib, strongly reduced pAkt in gastric cancer cells, although both drugs given separately did not [58]. The authors suggested that SFN may sensitize the tumor cells to lapatinib. Whether a similar scenario holds true for A498 cells is unclear. We assume that the dualistic action of amygdalin and SFN on A498 cells might be (at least partially) initiated via the cdk-Cyclin axis. Indeed, cdk1, cdk2 along with cyclin A and B were all potently diminished by the combination protocol in this cell line.

Further modes of amygdalin-SFN interaction should be considered to explain synergism. Molecular docking studies have shown that amygdalin may interact with broccoli myrosinase which catalyzes the conversion from glucorapharin to SFN [59]. This seems unlikely, however, since pure SFN and not its precursor, glucoraphanin, was used in our study. Jurkowska recently discovered that the isothiocyanate, 4-hydroxybenzyl isothiocyanate, down-regulates the level of mitochondrial rhodanese, forcing increased reactive oxygen species (ROS) production and inhibition of tumor cell growth [60]. This mechanism has not been proven for SFN, although SFN's impact on ROS has been documented [61]. Since amygdalin suppresses rhodanese as well [8], the synergism of SFN and amygdalin might be traced back to the blockade of rhodanese. It has been suggested that malignant cells have lower levels of the enzyme rhodanese or may even be deficient in rhodanese [8]. Rhodanese functions as an HCN detoxifier by catalysing the formation of thiocyanate from HCN and thiosulfate [8]. Given that rhodanese is lowered or lost in cancer cells, HCN increase could make the cancer cells more vulnerable to other anti-tumor drugs. Christodoulou et al. have documented that amygdalin and cisplatin co-treatment exerts cytotoxic effects on breast cancer but induces chemoprotective effects on normal breast cells [43]. The investigators hypothesized that different expression levels of rhodanese in healthy versus tumor cells might (partially) account for this. This theory may explain why amygdalin has been shown to exert synergism under differing conditions, including drugs with different modes of action.

## 5. Conclusions

Overall, evidence is provided that low-dosed amygdalin and SFN, when applied simultaneously, exert synergistic effects on RCC cell growth in vitro. Embedding both drugs into standard treatment protocols may, therefore, increase tumor response and delay tumor progression. Further attention should be paid to improving amygdalin's and SFN's bioavailability and specificity. Nanoencapsulation has been shown to be an innovative technique to enhance drug efficacy. In fact, inclusion complexes of amygdalin with  $\beta$ -cyclodextrin have already been shown to significantly enhance amygdalin's anti-cancer activity of [62]. Biocompatible and biodegradable alginate-chitosan nanoparticles have also been developed for cancer-specific amygdalin delivery with improved cytotoxic effects and simultaneous protection of healthy cells [63]. A novel investigation points to the synthesis of silk fibroin protein nanoparticles that may allow controlled and long-term SFN release [64]. The in vitro model presented here should now be transferred to an in vivo model to evaluate the clinical feasibility of applying low-dose amygdalin and SFN to treat RCC. It might also be worthwhile to explore the interaction of SFN/amygdalin with immune checkpoint inhibitors (ICI) which have been approved for cancer immunotherapy. In fact, few data published so far are discrepant. Application of SFN to glioblastoma ICI therapy may provide a significant therapeutic effect as stated out by Lee et al. [65]. However, others assume that SFN may impair the anticancer effect of ICI on gastric cancer cells [66]. In this context, SFN may act as a double-edged sword, reducing carcinogenesis but also blocking T cell-mediated immune response [67]. Therefore, ongoing experiments should deal with the relevance of the amygdalin-SFN combination embedded into an ICI treatment protocol in RCC.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu16213750/s1>, Supplement S1: Vendor/Catalog No and RRID codes of the antibodies used. Supplement S2: Western Blots.

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## Review

# Shikonin as a Dietary Phytochemical with Multi-Target Anti-Cancer Activities: From Molecular Mechanisms to Translational Applications

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**Abstract:** Shikonin, a dietary naphthoquinone phytochemical from the roots of *Lithospermum erythrorhizon*, has gained attention for its anticancer potential. Preclinical studies show that shikonin regulates multiple programmed cell death pathways, including apoptosis, necroptosis, ferroptosis, and pyroptosis, through mechanisms involving reactive oxygen species (ROS) accumulation, mitochondrial dysfunction, and kinase-mediated signalling. Beyond cytotoxicity, shikonin suppresses metastasis by blocking epithelial–mesenchymal transition (EMT) and downregulating matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). It also disrupts tumour metabolism by targeting pyruvate kinase isoform M2 (PKM2) and modulating the Warburg effect. Evidence further indicates that shikonin can enhance the efficacy of chemotherapy, targeted therapy, immunotherapy, and radiotherapy, thereby contributing to the reversal of therapeutic resistance. To address limitations related to solubility and bioavailability, novel formulations such as nanoparticles, liposomes, and derivatives like  $\beta,\beta$ -dimethylacrylshikonin have been developed, showing improved pharmacological profiles and reduced toxicity in experimental models. Overall, the current literature identifies shikonin as a promising dietary phytochemical with diverse anticancer activities, therapeutic synergy, and formulation advances, while highlighting the need for clinical studies to establish its translational potential.

**Keywords:** shikonin; dietary phytochemicals; anticancer mechanisms; drug resistance; combination therapy

## 1. Introduction

According to the global cancer statistics in 2022, an estimated 20 million new cancer cases and 9.7 million cancer deaths were reported worldwide [1]. Despite decades of advancement in oncology research, cancer remains a major global health challenge, particularly in advanced or metastatic stages. Conventional treatments, such as surgery, chemotherapy, radiotherapy and immunotherapy, have significantly improved survival outcomes and have reduced mortality rates in cancer patients [2]. However, drug resistance driven by genetic mutations, tumour heterogeneity, and tumour microenvironment

(TME) remains a critical barrier that hinders the effectiveness of conventional therapies and compromises long-term treatment outcomes [3]. Besides that, these therapies are often associated with systemic and off-target toxicities, characterised by fatigue, dermatological reactions, hepatotoxicity, cardiotoxicity and nephrotoxicity [4,5]. Therefore, natural compounds, especially dietary phytochemicals, have emerged as promising alternative therapeutic approaches due to their diverse biological activities, lower toxicity and accessibility from medicinal plants [6].

Phytochemicals are bioactive compounds derived from plants, including grains, fruits, vegetables, and herbs, recognised for their beneficial effects on human health. They can be categorised into several major classes, such as carotenoids, polyphenols, naphthoquinones and terpenoids, based on their chemical structures [7]. Since antiquity, plant-derived products have been incorporated into traditional medical systems, such as Traditional Chinese Medicine (TCM) and Ayurveda, serving both preventive and therapeutic purposes [8]. For instance, ginger (*Zingiber officinale*) exhibits thermogenic properties that stimulate blood circulation and has long been used to treat respiratory disorders such as coughs and the common cold [9]. In addition, *Lithospermum erythrorhizon* has been traditionally used to reduce body surface heat, relieve congestion and treat skin diseases, burns and injuries [10]. More recently, numerous studies have elucidated the molecular mechanisms through which phytochemicals regulate physiological processes and promote human health. Among these, bioactive compounds such as resveratrol, derived from grapes, and beta-carotene ( $\beta$ -carotene), found in carrots, exhibit antioxidant and anti-inflammatory effects in the treatment of acute pancreatitis [11,12].

With the growing interest in exploring dietary phytochemicals, shikonin has gained significant attention as a bioactive naphthoquinone pigment with both pharmacological and therapeutic potential, particularly in cancer treatment. It is extracted from the roots of *Lithospermum erythrorhizon* (purple gromwell, Zicao), an edible and medicinal plant long used in TCM and occasionally incorporated into functional foods [13,14]. The roots contain dietary fibre, essential fatty acids, and polyphenolic compounds with antioxidant capacity, supporting its nutritional relevance alongside pharmacological use. Its inclusion in medicinal teas, ointments, and decoctions underscores its dual role as both a therapeutic agent and a dietary phytochemical [15]. This traditional use supports its classification as a dietary phytochemical, reinforcing both its reputation for safety and its promise in long-term chemoprevention and integrative cancer care [15,16]. Recent studies show that shikonin suppresses tumour growth in lung, ovarian, and other cancers through diverse mechanisms, including apoptosis induction, cell cycle arrest, inhibition of metastasis, necroptosis, and disruption of tumour metabolism [17]. Its multi-targeted activity and relatively low systemic toxicity highlight shikonin as a compelling candidate for development as both a preventive agent and an adjunct to conventional cancer therapies [18].

In this review, we aim to provide an overview of the chemical and pharmacological profile of shikonin and its derivatives, along with their anticancer mechanisms in regulating tumour progression. This review also discusses the potential of shikonin and its derivatives in combination therapy and the translational studies relevant to future research and clinical applications in cancer treatment.

## 2. Chemistry and Pharmacokinetics

Shikonin is a naphthoquinone compound found abundantly in the roots of the Boraginaceae family, giving them a red-purple appearance [19]. The chemical structure of shikonin consists of a 1, 4-naphthoquinone core substituted with hydroxyl groups at positions 5 and 8 and a hydroxypentenyl side chain at position 2. Its molecular formula is  $C_{16}H_{16}O_5$ , with a molecular weight of 288.29 g/mol [10]. Shikonin has poor aqueous

solubility but is highly soluble in organic solvents such as ethanol and chloroform [14]. Besides that, shikonin is sensitive to light and heat, and it can be easily degraded when exposed to temperatures above 60 °C [20]. Shikonin also exhibits redox activity, which involves the transfer of two protons and two electrons at the naphthazarin moiety [21]. This property enables shikonin to function as a pro-oxidant agent by inducing oxidative stress in cancer cells to suppress tumour growth [22].

### 2.1. Methods of Isolation and Production of Shikonin

Shikonin is commonly isolated using maceration or Soxhlet extraction techniques with organic solvents like ethanol, methanol, hexane, ethyl acetate or chloroform [23,24]. Sagra-tini et al. reported that Soxhlet extraction using ethyl acetate for 6 h produced the highest yield of alkannin/shikonin mixture (80%) from the roots of *Onosma echiooides* [23]. Ethanol is often preferred due to low toxicity, ease of removal and high extraction yield. However, these methods have disadvantages such as long processing times, high energy consumption, risk of compound degradation and low extraction efficiency [25]. To overcome these drawbacks, alternative extraction techniques have been developed. Ultrasonic-assisted extraction (UAE) applies mechanical, thermal and cavitation effects to disrupt cell walls and enhance the release of bioactive compounds [26]. Huang et al. reported the maximum yield of shikonin (1.26%) was extracted using the UAE procedure under optimal conditions of 39 °C with 95% ethanol and 93 W ultrasound power for 87 min [20]. Furthermore, microwave-assisted extraction (MAE) is another effective extraction method, which involves the use of electromagnetic waves to heat the solvent and plant matrix within a closed system [27]. The optimal extraction conditions for shikonin using the MAE technique are 330 W of microwave power with 80% methanol for 12 min [28]. Supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction uses CO<sub>2</sub> above its critical temperature and pressure to dissolve target compounds from plant material [29]. Shen et al. optimised the conditions of SC-CO<sub>2</sub> extraction of shikonin at 3 MPa and 40 °C, with a CO<sub>2</sub> flow rate of 27 L/h for 2 h [30]. These advanced extraction technologies are widely used, particularly in pharmaceutical and food industries, for their high extraction yield and purity, eco-friendly nature and short operation time [31,32].

Following the isolation of shikonin from plants, purification is essential to remove impurities and residual organic solvents. Common separation techniques include high-performance liquid chromatography (HPLC), high-speed counter-current chromatography (HSCCC) and column chromatography [23,33]. Besides that, plant cell culture technique has been employed to produce large quantities of shikonin. For instance, callus culture of *Onosma bulbotrichum* in Murashige and Skoog (MS) medium supplemented with growth hormones yielded 2.6 times more shikonin than wild plant roots [34]. Another study conducted by Yazaki et al. demonstrated that the hairy root culture of *Lithospermum erythrorhizon* infected with *Agrobacterium rhizogenes* produced a similar amount of shikonin as the cell suspension culture [35]. In addition, multiple studies have explored chemical synthesis pathways to generate shikonin and its derivatives [36,37]. Wang et al. developed a total synthesis pathway, which involves the asymmetrical hydrogenation of the Ru (II) catalyst followed by the removal of methyl groups, and results in a high yield (47%) of shikonin production [38].

### 2.2. Shikonin Derivatives and Their Anticancer Activities

Apart from natural extraction, shikonin derivatives are obtained via semi-synthetic routes by chemically modifying the side chain attached to the naphthazarin structure. For instance, Zhou et al. demonstrated the methylation of the phenolic hydroxyl group of shikonin produced its derivative, 5, 8-O-dimethylacrylshikonin. This study reported that

5, 8-*O*-dimethyl acylshikonin inhibited the tumour growth in Kunming (KM) mice with subcutaneous injection of Sarcoma S-180 cells [39]. Another research study conducted by Huang et al. found that shikonin oxime derivatives incorporating a sulphur atom exhibited cytotoxic effects against breast cancer, leukaemia and prostate cancer cells [40]. Other derivatives, including  $\alpha$ -methylbutyrylshikonin and  $\beta$ ,  $\beta$ -dimethylacrylshikonin, exhibited potent cytotoxicity against melanoma, leukaemia, colorectal cancer, gastric cancer, and medullary thyroid carcinoma cells [41–45]. In addition, acetylshikonin produced via acetylation of shikonin showed antitumour effects on oral squamous cell carcinoma (OSCC), non-small cell lung cancer (NSCLC) and colorectal cancer [46–48]. Other examples of shikonin derivatives, such as  $\beta$ -hydroxyisovalerylshikonin, deoxyshikonin and isobutyrylshikonin, demonstrated various anticancer activities on pancreatic cancer, cervical cancer and oral cancer [49–52]. Table 1 summarises the chemical structures, modifications and the anticancer effects of various shikonin derivatives. These findings highlight that the structural modifications of shikonin can significantly improve its pharmacological effects, facilitating the development of novel shikonin-based anticancer drugs.

**Table 1.** Shikonin derivatives and associated cancer targets.

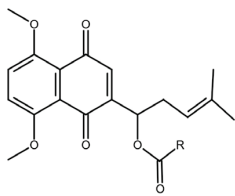
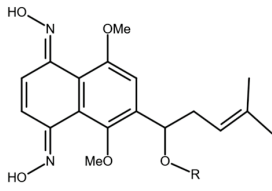
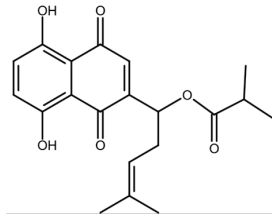
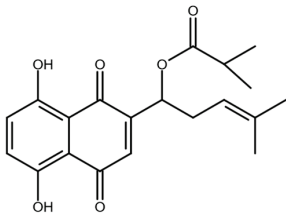
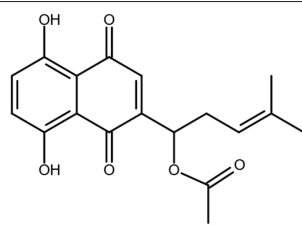
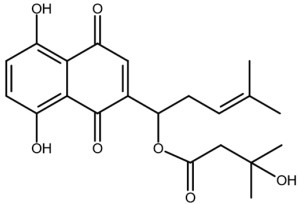
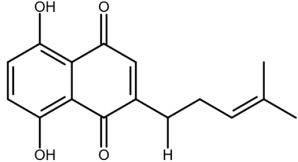
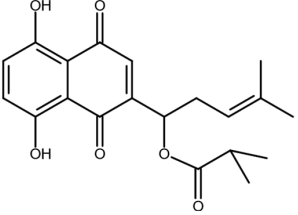
| Shikonin Derivatives                     | Chemical Structure                                                                  | Structural Modifications                                            | Cancer Types                                                                                   | Ref.    |
|------------------------------------------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------|------------------------------------------------------------------------------------------------|---------|
| 5, 8- <i>O</i> -dimethyl acylshikonin    |   | Methylation of phenolic hydroxyl group                              | Colon cancer<br>Leukaemia<br>Breast cancer                                                     | [39]    |
| Shikonin oxime derivative                |  | Conversion of carbonyl to oxime group                               | Breast cancer<br>Leukaemia<br>Prostate cancer                                                  | [40]    |
| $\alpha$ -methylbutyrylshikonin          |  | Esterification of hydroxyl group with $\alpha$ -methylbutyryl group | Melanoma<br>Leukaemia                                                                          | [41,42] |
| $\beta$ , $\beta$ -dimethylacrylshikonin |  | Side chain esterified with dimethylacrylic group                    | Colorectal cancer<br>Gastric cancer<br>Medullary thyroid cancer                                | [43–45] |
| Acetylshikonin                           |  | Acetylation of hydroxyl group                                       | Oral squamous cell carcinoma (OSCC)<br>Non-small cell lung cancer (NSCLC)<br>Colorectal cancer | [46–49] |

Table 1. Cont.

| Shikonin Derivatives               | Chemical Structure                                                                | Structural Modifications                             | Cancer Types                         | Ref.    |
|------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------|--------------------------------------|---------|
| $\beta$ -hydroxyisovalerylshikonin |  | Esterification with $\beta$ -hydroxyisovaleryl group | Pancreatic cancer<br>Cervical cancer | [49,50] |
| Deoxyshikonin                      |  | Removal of hydroxyl group                            | Cervical cancer                      | [51]    |
| Isobutyrylshikonin                 |  | Esterification with isobutyryl group                 | Oral cancer                          | [52]    |

### 2.3. Pharmacokinetics of Shikonin and Its Pharmaceutical Formulations

The pharmacokinetics of shikonin has been primarily explored using in vivo animal models, such as rats, mice and dogs. Shikonin exhibits relatively low bioavailability due to its poor aqueous solubility and high metabolism rate. For example, a study on the oral administration of Zi-Cao-Cheng-Qi decoction (25 g/kg) in rats reported a blood concentration of shikonin at 0.5 h post-administration of 0.48  $\mu\text{g/mL}$ , with a total plasma protein binding rate of 64.6% [53]. However, another study reported that shikonin was undetectable in the plasma of Sprague Dawley (SD) rats 10 h after an intravenous (IV) injection of shikonin at a dose of 1.5 mg/kg [54]. This evidence suggests that shikonin undergoes a substantial first-pass effect (FPE) and rapid clearance in rats. After that, shikonin undergoes two phases of metabolism, involving the hydroxylation of the naphthoquinone core and glucuronic acid conjugation [55]. Huang et al. found that shikonin upregulated the expression of drug-metabolising enzymes and transporters in primary rat hepatocytes, thereby modulating the detoxification and elimination pathways [56]. Finally, shikonin is excreted via both bile and urine [57].

Shikonin has been incorporated into various pharmaceutical formulations to improve its stability, bioavailability and therapeutic potential. A liposome is a spherical lipid-based drug vehicle that exhibits excellent permeability and retention effects, which allow the drug to accumulate in target tissues and promote drug efficacy [58]. For instance, shikonin-loaded liposomes modified with RGD peptide promoted intracellular uptake and enhanced cytotoxicity in cancer cells due to the specific binding between RGD and  $\alpha_v\beta_3$  receptors on the tumour cell surface [59]. Moreover, Guo et al. found that Zinc-Shikonin-Polyethylene Glycol (Zn-SHK-PEG) nanoparticles showed long retention time in the lung, kidney, liver and spleen without systemic toxicity. Zn-SHK-PEG showed significant inhibition of intracellular ROS generation and strong anti-inflammatory effects in a sepsis model [60]. Besides that, nanogel is a type of hydrogel widely used in drug delivery due to its strong water absorption, high drug-loading capacity and long circulation half-life [61]. Sarcoma-targeting-peptide (STP)-modified nanogel increased the delivery of shikonin in osteosarcoma cells and inhibited tumour progression and metastasis through



necroptosis. Treatment with shikonin-loaded STP nanogel exhibited high antitumor activity with low systemic toxicity compared to free shikonin [62].

### 3. Mechanisms of Anticancer Action

#### 3.1. Induction of Apoptosis by Shikonin

##### 3.1.1. Shikonin-Induced Intrinsic Apoptosis via MAPK Signalling and ROS-Mediated ER Stress

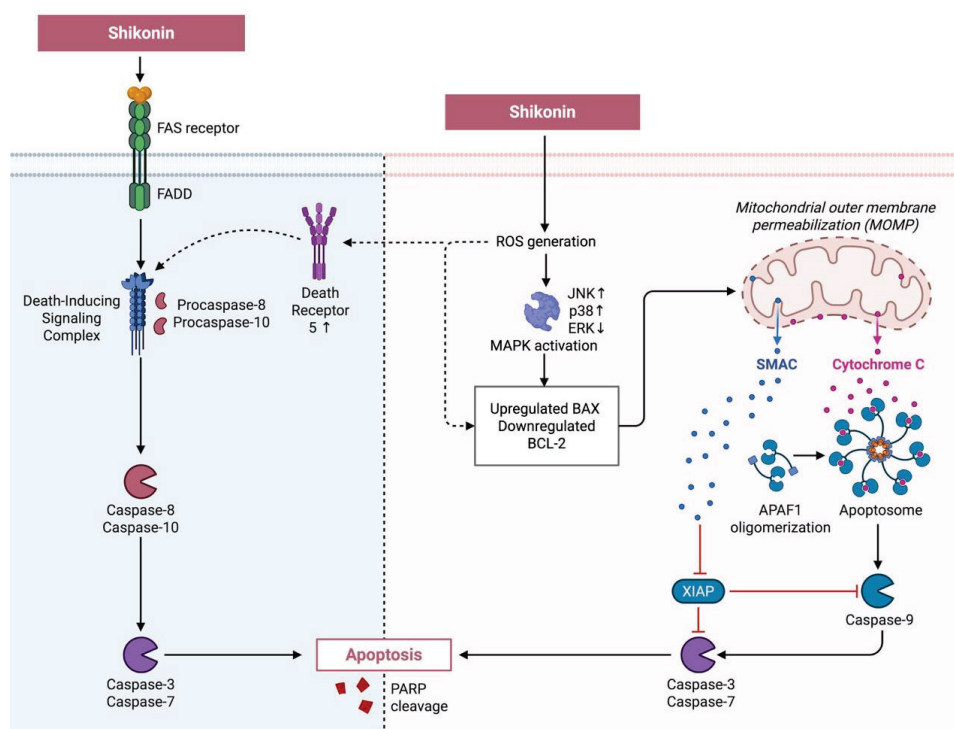
The intrinsic apoptotic pathway is primarily regulated by mitochondrial events and is characterised by the activation of caspases, the key executioners of apoptosis. Caspases such as caspase-3, -7, and -9 degrade cytoskeletal proteins, activate deoxyribonucleases (DNases), and cleave regulatory proteins such as poly (ADP-ribose) polymerase (PARP), thereby preventing DNA repair and committing cells to programmed cell death (PCD) [63–65]. Numerous studies have demonstrated that shikonin triggers intrinsic apoptosis through activation of the mitogen-activated protein kinase (MAPK) cascade, leading to downstream caspase-dependent apoptosis [66–71].

Shikonin-induced activation of MAPK pathways has been observed in multiple cancer types, including colon carcinoma, chondrosarcoma, blood cancers, melanoma, and renal cancer [66–69,71–75]. In colon carcinoma, shikonin treatment activated c-Jun N-terminal kinase (JNK) and promoted galectin-1 dimer formation to induce apoptosis in SW-620 cells, whereas radiation-resistant SNU-C5RR cells exhibited apoptosis associated with phosphorylation of JNK, p38, and extracellular signal-regulated kinase (ERK) [67,73]. In colorectal cancer (CRC) HCT-116 and HCT-15 cells, 1.5  $\mu$ M concentration of shikonin effectively activated the PERK/CHOP and IRE1 $\alpha$ /JNK apoptotic pathways, indicating its involvement in oestrogen stress responses [76].

In bone cancer research, acetylshikonin and cyclopropylshikonin dose-dependently (0.5, 2.5, 5, or 10  $\mu$ M) increased phosphorylation of ERK, JNK, and p38 in Ca-78 and SW-1353 chondrosarcoma cells, while shikonin downregulated ERK activity to induce apoptosis in U2OS osteosarcoma cells [68,75]. Similarly, in blood cancers, shikonin enhanced JNK activation and triggered intrinsic apoptosis in primary effusion lymphoma via ROS generation and significantly elevated p38 and JNK phosphorylation in NB4 cells [72,77]. In melanoma A375M cells, apoptosis was associated with MAPK activation through a 2- to 7-fold increase in JNK and p38 phosphorylation in a dose-dependent manner (0, 2, or 4  $\mu$ M) [71]. In renal cancer, shikonin was also found to induce apoptosis through downregulating p44/42 MAPK protein in Caki-2 and A-498 cells, but shikonin induces apoptosis by enhancing p38 phosphorylation in Caki-1 and ACHN cells [69,74]. Other cancer models, including triple-negative breast cancer (TNBC) MDA-MB-231 cells, lung adenocarcinoma A546 cells, and pancreatic cancer PANC-1 cells, also demonstrated ERK inhibition and apoptosis induction following shikonin treatment [75]. In summary, shikonin-induced activation of the MAPK-mediated intrinsic apoptotic pathway exhibits pronounced potency in CRC cell lines.

Furthermore, shikonin has been found to induce ROS generation and activate apoptotic proteins, resulting in activation of caspase-dependent apoptosis in blood cancer, epidermoid carcinoma, brain cancer, and oral cancer [70,78]. In adult T-cell leukaemia/lymphoma ED- and TL-Om1 cells, shikonin rapidly upregulated ER stress proteins, including activating transcription factor 4 (ATF4), X-box binding protein 1 (XBP-1), and p38 MAPK, leading to apoptosis [70]. In glioma Hs683 cells, shikonin induced apoptosis via increased caspase-3, PERK, and CHOP expression, indicating ER stress-mediated cell death [78]. Similarly, shikonin increased the late apoptosis percentage from 0.82% to 7.23% in oral cancer EC9706 cells by increasing Bax, cleaved caspase-3, and cleaved PARP levels, confirming caspase-dependent apoptosis [79]. Collectively, these findings demonstrate that shikonin

effectively triggers intrinsic apoptotic pathways through MAPK activation, ROS generation, and ER stress signalling (Figure 1).



**Figure 1.** Shikonin induce intrinsic and extrinsic apoptotic pathways in various types of cancer. Upregulated genes (↑) and downregulated genes (↓) are indicated.

### 3.1.2. Extrinsic Apoptotic Pathways Triggered by Shikonin: Death Receptor and Caspase-8 Activation

In contrast to the intrinsic apoptotic pathway, the extrinsic apoptotic pathway induces p53-independent apoptosis through the activation of Apo2 ligand/tumour necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL) and Apomab in cancer therapy. These ligands bind to death receptors (DR), such as DR4 and DR5, triggering the death-inducing signalling complex (DISC) by recruiting the adapter Fas-associated death domain (FADD) and procaspases -8 and -10, ultimately leading to caspase-dependent apoptosis [80,81]. Shikonin has been shown to engage this extrinsic apoptotic machinery in multiple cancer models. In GTO primary effusion lymphoma cells, shikonin induced caspase-8-mediated apoptosis, accompanied by increased expression of caspases-3, -8, and -9 [72]. In QBC939 oral cancer cholangiocarcinoma cells, shikonin treatment enhanced cleaved caspase-3 and -8 expression [82]. Similarly, in another cholangiocarcinoma cell line, shikonin also upregulated DR5 and caspases-3, -8, and -9 through ROS-mediated activation of the JNK signalling cascade [22]. In ovarian cancer cell lines, including KURAMOCHI, OVSAHO, CP70, and ascites-derived E04 cells, shikonin activated Fas ligand (FasL)/caspase-8 signalling, as evidenced by increased FasL and cleaved caspase-8, -3, and -7 levels to induce extrinsic apoptosis [83]. These findings suggest that shikonin is capable of triggering extrinsic apoptotic pathways, particularly in ovarian and cholangiocarcinoma models, through death receptor activation and caspase-8 signalling (Figure 1).

### 3.1.3. Induction of Apoptosis Through Unique Signalling Pathways

Shikonin has been reported to target specific signalling pathways to induce caspase-dependent apoptosis in a variety of cancer cell lines. In liver cancer, shikonin dose-dependently targets pyrroline-5-carboxylate reductase 1 (PYCR1), leading to suppression

of the PI3K/Akt/mTOR signalling pathway in hepatocellular carcinoma (HCC) SNU-449 and Hep-3B cells. Western blot analyses revealed reduced phosphorylation of PI3K, Akt, and mTOR, accompanied by increased levels of cleaved caspase-3, -9, and PARP, indicating that shikonin activates the caspase cascade through inhibition of PI3K/Akt/mTOR signalling [84]. Similarly, in SMMC-7721 HCC cells, shikonin treatment resulted in up-regulation of p53 expression and downregulation of phosphorylated Akt and PI3K [85]. Another study reported that shikonin significantly reduced the protein expression of pyruvate kinase M2 (PKM2), HIF-1 $\alpha$ , and PHD3, as well as the nuclear translocation of PKM2 and HIF-1 $\alpha$  in SMMC-7721 cells [86]. In oral cancer, shikonin inhibited the Akt/mTOR signalling pathway, inducing apoptosis in TE-1 oesophageal cancer cells [87]. In lung cancer, shikonin triggered apoptosis in NSCLC A549 and PC-9 cell lines via the p53/miR-628-3p signalling axis, while in H1299 cells, apoptosis was mediated by downregulation of the survivin signalling pathway [88,89]. In ovarian cancer, shikonin induced apoptosis in SKOV3 and A2780 cell lines by inhibiting the oestrogen signalling pathway and down-regulating the expression of its downstream gene, G protein-coupled oestrogen receptor (GPER) [90]. In skin cancer, shikonin inhibited STAT3 signalling, leading to apoptosis in A375 and A2058 melanoma cells [91]. In colon cancer, shikonin induced apoptosis by inhibiting the IL-6/STAT3 signalling pathway in HCT116 and SW480 cells, supported by the downregulation of JAK1 and JAK2 proteins after treatment [92]. Overall, these studies demonstrate that shikonin induces apoptosis across multiple cancer types by targeting unique oncogenic signalling pathways, including PI3K/Akt/mTOR, STAT3, oestrogen, and survivin pathways, ultimately converging on caspase-dependent cell death.

#### 3.1.4. Shikonin and Its Derivatives Induce Apoptosis in Drug-Resistant Cancer Cells

Shikonin also demonstrated incredible antitumor effects in various drug-resistant cancer cell lines. In the epidermal growth factor receptor (EGFR)-T790M-mutant drug-resistant NSCLC cell line H1975, shikonin treatment induced caspase-dependent apoptosis [93]. Similarly, in EGFR-mutated NSCLC H1299 cells, the shikonin derivative E5 inhibited the nuclear translocation of PKM2, thereby suppressing transcriptional activation of oncogenes and triggering apoptosis [94]. In colon cancer, shikonin induced apoptosis in oxaliplatin (OXA)-resistant HCT116 colorectal cancer cells through a ROS-mediated endoplasmic reticulum (ER) stress pathway [95].

Additionally, in 5-fluorouracil (5-FU)-resistant colorectal cancer SNU-C5/5-FUR cells, shikonin regulated glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) expression, increased ROS generation, elevated mitochondrial calcium (Ca<sup>2+</sup>) levels about 1.5-fold, and ultimately induced apoptosis [96]. Moreover, the shikonin ester derivative 4-aminophenoacetic acid induced apoptosis in Kirsten rat sarcoma virus (KRAS)-mutant HCT116 colon cancer cells by deactivating the Akt signalling pathway [97]. Cumulatively, these findings highlight that shikonin and its derivatives exert significant antitumor activity against drug-resistant cancers, including NSCLC and colorectal cancer, primarily through apoptosis induction mediated by diverse molecular mechanisms.

#### 3.1.5. Apoptosis Effects of Shikonin Derivatives

Shikonin derivatives have also demonstrated potent antitumor effects by activating both intrinsic and extrinsic apoptotic pathways in various cancer cell lines (Table 2) [68,98–100]. In blood cancer, acylshikonin induced apoptosis in leukaemia K562 cells via the intrinsic pathway, as evidenced by increased levels of cleaved caspase-3, -9 and cleaved PARP [101]. In ovarian cancer,  $\beta$ -hydroxyisovaleryl-shikonin induced apoptosis in HeLa cells through inhibition of the PI3K/AKT/mTOR signalling pathway [50]. In colorectal cancer, the semi-synthetic derivative M12 enhanced ROS generation and dis-

rupted mitochondrial membrane potential (MMP), resulting in intrinsic apoptosis, while  $\beta$ ,  $\beta$ -dimethylacrylshikonin induced apoptosis in HCT-116 cells by downregulating B-cell lymphoma (Bcl)-2 and Bcl-xL and upregulating Bcl-2-associated X protein (Bax) and BH3-interacting domain death agonist (Bid) [43,50]. In skin cancer, cyclopropylacetylshikonin triggered apoptosis in WM9 and WM164 melanoma cells through activation of the caspase-3/7 cascade, whereas  $\beta$ ,  $\beta$ -dimethylacrylshikonin induced NOXA-mediated, caspase-3-dependent apoptosis in BRAF- and NRAS-mutated melanoma subtypes [98,102]. In bone cancer, both acetylshikonin and cyclopropylshikonin upregulated caspase-7, -9, pro-apoptotic NOXA, and the DNA damage marker  $\gamma$ H2AX within 24 h of treatment in chondrosarcoma Cal78 and SW-1353 cells [68]. Furthermore,  $\beta$ ,  $\beta$ -dimethylacrylshikonin effectively induced apoptosis in chordoma MUG-Chor1 and U-CH2 cell lines through upregulation of pro-apoptotic genes, including NOXA and p53 upregulated modulator of apoptosis (PUMA) [103]. In TNBC MDA-MB-231 cells, treatment with the shikonin derivative E2 modulated the pyruvate dehydrogenase kinase 1 (PDK1) and PDHC/PDK axis, leading to ROS accumulation, upregulation of Bax and Fas proteins, and subsequent apoptosis [99]. In essence, shikonin derivatives demonstrated strong pro-apoptotic activity across multiple cancer types, with particularly notable efficacy in colorectal, bone, skin, and chordoma subtypes, while  $\beta$ ,  $\beta$ -dimethylacrylshikonin showed broad-spectrum antitumor activity.

**Table 2.** Shikonin derivatives induced apoptosis through multiple pathways in various cancers.

| Shikonin Derivatives                     | Target Cancer Types              | Pathways       | Results                                                                          | Ref.  |
|------------------------------------------|----------------------------------|----------------|----------------------------------------------------------------------------------|-------|
| Acetylshikonin                           | Leukaemia (K562)                 | Intrinsic      | $\uparrow$ Cleaved caspase-3; $\uparrow$ PARP; $\uparrow$ Caspase-9              | [101] |
| $\beta$ -hydroxyisovalerylshikonin       | Ovarian (HeLa)                   | PI3K/AKT/mTOR  | $\downarrow$ PI3K; $\downarrow$ AKT; $\downarrow$ mTOR; $\downarrow$ P70S6K mRNA | [50]  |
| Shikonin M12                             | Colorectal                       | Intrinsic      | $\uparrow$ ROS; $\downarrow$ MMP                                                 | [100] |
|                                          | Colorectal (HCT-116)             | Intrinsic      | $\downarrow$ Bcl-2; $\uparrow$ Bax                                               | [43]  |
| $\beta$ , $\beta$ -Dimethylacrylshikonin | Skin (BRAF/NRAS-mutated)         | Intrinsic      | $\uparrow$ NOXA; $\uparrow$ Caspase-3 activation                                 | [102] |
|                                          | Chordoma (MUG-Chor1, U-CH2)      | Intrinsic      | $\uparrow$ NOXA; $\uparrow$ PUMA                                                 | [103] |
| Cyclopropyl-acetylshikonin               | Skin (WM9, WM164)                | Intrinsic      | $\uparrow$ Caspase-3/7                                                           | [98]  |
| Acetylshikonin and cyclopropylshikonin   | Chondrosarcoma (Cal 78, SW-1353) | Intrinsic      | $\uparrow$ Caspase-7/9; $\uparrow$ NOXA; $\uparrow$ $\gamma$ H2AX                | [68]  |
| E2                                       | TNBC (MDA-MB-231)                | PDK1/PDHC axis | $\uparrow$ ROS; $\uparrow$ Bax; $\uparrow$ Fas                                   | [99]  |

**Abbreviations:** ROS: reactive oxygen species; MMP: mitochondrial membrane potential; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; NOXA and PUMA: pro-apoptotic proteins; PARP: poly (ADP-ribose) polymerase; TNBC: triple-negative breast cancer; PDK1: pyruvate dehydrogenase kinase 1; PDHC: pyruvate dehydrogenase complex;  $\gamma$ H2AX: DNA damage marker;  $\uparrow$ : up-regulated genes;  $\downarrow$ : downregulated genes.

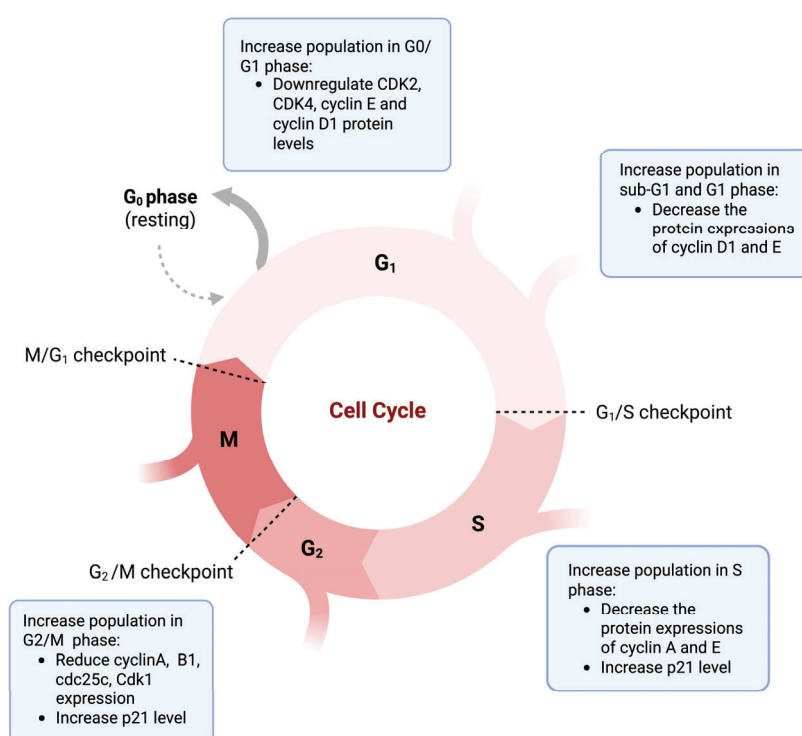
### 3.2. Cell Cycle Arrest

Cell cycle regulation is fundamental for maintaining controlled cell proliferation, and its dysregulation is a hallmark of tumorigenesis [104,105]. The cell cycle comprises the G1, S, G2, and M phases, which are primarily regulated by the cyclin-dependent kinase (CDK) family and controlled by restriction points and checkpoints [106,107]. In cancer therapy, induction of cell cycle arrest represents an important protective mechanism, as it allows cancer cells to respond to oxidative stress and DNA damage, ultimately leading to regulated cell death [104–107]. Several studies have demonstrated that shikonin induces

cell cycle arrest concomitant with regulated cell death pathways in prostate cancer, renal cell carcinoma, osteosarcoma, melanoma, and other cancers [91,108–111].

Many findings have reported that shikonin and its derivatives induce cell cycle arrest in the sub-G1 and G2/M phases in various cancers. For example, in melanoma A375 and A2058 cell lines, shikonin dose-dependently inhibited cell proliferation and increased cell accumulation in the sub-G1 and G2/M phases. Treatment of melanoma B16F10 cells with the derivative 2-methylbutyryl shikonin also induced G2/M phase arrest, accompanied by reduced cyclin B1 and Cdk1 expression and increased p21 levels [91,111]. Another example of a shikonin derivative, acetylshikonin, induced sub-G1 arrest in osteosarcoma U2OS cells, increasing the sub-G1 population from 5.4% to 16.8% [110]. In prostate cancer models, shikonin exposure increased the G2/M phase population in both parental PC3 and docetaxel-resistant DU145 cells, accompanied by decreased levels of checkpoint proteins cyclin A, cyclin B, and CDK1 [108]. In A549 lung cancer and PANC-1 pancreatic cancer cells, shikonin time-dependently promoted G2/M arrest, while shikonin and cyclopropylshikonin induced G2/M arrest in human chondrosarcoma cells via suppression of Cdc25C expression [68,75].

On the other hand, shikonin can also induce cell cycle arrest at the G1 and S phases. In NSCLC H1299 cells, shikonin dose-dependently increased the cell population in the G0/G1 phase, which was associated with downregulation of CDK2, CDK4, cyclin E, and cyclin D1 protein levels [89]. Shikonin also induced cell cycle arrest at the G1 phase in leukaemia NB4 cells [77]. Additionally, acetylshikonin induced S-phase arrest in leukaemia K562 cells via upregulation of the CDK inhibitor p21 [101], while  $\beta$ -hydroxyisovalerylshikonin treatment increased the proportion of ovarian cancer HeLa cells in the S phase [50]. In colorectal cancer,  $\beta$ ,  $\beta$ -dimethylacrylshikonin induced G0/G1 arrest in HCT-116 cells [43]. Together, these findings indicate that shikonin and its derivatives can induce cell cycle arrest at multiple phases, including G1, S, G2/M, and sub-G1, across diverse cancer types, supporting their potential as modulators of cell cycle progression in cancer therapy (Figure 2).



**Figure 2.** Shikonin induces cell cycle arrest pathways in various cancer.



### 3.3. Suppression of Metastasis and Invasion

Tumour metastasis remains the primary cause of cancer-related mortality and is a critical determinant of cancer prognosis. Aberrant epithelial–mesenchymal transition (EMT) is one of the hallmarks of metastasis and is commonly associated with invasion due to the development of motility-invasive properties [112]. EMT is characterised by the loss of epithelial phenotypes and the acquisition of mesenchymal cell properties [113]. EMT is a multistep process regulated via various mechanisms such as the expression of EMT-translational factors such as SNAIL, which upregulates the mesenchymal gene expression and downregulates the epithelial gene expression [112,113]. Subsequently, this can result in the “cadherin switch”, in which expression of the epithelial adhesion molecule E-cadherin is reduced while the mesenchymal marker N-cadherin is increased, promoting cell motility and proliferation [112]. Additionally, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are commonly upregulated during EMT. These enzymes degrade the extracellular matrix, facilitating cell detachment from the primary tumour site, and act as angiogenesis modulators by activating integrin signalling and promoting angiogenic factors such as angiostatin and endostatin [114].

Shikonin has demonstrated the ability to inhibit multiple steps of the metastatic cascade through various signalling pathways. Liu et al. reported that shikonin treatment (2.5 and 5.0  $\mu$ M) suppressed cell proliferation and induced dose-dependent apoptosis in QBC939 cholangiocarcinoma cells. Cell invasion was reduced, and apoptosis and necrosis were increased, as assessed by Annexin V/propidium iodide flow cytometry [82]. Chen et al. showed that shikonin inhibited metastasis in prostate cancer PC-3 and DU145 cells by downregulating MMP-9 and MMP-2 expression, as observed via gelatine zymography. This effect was mediated by shikonin-induced ROS generation, which activated ERK1/2 and subsequently suppressed MMP-9 and MMP-2 expression [115]. Bhat et al. observed similar effects in shikonin-treated melanoma cells, alongside attenuated EMT via the regulation of the 1- $\kappa$ B $\alpha$ /NF $\kappa$ B signalling pathways. I kappaB alpha (1- $\kappa$ B $\alpha$ /) acts as an inhibitor of nuclear factor kappa B (NF- $\kappa$ B), a factor promoting tumour angiogenesis and metastasis [111].

Huang et al. reported that shikonin, when administered at a dose of 5.0  $\mu$ M, downregulated tumour necrosis factor receptor-associated protein 1 (TRAP1) expression, resulting in decreased TE-1 human oesophageal cancer cell viability as observed in cell viability assays [87]. Consistent with previous studies, the shikonin-treated TE-1 cells were also found to have decreased MMP2 and MMP9 expression, consequently inhibiting cell migration and proliferation [82,87,115]. Zeng et al. demonstrated in an in vitro pancreatic cell model that  $\beta$ -hydroxyisovaleryl-shikonin was able to suppress EMT progression via inhibiting the phosphorylation of the PI3K/Akt pathway and reducing the expression of N-cadherin, MMP-9, and MMP-2 [49,116]. Similarly, Zhang et al. reported similar effects in shikonin-treated nasopharyngeal carcinoma cells with suppressed cell proliferation and migration [116]. Bao et al. reported findings that shikonin was able to exert antimetastatic properties on TNBC cells as demonstrated on wound healing and Transwell invasion assays. Western blots revealed that shikonin downregulated mesenchymal markers N-cadherin and vimentin expression and upregulated epithelial E-cadherin expression in the treated cells [113]. This was also observed by Mo et al. in human bladder cancer cells, linking EMT suppression with the inhibition of proton efflux pump sodium–hydrogen exchanger 1 (NHE1) by shikonin, though the linking mechanism between shikonin’s inhibitory effects of NHE1 and EMT suppression has yet to be further elucidated [117].

Shikonin’s involvement in EMT inhibition occurs not only with protein signalling pathways but also in gene expression of tumour cells. For instance, it suppresses gene miR-15-5p expression, a microRNA found to promote breast cancer cell EMT and inhibit the

expression of tumour suppressor gene phosphatase and tensin homologue (PTEN) [113]. Tabari et al. reported that co-treatment with shikonin and metformin upregulated PTEN expression in MCF-7 breast cancer cells, completely inhibiting cell migration within 24 h at 3.0  $\mu$ M shikonin and 7.0 mM metformin. Real-time polymerase chain reaction (RT-PCR) analysis revealed decreased expression of pro-EMT genes, such as SNAIL, and increased expression of the anti-EMT gene CDH1 (E-cadherin) [118]. Zhang et al. also elucidated that shikonin suppressed tumour cell metastasis via the p53/miR-361-5p/ZEB1 axis in glioblastoma cells. Western blot studies showed how shikonin upregulates the expression of p53, a miR-361-5p promoter, which results in miR-361-5p inhibiting ZEB1 overexpression [119]. ZEB1 transcription factors play a major role in promoting EMT by inhibiting E-cadherin and CDH1 expression by binding to their promoters, histone deacetylase (HDAC)1 and HDAC2 [120]. In summary, shikonin exerts anti-metastatic effects by interfering with multiple EMT regulatory mechanisms. It downregulates pro-EMT transcription factors (e.g., SNAIL) and microRNAs (e.g., miR-15-5p), while upregulating tumour suppressors and anti-EMT genes (e.g., CDH1 and PTEN). These changes suppress mesenchymal markers (N-cadherin, vimentin) and enhance epithelial markers (E-cadherin). In parallel, shikonin inhibits MMP expression through modulation of PI3K/Akt and activation of ERK1/2 signalling. Together, these molecular events translate into reduced migration, invasion, and metastatic potential in multiple tumour cell types.

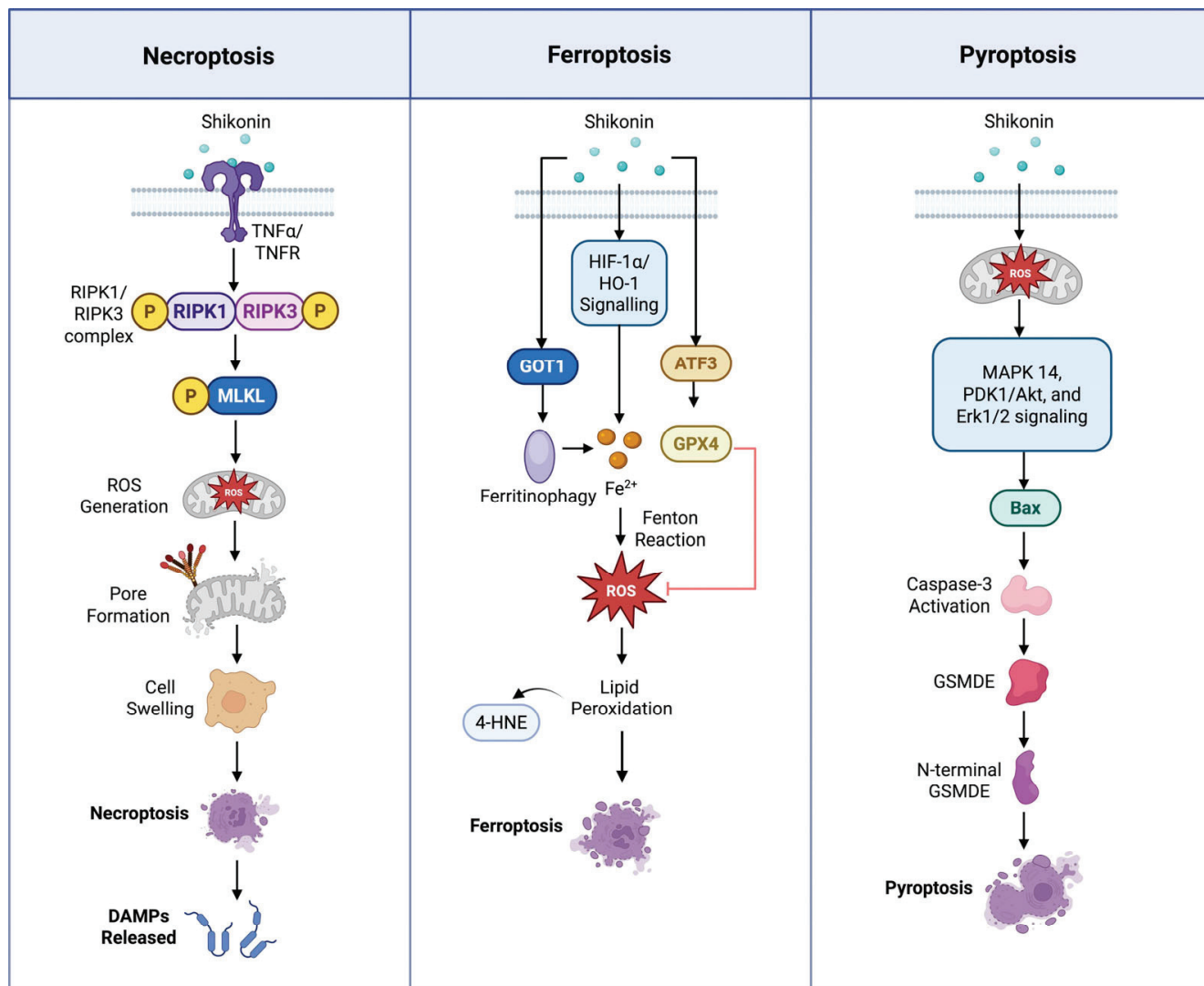
### 3.4. Induction of Necroptosis

Necroptosis is a regulated form of cell death typically triggered by tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) or Toll-like receptors (TLRs) under conditions of caspase-8 inhibition (Figure 3). It is primarily mediated by the phosphorylation of receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3), which subsequently phosphorylate mixed lineage kinase domain-like protein (MLKL), resulting in necrosome assembly, plasma membrane rupture, and the release of damage-associated molecular patterns (DAMPs) [121–123].

Recent studies have shown that shikonin induces necroptosis in various cancer cell types, including osteosarcoma, glioma, and chronic myeloid leukaemia, through mechanisms involving ROS generation, loss of MMP, and RIPK1/RIPK3 activation [124,125]. In glioma cells (SHG-44, U87, U251), shikonin upregulated RIPK1 and RIPK3 expression via excessive ROS production. By treating mitochondrial superoxide cleaner MnTBAP in these glioma cell lines, the ROS generation decreased, while the combination treatment of RIP1/RIP3 inhibitor and shikonin caused RIP1/RIP3 activation and necrosome assembly decreased, suggesting shikonin induces RIP1/RIP3 activation, which affects the downstream of ROS generation leading to glioma necroptosis [125].

Other than the SHG-44, U87, and U251 glioma cell lines, shikonin also showed necroptotic effects in the A172 and T98G glioma cell lines by increasing RIP1/RIP3 and MLKL levels [125,126]. In chronic myeloid leukaemia (CML) resistant to tyrosine kinase inhibitors, shikonin downregulated miR-92a-1-5p, a negative regulator of MLKL, thereby activating the RIPK1/RIPK3/MLKL signalling axis to induce necroptosis [124].

In Jurkat T-cell leukaemia cells, shikonin triggered a RIP-1-dependent pathway during early apoptosis, suggesting that shikonin induces an RIP1-dependent necroptotic pathway, which will affect the downstream of ROS generation leading to apoptosis [127]. In pancreatic cancer cells (AsPC-1 and PANC-1), shikonin dose-dependently induced both apoptosis and necroptosis. Interestingly, combined treatment with the necroptosis inhibitor Nec-1 and the apoptosis inhibitor zVAD in AsPC-1 cells shifted cell death predominantly toward necroptosis, indicating that shikonin can activate both pathways simultaneously [128].



**Figure 3.** Shikonin-induced regulated cell death through necroptosis, ferroptosis and pyroptosis.

Another research showed that shikonin induced more predominant necroptotic death in doxorubicin (DX)-resistant prostate cancer cell lines. They found that the combined administration of the Nec-1 inhibitor and shikonin results in reversed anti-growth effects of shikonin in all parental and DX-resistant prostate cancer cell lines [108]. In breast adenocarcinoma MCF-7 cells, shikonin irreversibly inhibited the antioxidant enzyme thioredoxin reductase 1 (TrxR1), inducing oxidative stress and necroptosis, which was prevented by Nec-1 co-treatment. This suggests that targeting TrxR1 may sensitise cancer cells to shikonin-induced necroptosis [129].

Briefly, these findings indicate that shikonin can trigger necroptosis, often in conjunction with apoptosis, across diverse cancer types through pathways involving RIPK1/RIPK3/MLKL activation, ROS generation, and redox system disruption.

### 3.5. Induction of Ferroptosis

Ferroptosis is an iron-dependent form of regulated cell death mechanism that is mediated by iron, lipids, and ROS, which will affect mitochondrial membrane structure and mitochondrial volume [121–123]. Shikonin's antitumor effects extend beyond apoptosis and necroptosis, as it has also been shown to induce ferroptosis in multiple myeloma (MM) through glutamic-oxaloacetic transaminase 1 (GOT-1)-mediated ferritinophagy, resulting in lactate dehydrogenase release and subsequent cell death [130]. In small cell lung cancer

(SCLC), shikonin triggered ferroptosis by increasing oxidative stress and suppressing the antioxidant enzyme GPX4 via an ATF3-centred, epigenetically regulated pathway [131]. Moreover, in osteosarcoma MC63 cells, shikonin simultaneously induced apoptosis and ferroptosis, with its ferroptotic effect mediated by mitochondrial ROS (MitoROS)-triggered HIF-1 $\alpha$ /HO-1 signalling. This led to iron overload, oxidative stress, and depletion of mitochondrial antioxidant defence enzymes [132]. In short, these findings indicate that shikonin can induce ferroptosis in osteosarcoma and small cell lung cancer, expanding its spectrum of regulated cell death mechanisms beyond apoptosis and necroptosis (Figure 3).

### 3.6. Induction of Pyroptosis

Pyroptosis is a proinflammatory form of regulated cell death pathway that can be triggered by caspase -1, -3, -4, and -5 and is mediated through three cellular pathways, which are granzyme-mediated, canonical, and non-canonical pathways [121–123]. Shikonin has demonstrated broad antitumor effects, including the induction of pyroptosis. In human gastric cancer (GC) cell lines SGC-7901 and BGC-823, shikonin induced pyroptosis in a time- and dose-dependent manner via a gasdermin E (GSDME)-mediated pathway. Mechanistically, shikonin increased intracellular ROS, resulting in Bax/caspase-3 activation while concurrently inhibiting MAPK14/p38 $\alpha$ -regulated autophagy, thereby amplifying pyroptosis in gastric cancer cells [133].

Additionally, in the EGFR-T790M-mutant drug-resistant NSCLC cell line H1975, shikonin induced both pyroptosis and apoptosis by inhibiting PGE2-induced downstream signalling pathways, including PDK1/Akt and Erk1/2. Co-treatment with shikonin, the necroptosis inhibitor Nec-1, the apoptosis inhibitor zVAD, and the ferroptosis inhibitor Fer-1 revealed compromised cell membranes, confirming that shikonin triggers both pyroptosis and late-stage apoptosis in H1975 cells [93]. In brief, these findings indicate that shikonin is capable of inducing pyroptosis in gastric cancer and non-small cell lung cancer, further expanding its spectrum of regulated cell death mechanisms.

### 3.7. Inhibition of Tumour Metabolism

Tumour cells undergo metabolic reprogramming to meet the increased energy and biosynthetic demands required for rapid proliferation and metastasis [134]. One of the main altered metabolic processes is aggravated aerobic glycolysis, also known as the Warburg effect. It is characterised by increased glucose uptake in cancer cells that preferentially undergo aerobic glycolysis despite sufficient oxygen availability, leading to rapid ATP and lactate production [135]. The resulting acidic TME promotes metastatic cell proliferation and activates proliferation-associated factors such as transforming growth factor  $\beta$  (TGF- $\beta$ ) [136].

PKM2, a key glycolytic enzyme isoform, is frequently upregulated in cancer and plays a central role in metabolic and transcriptional regulation [137]. PKM2 exists in multiple conformations, including inactive monomers, active tetramers, and nuclear dimers. The dimeric form mainly plays a role in modulating cellular proliferation but is also able to stimulate the expression of transcription factors associated with glycolysis [138,139]. It is also able to promote the Warburg effect by favouring glucose-derived carbon utilisation for macromolecular biosynthesis while tetramer PKM2 is highly active and stimulates rapid ATP production via oxidative phosphorylation [140].

Shikonin has been shown to disrupt tumour metabolic reprogramming by directly inhibiting PKM2 activity and expression. Long et al. demonstrated that shikonin-loaded nanoparticles optimised with hyaluronic acid reduced lactate production in CT26 colorectal cancer cells through PKM2 inhibition. Shikonin was able to remodel the tumour

immune microenvironment (TIME) by inhibiting lactate-induced immunosuppression and stimulating cancer cell apoptosis [136].

In NSCLC lines A549 and PC9, shikonin at doses of 4.0  $\mu$ M, 6.0  $\mu$ M, and 8.0  $\mu$ M inhibited glucose uptake and lactate production. In the same study, Dai et al. confirmed that shikonin's antiglycolytic properties were exerted via inhibiting PKM2 by observing its effectiveness in PKM2-deficient NSCLC cells [141]. Another study by Zhou et al. further proved shikonin's involvement in inhibiting PKM2 activity by attenuating the guanosine triphosphate binding protein 4 (GTPBP4)-PKM2-dependent regulatory axis in glycolytic reprogramming in HCC. Tumours collected from mice xenografted with HCC cell lines showed upregulated levels of GTPBP4, an inducer of dimer PKM2 production, which was significantly correlated with poor prognosis, increased metastasis and tumour progression, and increased aerobic glycolysis contributing to the Warburg effect. However, Western blot and immunoprecipitation tests demonstrated that shikonin was able to completely inhibit GTPBP4-PKM2-induced glycolytic activity, thus suppressing HCC growth and metastasis [140].

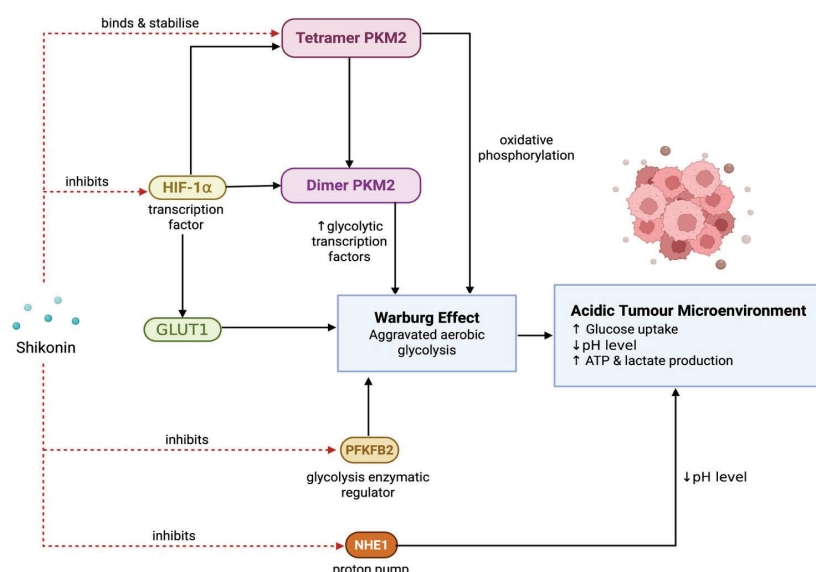
Consistent with previous studies, Zhang et al. found that shikonin inhibited PKM2 activity in a dose-dependent manner in nasopharyngeal carcinoma cells, accompanied by significantly reduced ATP levels, lactate production, and glucose uptake. This demonstrates shikonin's ability to alter the glycolytic flux and TME by inhibiting PKM2 [116]. Huang et al. observed via size-exclusion chromatography that shikonin inhibited the formation of PKM2 dimer and tetramer isoforms. Shikonin binds to the PKM2 isoforms and modulates PKM2 aggregation into a polymer, thus inhibiting its activity [138].

Additionally, it was also found that shikonin could selectively inhibit the formation of dimer PKM2 forms by binding to the tetramer forms and stabilising them [138,139]. One such factor is hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ), which encodes for the production of glycolytic enzymes, glucose transporters GLUT1, and PKM2 itself [139,141]. The inhibition causes downregulated expression of prolyl hydroxylase 3 (PHD3), a HIF-1 $\alpha$  coactivator, thus dysregulating the PHD3/HIF-1 $\alpha$  positive feedback loop [86]. Other than via the direct inhibition of PKM2, shikonin also acts on endocan, also known as the endothelial cell specific molecule 1 (ESM1), which facilitates the dimerisation of PKM2 [142].

Shikonin has also been found to inhibit aerobic glycolysis via the inhibition of NHE1, a proton efflux pump, resulting in an acidic tumour cell intracellular microenvironment [117]. While an acidic intercellular microenvironment further aggravates metastasis, an acidic intracellular microenvironment inhibits DNA synthesis initiation, a process requiring an alkaline environment, consequently suppressing cell proliferation [117,136]. Additionally, shikonin exerts inhibitory properties on 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate (PFKFB2) expression, an enzymatic regulator of the fructose-2,6-bisphosphate synthesis and degradation in glycolysis [143].

Overall, shikonin is able to suppress tumour metabolism by acting on various mechanisms inhibiting the Warburg effect. It mainly acts on the glycolytic enzyme PKM2 via different approaches: direct inhibition via aggregation, preventing the formation of its tetramer and dimer isoforms, and targeting its transcriptional factor, HIF- $\alpha$  (Figure 4). Additionally, shikonin interferes with other factors of the glycolytic cycle as well, such as the inhibition of NHE1 and PFKFB2. This inhibition allows shikonin to alter the tumour's intracellular and intercellular microenvironment, specifically by altering the pH levels against favouring tumour growth. Shikonin's mechanism in suppressing tumour metabolism is well established and has consistently shown improved prognosis over multiple studies, with lowered production of ATP and lactate and glucose uptake.





**Figure 4.** Shikonin suppresses tumour metabolism by inhibiting PKM2 and glycolysis. Key in-creases (↑) and decreases (↓) in molecular markers are indicated.

## 4. Combination Therapy

### 4.1. Shikonin as a Chemosensitiser: Enhancing Chemotherapy and Overcoming Resistance

The therapeutic limitations of single-agent chemotherapy have driven widespread interest in combination strategies that can simultaneously target multiple cancer pathways [144]. Shikonin functions as a potent chemosensitiser, enhancing the efficacy of chemotherapeutic agents while overcoming drug resistance.

#### 4.1.1. Inhibition of PKM2 Expression in Non-Small Cell Lung Cancer

In NSCLC, Dai et al. reported that shikonin significantly enhanced cisplatin sensitivity through dual inhibition of PKM2-mediated glycolysis and exosome signalling [141]. PKM2 is a central regulator of the Warburg effect, sustaining tumour growth through aerobic glycolysis and biosynthesis, while also promoting exosome release via non-metabolic activation of SNAP-23. These exosomes carry oncogenic cargo that remodels the TME, driving angiogenesis, metastasis, and immune evasion. Targeting PKM2 could therefore disrupt both metabolic reprogramming and exosome-mediated signalling, providing a dual approach to suppress tumour progression [145,146]. Shikonin suppresses PKM2 expression by inhibiting HIF-1 $\alpha$  transcription and the splicing factors that drive PKM2 isoform selection. This disruption of the PKM2/HIF-1 $\alpha$  axis reduces inflammation and oxidative stress [138]. Notably, shikonin exerted potent anti-tumour effects in NSCLC cells, with IC<sub>50</sub> values at 24 h of 5.739  $\mu$ M in A549 and 6.302  $\mu$ M in PC9 cells. At 8  $\mu$ M, shikonin reduced PK activity by ~33% within 20 min. In A549 cells, it markedly suppressed glucose uptake (~67%) and lactate production (~57%), compared with more modest reductions in PC9 cells (~24% and ~40%), reflecting greater sensitivity in A549. This metabolic inhibition corresponded with decreased PKM2 expression and activity, without changes in MCT1 or LDHA. PKM2 knockdown abolished these anti-glycolytic and anti-proliferative effects, confirming the dependence of shikonin's action on PKM2 inactivation [141].

Shikonin also impaired exosome release, reducing exosomal PKM2 secretion by ~69%, while exosome blockade with GW4869 produced similar restoration of cisplatin sensitivity. This decrease in exosome signalling coincided with a strong pro-apoptotic effect, with ~650% and ~525% increases in apoptosis at 6  $\mu$ M observed in A549 and PC9 cells, respectively. Importantly, cisplatin-resistant NSCLC cells transferred resistance to cisplatin-sensitive A549 cells through exosomal PKM2, as shown by a ~72% reduction in cisplatin

efficacy when A549 cells were exposed to cisplatin alone. Shikonin effectively reversed this acquired resistance in a dose-dependent manner, restoring up to ~100% of cisplatin sensitivity in cells cultured in conditioned media, confirming its ability to block PKM2-mediated resistance transfer. In nude mice inoculated with A549 cells, intraperitoneal co-treatment with shikonin and cisplatin markedly enhanced cisplatin sensitivity through metabolic reprogramming, resulting in the greatest reductions in tumour burden and  $^{18}\text{F}$ -FDG uptake compared with monotherapies. Shikonin downregulated PKM2 and Glut1 expression, as confirmed by immunohistochemistry (IHC), corresponding with a >50% decrease in glucose uptake on  $^{18}\text{F}$ -FDG PET/CT (SUVmax), and suppressed exosomal PKM2 release. Notably, inhibition of exosome secretion with GW4869 or direct targeting of exosomal PKM2 restored cisplatin responsiveness, underscoring shikonin's dual action on intracellular metabolism and intercellular resistance transmission. These combined effects led to >70% reductions in tumour volume and weight [141].

#### 4.1.2. Upregulation of HMOX1-Induced Ferroptosis in Ovarian Cancer

Ni et al. demonstrated that shikonin synergises with cisplatin to overcome resistance in ovarian cancer by enhancing ferroptosis, an iron-dependent form of cell death driven by lipid peroxidation [147]. This approach is particularly effective against aggressive, apoptosis-resistant tumour cells, exploiting their dependence on iron and dysregulated lipid metabolism to induce lethal oxidative damage [148]. The study employed three cisplatin-resistant ovarian cancer cell lines, A2780/DDP, SKOV3/DDP, and OVCAR4/DDP, with average  $\text{IC}_{50}$  values of 23.46  $\mu\text{M}$ , 50.06  $\mu\text{M}$ , and 18.06  $\mu\text{M}$ , respectively. Compared with cisplatin alone, shikonin in combination therapy significantly upregulated heme oxygenase 1 (HMOX1), as shown by volcano plot analysis, where 23 proteins were downregulated and 35 were upregulated [147]. Increased HMOX1 expression promoted heme breakdown [149] and increased intracellular ferrous iron ( $\text{Fe}^{2+}$ ) levels by 50% in A2780/DDP and 100% in SKOV3/DDP cells [150]. Higher  $\text{Fe}^{2+}$  facilitated ROS production through Fenton reactions, generating hydroxyl radicals that attacked polyunsaturated fatty acids in cell membranes [151]. This triggered ~34% and ~50% increases in ROS levels in A2780/DDP and SKOV3/DDP cells, respectively, leading to ~33% and ~90% increases in lipid peroxidation [147]. The resulting oxidative stress overwhelmed antioxidant defences, compromised membrane integrity, and induced ferroptosis [152,153], thereby restoring cisplatin sensitivity in resistant ovarian cancer cells.

In vivo, the therapeutic synergy was confirmed in a cisplatin-resistant ovarian cancer xenograft model using BALB/c nude mice inoculated with A2780/DDP cells. Shikonin at 0.8 mg/kg combined with cisplatin at 3.0 mg/kg suppressed tumour growth by over 67% compared to monotherapies, without causing weight loss or hepatorenal toxicity. IHC confirmed 3-fold upregulation of ferroptosis markers, including HMOX1, TFRC, and POR. This dual-action mechanism, inducing iron-dependent cell death while maintaining metabolic safety, highlights shikonin as an effective adjunct to platinum-based chemotherapy in overcoming resistance in ovarian cancer [147].

#### 4.1.3. Upregulation of p53-Mediated Apoptosis in Oesophageal Cancer

Besides cisplatin, shikonin also demonstrates synergistic effects with other chemotherapeutic drugs, such as paclitaxel. Du et al. investigated shikonin's ability to sensitise oesophageal squamous cell carcinoma (ESCC) to paclitaxel using KYSE270 and KYSE150 cell lines. In vitro, co-treatment with shikonin and paclitaxel increased p53 expression by approximately 200% [154]. P53 is a tumour suppressor that regulates genes involved in cell cycle arrest, apoptosis, senescence, autophagy, and DNA repair, and its upregulation led to markedly higher apoptosis, with a ~367% increase compared to either agent alone [154,155].

This was supported by enhanced caspase-3 cleavage, detected by Western blotting, and by nuclear fragmentation, both of which are established hallmarks of apoptosis. Caspase-3 acts as a key executioner protease, mediating programmed cell death by cleaving critical structural and regulatory proteins, while nuclear fragmentation reflects chromatin breakdown during late-stage apoptosis [156–158]. Moreover, p53 upregulation suppressed BCL2 expression by about 38% [154]. BCL2 is an anti-apoptotic protein that confers resistance to chemotherapy, and its reduction enhances sensitivity to treatment [159,160]. Whereas either shikonin or paclitaxel alone had minimal effects on p53 and BCL2, their combination significantly increased p53 and decreased BCL2, underscoring their cooperative role in promoting apoptosis [154].

#### 4.1.4. Inhibition of PAK1 Expression in Pancreatic Cancer

Pancreatic cancer has a poor prognosis, with gemcitabine-based therapy providing only limited survival benefits. Ji et al. investigated whether combining shikonin with gemcitabine could enhance therapeutic efficacy by targeting p21-activated kinase 1 (PAK1) [161], an oncogenic kinase frequently overexpressed in pancreatic cancer that drives proliferation, survival, invasion, and therapy resistance [162,163]. Using the PANC-1 and BxPC-3 cell lines, shikonin was identified as a novel PAK1 inhibitor, with  $IC_{50}$  values of  $1.800 \pm 0.013 \mu\text{M}$  in PANC-1 and  $3.183 \pm 0.321 \mu\text{M}$  in BxPC-3 cells. Western blot analysis confirmed that shikonin inhibited PAK1 phosphorylation and suppressed its downstream signalling pathways, including mTOR, MEK1, and c-RAF. Apoptosis was induced dose-dependently, with 3, 5, and 10  $\mu\text{M}$  treatments causing 14.66%, 83.35%, and 90.50% apoptosis in BxPC-3 cells and approximately 7%, 14%, and 17% in PANC-1 cells, respectively. Notably, shikonin markedly sensitised BxPC-3 cells to gemcitabine, reducing the  $IC_{50}$  values from 14.22 to 2.18  $\mu\text{M}$ , with strong synergism demonstrated by combination index values of 0.18. These findings highlight shikonin as a potent bioactive inhibitor of PAK1 and a promising candidate for combination therapy in pancreatic cancer [161].

Taken together, these studies underscore shikonin's broad capacity to enhance chemotherapeutic efficacy by targeting diverse resistance mechanisms across multiple malignancies. Mechanistically, it disrupts tumour survival through dual blockade of PKM2-mediated glycolysis and exosome signalling in NSCLC, ferroptosis induction in cisplatin-resistant ovarian cancer, p53-dependent apoptosis in ESCC, and PAK1 inhibition in pancreatic cancer. Clinically, shikonin consistently restores drug sensitivity and amplifies cytotoxicity, highlighting its potential as a versatile adjunct to combination chemotherapy. These findings warrant further preclinical and clinical evaluation to establish its translational value in overcoming therapy resistance and improving patient outcomes.

#### 4.2. Endocrine-Targeted Therapy and Resistance Modulation by Shikonin

Targeted therapies have transformed breast cancer treatment, yet their efficacy is often limited by the emergence of resistance mechanisms that diminish clinical benefit. Resistance can develop through on-target genetic alterations that impair drug binding, such as EGFR T790M and ALK mutations, or through activation of alternative survival pathways, including MET amplification and KRAS mutations, which circumvent the inhibited signalling axis [164,165].

##### 4.2.1. Activation of Mitochondrial-Mediated Apoptosis in Breast Cancer

Shikonin has been shown to enhance the efficacy of endocrine-targeted therapy in both oestrogen receptor-positive ( $ER^+$ ) and -negative ( $ER^-$ ) breast cancers by modulating apoptotic and survival pathways. Lin et al. reported that combining shikonin with 4-hydroxytamoxifen (4-OHT) in MCF-7 ( $ER^+$ ) and MDA-MB-435S ( $ER^-$ ) cells produced maximum inhibition rates of 47% and 66%, respectively, surpassing the effects of either

drug alone [166]. Mechanistically, shikonin targeted mitochondrial complex II (succinate dehydrogenase), causing electron transport chain dysfunction and excessive superoxide production, which increased ROS levels 2.3-fold in MCF-7 and 1.8-fold in MDA-MB-435S [166,167]. The resulting oxidative stress damaged mitochondrial components and triggered mitochondrial permeability transition pore opening, leading to loss of MMP by ~77% in MCF-7 and ~60% in MDA-MB-435S [166,168]. Dissipation of MMP initiated intrinsic apoptosis through cytochrome c release and Apaf-1/caspase-9 apoptosome formation, with concurrent Smac/DIABLO release neutralising IAPs and activating the caspase cascade [169].

Combination treatment increased early apoptosis by 22.9% in MCF-7 and late apoptosis by 26.3% in MDA-MB-435S. Additionally, shikonin suppressed PI3K expression by ~33% in MCF-7 and ~20% in MDA-MB-435S, while reducing AKT activity by ~68% only in MCF-7, implicating the PI3K/AKT/caspase-9 axis as a key mechanism in ER<sup>+</sup> cells. In vivo, in a BALB/c mouse model bearing MCF-7 xenografts, the combination of shikonin (1.5 mg/kg) and 4-OHT (3 mg/kg) produced superior antitumour efficacy, achieving 76.65% tumour growth inhibition versus 57.20% for shikonin and 45.44% for 4-OHT alone. Tumour weight was reduced by 58% and 47% compared to 4-OHT and shikonin monotherapies, respectively. While shikonin alone caused mild adverse effects, the combination treatment maintained a favourable safety profile, with no significant body weight loss or renal toxicity. Histopathological analysis showed enhanced apoptosis and reduced malignancy in the combination group, as confirmed by H&E staining and IHC. These findings highlight the synergistic potential of shikonin to overcome endocrine resistance in ER<sup>+</sup> breast cancer, with a 1:2 dose ratio (shikonin:4-OHT) emerging as an effective and tolerable regimen [166].

#### 4.2.2. Restoration of the uc.57–BCL11A Axis Overcomes Tamoxifen Resistance

Complementing these findings, Zhang et al. investigated shikonin as a potential modulator of tamoxifen (TAM) resistance by targeting resistance-associated pathways and ultra-conserved long non-coding RNAs (lncRNAs) [170]. lncRNAs are non-protein-coding transcripts longer than 200 nucleotides that regulate gene expression and contribute to cancer progression by modulating chromatin structure, transcriptional programmes, and signalling pathways. Certain lncRNAs also drive tumour metastasis and therapy resistance through interactions with epigenetic complexes and hormone receptors [171]. Using TAM-resistant MCF-7R and parental MCF-7 cell lines, the study showed that MCF-7R cells expressed ~80% less of the ultra-conserved lncRNA uc.57 and ~280% more of its target gene BCL11A compared with TAM-sensitive cells, linking uc.57 downregulation and BCL11A upregulation to TAM resistance. Shikonin treatment restored uc.57 expression, which suppressed BCL11A and consequently inhibited the PI3K/AKT and MAPK pathways by ~50% [170].

The MAPK pathway promotes growth and angiogenesis through RAS/RAF/ERK signalling, while PI3K/AKT enhances survival and epithelial–mesenchymal transition through lipid-mediated signals. Frequent activation and crosstalk of these pathways contribute to tumour aggressiveness and endocrine therapy resistance, making their dual inhibition an important therapeutic goal [172–174]. Functionally, shikonin plus TAM suppressed MCF-7R cell growth by 69% in vitro, whereas TAM alone had no effect. In human breast tissue-derived SCID mouse models of TAM-resistant ER-positive breast cancer (MCF-7R xenografts), shikonin at 1.5 mg/kg combined with TAM (3 mg/kg) reduced tumour volume by over 50% compared to shikonin alone, effectively overcoming TAM resistance. Parallel experiments with uc.57-overexpressing MCF-7R-lv-uc.57 xenografts confirmed this mechanism, as TAM monotherapy significantly inhibited tumour growth in



uc.57-positive tumours, recapitulating the effect of the shikonin–TAM combination. The five-week treatment caused no observable toxicity, with stable body weight and normal ALT/AST levels [170].

Together, these findings demonstrate shikonin's ability to enhance endocrine-targeted therapies by disrupting both mitochondrial metabolism and transcriptional resistance pathways. Mechanistically, it induces mitochondrial dysfunction, elevates ROS production, and suppresses PI3K/AKT signalling in ER<sup>+</sup> breast cancer, while restoring the uc.57–BCL11A axis and dual blockade of PI3K/AKT and MAPK pathways in tamoxifen-resistant models. Clinically, shikonin potentiates 4-OHT efficacy, restores tamoxifen responsiveness, and reduces tumour burden, supporting its role as a promising adjuvant strategy. These results highlight the translational value of shikonin in overcoming endocrine resistance, with uc.57 and BCL11A emerging as candidate biomarkers for future therapeutic development.

#### 4.3. Shikonin Enhances Immunotherapy via Immunogenic Cell Death

Immunogenic cell death (ICD) is a regulated form of tumour cell death that stimulates adaptive immunity through the release of DAMPs such as calreticulin (CRT), ATP, HMGB1, and heat shock proteins. These signals recruit and mature dendritic cells (DC), enhance tumour antigen presentation, and activate cytotoxic T lymphocytes (CTL), establishing long-term immune memory. Shikonin induces ICD via ROS-driven apoptosis and necroptosis, marked by CRT exposure, ATP release, and HMGB1 secretion. Importantly, tumour lysates from shikonin-treated cells, especially when combined with pathogen-associated molecular patterns (PAMPs), further promote dendritic cell maturation and stimulate Th1 and Th17 responses, thereby reinforcing anti-tumour immunity [175,176].

In TNBC, Shahsaari et al. reported that shikonin enhances anti-PD-1 immune checkpoint therapy by inducing RIPK1/RIPK3-dependent necroptosis [177]. Necroptosis is a caspase-independent programmed cell death triggered when death receptors such as TNFR1 or cellular stress activate RIPK1, which phosphorylates RIPK3 to form the necrosome complex. This drives MLKL oligomerisation, membrane permeabilisation, and inflammatory necrosis [178–180]. Shikonin showed an IC<sub>50</sub> of 3.586 µM at 12 h and acted by generating ROS, disrupting MMP (~83%), and upregulating RIPK1 (~35%) and RIPK3 (~40%). It demonstrated plasticity between death pathways, with necroptosis dominating when apoptosis was blocked by Z-VAD-FMK (necrosis ~20%, apoptosis ~2%) and apoptosis prevailing when necroptosis was inhibited by Nec-1 (necrosis ~3%, apoptosis ~39%). Caspase-3 and -8 activity increased by ~633% and ~288%, respectively, confirming strong apoptotic induction. Notably, shikonin's capacity to trigger immunogenic necroptosis enhanced the efficacy of PD-1 blockade by reshaping the TME to favour immune infiltration and recognition. By targeting mitochondrial dysfunction and activating RIPK1/RIPK3/MLKL while preserving caspase-dependent apoptosis, shikonin presents a dual cytotoxic and immunomodulatory strategy for treating aggressive breast cancers [177].

Supporting evidence from Chen et al. demonstrated that shikonin synergises with PD-1 blockade to overcome immunotherapy resistance via a PKM2-ROS-Hsp70 immunogenic axis [181]. Hsp70 acts as a dual-function chaperone, maintaining proteostasis and inhibiting apoptosis intracellularly while serving extracellularly as a DAMP to activate antigen-presenting cells (APCs), enhance NK cell cytotoxicity, and stimulate pro-inflammatory cytokine production. Its context-dependent functions, both promoting tumour growth and facilitating immune recognition, make Hsp70 a compelling therapeutic target for inhibition to disrupt cancer survival or exploitation to boost ICD and anti-tumour immunity [182–184]. Shikonin induced dual cell death in CT26 cells, combining dose-dependent apoptosis (12.47% at 5 µM and 20.17% at 10 µM) with PKM2-dependent ROS-mediated ICD, confirmed by reversal with PKM2 silencing and NAC inhibition. It specifically enhanced CRT



surface exposure ( $22.13 \pm 0.153\%$  at  $5 \mu\text{M}$ ;  $23.70 \pm 0.265\%$  at  $10 \mu\text{M}$ ) and Hsp70 expression ( $32.61 \pm 11.260$ ) without significant changes in total CRT or HMGB1 levels or soluble HMGB1 release, as shown by Western blot and ELISA. These changes remodelled the tumour microenvironment, increasing CD8<sup>+</sup> T-cell infiltration ( $2.52 \pm 0.659\%$ ) and dendritic cell recruitment ( $4.02 \pm 2.112\%$ ). In vivo, CT26 cells were subcutaneously inoculated into the right inguinal region of SPF female BALB/c mice, and treatment was initiated once tumours reached approximately  $62.5 \text{ mm}^3$ . Shikonin was administered intraperitoneally at  $3 \text{ mg/kg}$ , administered every 2 days for a total of seven doses, while anti-PD-1 antibody was given intraperitoneally at  $50 \mu\text{g}$  per mouse on days 5 and 9. This combination significantly enhanced anti-PD-1 efficacy, reducing tumour volume by 40% compared to PD-1 monotherapy ( $294.1 \pm 231.2 \text{ mm}^3$  vs.  $489.2 \text{ mm}^3$ ) and by 72% versus untreated controls ( $1048 \pm 1016 \text{ mm}^3$ ). The more than 3.5-fold reduction underscores shikonin's therapeutic potential as an adjuvant to checkpoint inhibitors for microsatellite-stable colorectal cancers where PD-1 monotherapy typically fails [181].

Shikonin represents a promising phytochemical for immunotherapy-resistant clear cell renal cell carcinoma (ccRCC), demonstrating a distinctive triple mechanism of action when combined with ipilimumab. In the SKRC17 and RCC53 cell lines, the latter derived from a patient with stage IV disease (pT2N1MxG2 to G3), Lyu et al. reported that shikonin directly suppressed cancer stem cells (CSCs), reducing migration and invasion by 50% and increasing apoptosis by approximately 344%, with an  $\text{IC}_{50}$  of  $1.32 \mu\text{M}$ . It also reprogrammed the immune microenvironment by decreasing FoxP3<sup>+</sup> regulatory T cells (Tregs) by 70% and enhancing CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation 2.5-fold in patient peripheral blood mononuclear cell (PBMC) co-cultures. In addition, it modulates CSC-immune biomarkers, including VCAM1, IL8, and CXCL1. Integrated NanoString profiling and network pharmacology identified 19 shared molecular targets underpinning the shikonin–ipilimumab synergy, with VCAM1 emerging as a predictive biomarker. High VCAM1 expression correlated with a 3-fold improvement in survival and increased NK/T-cell infiltration in TCGA data, although its prognostic significance was reversed under CTLA-4 blockade. Unlike current VEGF-TKI/ICI regimens limited to intermediate-poor risk IMDC patients, this combination uniquely addresses CSC-driven resistance through PKM2-mediated metabolic rewiring and ROS-dependent CSC elimination while enhancing tumour immunogenicity. Clinical translation is further supported by CSC marker downregulation (IL8, CXCL1, VCAM1), activation of immune and NK-cell signalling pathways, and stratification potential via Immunophenoscore, positioning the combination for future trials in ICI-refractory ccRCC [185].

In aggregate, these studies reveal that shikonin can enhance immunotherapy by reshaping the TIME. Mechanistically, it reduces lactate-induced immunosuppression, promotes DC activation, and stimulates cytotoxic T-cell responses while concurrently inhibiting immune checkpoints such as PD-1/PD-L1. In addition, shikonin has been reported to induce ICD, characterised by calreticulin exposure, ATP release, and HMGB1 secretion, thereby further enhancing antigen presentation and adaptive immune activation. Clinically, shikonin has shown the capacity to restore immune sensitivity and strengthen the efficacy of checkpoint inhibitors, highlighting its value in overcoming immune evasion. These findings warrant further preclinical validation and clinical trials to define optimal combination strategies and evaluate their safety and translational impact in immuno-oncology.

#### 4.4. Shikonin Derivative $\beta$ , $\beta$ -Dimethylacrylshikonin Enhances Radiotherapy

To investigate the radio-sensitising potential of shikonin, Kim et al. examined its analogue  $\beta$ ,  $\beta$ -dimethylacrylshikonin and found that it markedly enhanced cancer cell sensitivity to ionising radiation (IR) through ROS-mediated mechanisms. In vitro studies

across several cancer cell lines showed that HCT116 colorectal carcinoma cells exhibited the greatest reduction in viability (~44%) when pretreated with the analogue before IR, while LN428 glioma (~13%), H460 NSCLC (~26%), and A549 lung carcinoma (~18%) cells displayed only minor effects. Although the compound alone induced limited apoptosis, its combination with 5 Gy IR synergistically increased apoptotic cell death (~200%). This effect was linked to an approximately 123% increase in ROS and a ~196% increase in DNA damage, with Western blot and immunofluorescence confirming elevated  $\gamma$ -H2AX expression. The ROS scavenger N-acetylcysteine suppressed both ROS accumulation and apoptosis (~27%), with IC<sub>50</sub> confirming ROS dependence. In HCT116 xenograft models,  $\beta$ ,  $\beta$ -dimethylacrylshikonin showed remarkable radiosensitisation, with combination therapy using 5 Gy ionising radiation and the analogue achieving near complete tumour growth arrest (<500 mm<sup>3</sup>), compared to ~50% suppression with radiation alone and 3000 mm<sup>3</sup> in untreated controls. This 6-fold enhancement of radiation efficacy occurred without observable toxicity, indicating a favourable therapeutic window for colorectal cancer and potential for significant radiation dose reduction in clinical settings. The analogue's superiority over native shikonin underscores the value of structure activity optimisation for radiotherapy adjuvants [150].

Together, the evidence underscores  $\beta$ ,  $\beta$ -dimethylacrylshikonin as a potent radiosensitiser that augments the therapeutic efficacy of ionising radiation through ROS-mediated DNA double-strand breaks, repair pathway disruption, and apoptosis induction. Its pronounced activity in HCT116 colorectal carcinoma, particularly in p53 mutant models, highlights its potential to overcome radioresistance, while tumour type specificity observed in glioma and lung carcinoma lines suggests the need for biomarker-driven patient selection. Importantly, in vivo studies confirm its strong efficacy and favourable safety profile, reducing tumour burden without exacerbating normal tissue toxicity. These findings support further exploration of  $\beta$ ,  $\beta$ -dimethylacrylshikonin and related shikonin analogues as promising adjuvant strategies to enhance radiotherapy outcomes. Table 3 summarises the synergistic outcomes of shikonin in combination with various anticancer therapies across multiple cancer types.

**Table 3.** Synergistic outcomes of shikonin in cancer therapy.

| Cancer Type                              | Combination                   | IC <sub>50</sub> ( $\mu$ M) Reduction                                            | Apoptosis (% Annexin V+)                                                                                                 | Key Molecular Changes                                                                      | Ref.       |
|------------------------------------------|-------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|------------|
| Lung (A549, PC9)                         | Shikonin + Cisplatin          | A549 (5.74 $\mu$ M); PC9 (6.30 $\mu$ M)                                          | A549 (+650%); PC9 (+525%)                                                                                                | ↓ PKM2; ↓ Glycolysis; ↓ Exosome                                                            | [141]      |
| Ovary (A2780/DDP, SKOV3/DDP, OVCAR4/DDP) | Shikonin + Cisplatin          | A2780/DDP (23.46 $\mu$ M); SKOV3/DDP (50.06 $\mu$ M); OVCAR4/DDP (18.06 $\mu$ M) | Not reported                                                                                                             | ↑ HMOX1; ↑ Heme breakdown; ↑ Fe <sup>2+</sup> ; ↑ ROS; ↑ Lipid peroxidation; ↑ Ferroptosis | [147, 149] |
| Oesophagus (KYSE-150, KYSE-270)          | Shikonin + Paclitaxel         | Not reported                                                                     | KYSE270 (+ ~367%)                                                                                                        | ↑ p53 activation; ↓ Bcl-2 expression                                                       | [154]      |
| Pancreas (PANC-1, BxPC-3)                | Shikonin + Gemcitabine        | PANC-1 (1.800 $\mu$ M); BxPC-3 (3.18 $\mu$ M)                                    | PANC-1 (3 $\mu$ M—14.66%; 5 $\mu$ M—83.35%; 10 $\mu$ M—90.50%); BxPC-3: (3 $\mu$ M—~7%; 5 $\mu$ M—~14%; 10 $\mu$ M—~17%) | ↓ PAK1; ↓ Downstream signalling; ↑ Apoptosis                                               | [161]      |
| Breast (MDA-MB-435, MCF-7)               | Shikonin + 4-hydroxytamoxifen | Not reported                                                                     | MDA-MB-435S (26.3%); MCF-7 (22.9%)                                                                                       | ↑ ROS; ↓ MMP; ↑ Apoptosis                                                                  | [166]      |

Table 3. Cont.

| Cancer Type                       | Combination                     | IC <sub>50</sub> (μM) Reduction | Apoptosis (% Annexin V+)      | Key Molecular Changes                                                                     | Ref.  |
|-----------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------------------------------------------------------------------|-------|
| Breast (MCF-7R)                   | Shikonin + Tamoxifen            | Not reported                    | Not reported                  | ↑ lncRNA uc.57; ↓ BCL11A; ↓PI3K/AKT and MAPK pathways                                     | [170] |
| Breast (MDA-MB-468)               | Shikonin + anti-PD-1            | 3.59 μM                         | Z-VAD-FMK (~2%); Nec-1 (~39%) | ↑ RIP1K and RIP3K; ↑ ROS; ↓ MMP; Necroptosis                                              | [177] |
| Colon (CT26)                      | Shikonin + anti-PD-1            | Not reported                    | 12.47% (5 μM); 20.17% (10 μM) | ↑ Calreticulin exposure; ↑ Hsp70; APCs activation                                         | [181] |
| Colon (HCT116, LN428, H460, A549) | β, β-Dimethylacrylshikonin + IR | Not reported                    | ~200%                         | ↑ ROS; ↑ DNA damage; ↑ Apoptosis                                                          | [150] |
| Kidney (SKRC-17, RCC-53)          | Shikonin + Ipilimumab           | 1.32 μM                         | ~344%                         | ↓ FoxP3 <sup>+</sup> Tregs; ↑ Activation of CD8 <sup>+</sup> and CD4 <sup>+</sup> T cells | [185] |

**Abbreviations:** Bcl-2: B-cell lymphoma 2; ROS: reactive oxygen species; MMP: mitochondrial membrane potential; lncRNAs: long non-coding RNAs; MAPK: mitogen-activated protein kinase; RIPK1: receptor-interacting protein kinase1; RIPK3: receptor-interacting protein kinase3; Tregs: regulatory T cells; ↑: increased; ↓: decreased.

## 5. Preclinical and Clinical Studies

Several in vivo preclinical studies have been conducted to evaluate the potential of shikonin as an anticancer agent based on its well-characterised mechanisms. Shikonin has demonstrated consistent anticancer effects across diverse in vivo tumour models, supporting its potential for clinical application. Moreover, shikonin shows promise in enhancing the efficacy of existing anticancer therapies, particularly in overcoming drug resistance when used in combination strategies. Table 4 summarises the preclinical and clinical studies evaluating shikonin and its derivatives in various cancer models.

Table 4. Preclinical and clinical evaluation of shikonin and its derivatives in cancer models.

| Cancer Type/Model                       | Agent                               | Mechanism of Action                                             | Main Outcomes                                   | Ref.     |
|-----------------------------------------|-------------------------------------|-----------------------------------------------------------------|-------------------------------------------------|----------|
| Melanoma (A375 xenograft, mice)         | Shikonin (5.0 mg/kg)                | ↑ Apoptotic proteins                                            | Greater tumour suppression vs. dacarbazine      | [111]    |
| Melanoma (B16F10 lung metastasis, mice) | Shikonin + Quercetin                | PKM2 inhibition, ↓ platelet–tumour interaction, ↓ glycolysis    | Fewer metastatic nodules; no adverse effects    | [186]    |
| Breast cancer (Orthotopic; TNBC, MCF-7) | Shikonin                            | PDK1 inhibition, EMT suppression                                | ↓ Tumour invasion and growth; TNBC sensitivity  | [17,187] |
| Breast cancer (MCF-7 xenograft, mice)   | Shikonin + siRNA/TGF-β nanoparticle | ICD induction (calreticulin exposure), improved biodistribution | 21.8% ICD; tumour-specific targeting            | [188]    |
| HNSCC (xenograft, mice)                 | Shikonin                            | Suppression of FAM83A/PKM2 axis                                 | ↓ PKM2 expression; ↓ tumour progression         | [189]    |
| Cervical and ovarian cancer (xenograft) | Shikonin                            | Apoptosis, anti-metastatic activity                             | ↓ Tumour growth; ↑ cisplatin sensitivity        | [17,147] |
| Oral cancer (mouse model)               | Shikonin                            | Apoptosis induction                                             | Tumour growth inhibition                        | [190]    |
| Pancreatic cancer (cell models)         | β-hydroxyisovalerylshikonin         | ↑ ROS, apoptosis                                                | Enhanced cytotoxicity                           | [154]    |
| Clinical trial (lung cancer, 1991)      | Shikonin (dose unclear)             | Not reported                                                    | >25% tumour reduction; mean survival ~10 months | [191]    |

**Abbreviations:** PKM2: pyruvate kinase isoform M2; PDK1: pyruvate dehydrogenase kinase 1; EMT: epithelial–mesenchymal transition; TNBC: triple negative breast cancer; ICD: immunogenic cell death; ROS: reactive oxygen species ↑: increased; ↓: decreased.

Bhat et al. reported that shikonin significantly inhibited tumour growth in xenografted mice bearing human melanoma A375 cells, with Western blot analysis confirming increased expression of apoptotic proteins. At a dose of 5.0 mg/kg, shikonin-treated mice exhibited greater tumour suppression than those treated with dacarbazine, a standard chemotherapeutic agent [111]. Similarly, Zhu et al. demonstrated that combined treatment with shikonin and quercetin in a B16F10 melanoma mouse model significantly reduced the number of lung metastatic nodules without adverse effects, partially attributed to the inhibition of platelet–tumour interactions and glycolysis via PKM2 suppression [186].

Orthotopic mouse models of breast cancer treated with shikonin were found to have significant tumour growth inhibition and reduced tumour cell invasion in a study conducted by Pander et al. TNBC has also been found to be responsive to treatment with shikonin via mechanisms such as the inhibition of PDK1 [17]. TNBC cells were particularly sensitive to shikonin, which exerted antitumor effects via PDK1 inhibition and EMT suppression, two pathways closely associated with drug resistance and aggressive metastasis [187]. Li et al. developed an antigen-targeted nanoparticle system for co-delivery of shikonin, TGF- $\beta$ , and small interfering RNA. Flow cytometry and confocal microscopy revealed that 21.8% of treated cells underwent ICD, as indicated by calreticulin exposure. This nanoparticle system demonstrated optimal biodistribution, prolonged circulation time, and specific tumour cell targeting in vivo [188].

Zhang et al. conducted an in vivo study using BALB/c mice xenografted with head and neck squamous carcinoma cells targeting the FAM83A/PKM2 axis by suppressing PKM2 expression with shikonin. The study elucidated a newfound relationship between the involvement of FAM83A in PKM2 regulation and tumour progression and indicated that shikonin could be a promising therapeutic treatment targeting the FAM83A/PKM2 axis in treating head and neck squamous cell carcinomas (HNSCC) [189].

There have also been animal studies based on cervical and ovarian cancer treatment with shikonin, which showed shikonin's capabilities to suppress tumour progression by inhibiting metastasis and stimulating apoptosis [17]. Shikonin has also been found to improve treatment for cisplatin-resistant ovarian cancer cells by enhancing its cytotoxicity [147]. There have also been investigations into shikonin's effectiveness in oral cancer, where Min et al. showed that oral cancer growth was successfully inhibited in a mouse model [190]. Shikonin derivatives have also been investigated, such as  $\beta$ -hydroxyisovaleryl-shikonin by Zeng et al., who observed increased cell apoptosis and ROS production in pancreatic cancer cells [49]. Due to its glycolysis-inhibiting property, it induces weight loss in animal models and causes skin sensitisation [55]. Shikonin is also known to exert hepatotoxic effects due to its inhibitory effects against uridine 5'-diphosphate-glucuronosyltransferase, making it unsuitable for clinical usage despite its benefits as an anticancer therapeutic agent [36,55]. However, in vivo animal toxicity studies showed that the method of administration helped reduce the shikonin's toxicity greatly. Oral administration resulted in little to no toxicity, with a  $>1.0$  g/kg LD<sub>50</sub>, while intraperitoneal and intravenous injections yielded results indicating specific toxicity. These findings suggest that tissue-specific targeting and optimised administration routes are critical to mitigating toxicity, although the roles of metabolism and toxicokinetics require further clarification [36].

Another limiting factor is the vague bioavailability of shikonin, as it is easily influenced by patient-dependent factors such as metabolism and toxicokinetics. Establishing preclinical data has shown that shikonin may potentially damage normal tissues if not adequately targeted to cancer cells [55]. Prolonged shikonin treatment has been associated with nuclear membrane damage, although Tabari et al. demonstrated that co-treatment with metformin activated protective pathways such as AMP-activated protein kinase (AMPK), preventing this effect [118,192]. A popular method of targeted delivery of shikonin is

with the use of liposomes, such as by Zhu et al. in their shikonin-quercetin study, which also showed adverse effects due to its tendency to accumulate in the liver, resulting in hepatotoxicity rather than the intended targets [186]. A more optimal delivery approach may be via targeted nanoparticles, such as the Xu et al. shikonin-loaded mesoporous polydopamine-based nanoparticles in an emulsion, which allowed improved drug solubility and release rate [193]. Alternatively, Chen et al. utilised hyaluronic acid-modified Fe-MOF nanoparticles as a glycolysis-mediated agent to deliver shikonin to the tumour site and reported improved shikonin solubility alongside combating the toxic nature of shikonin towards other healthy cells exposed [194].

Despite having well-established data in preclinical *in vivo* trials, there are very few existing and ongoing clinical trials involving the treatment of cancer using shikonin [55]. To date, the only documented clinical trial of the implementation of shikonin as an anticancer therapeutic agent was conducted by Guo et al. in 1991, in which shikonin was prescribed to advanced-stage lung cancer patients [191]. The study reported a tumour reduction of over 25% in diameter and an average survival of 10 months but did not provide detailed information on dosage or adverse effects [191]. Much of these data are outdated and lack detailed information on dosing and administration. This lack of recent and comprehensive clinical data underscores the urgent need for modern clinical trials incorporating advanced drug delivery strategies and informed by preclinical findings to fully evaluate shikonin's therapeutic potential.

Overall, preclinical studies consistently demonstrate that shikonin and its derivatives exert potent anticancer effects across diverse tumour models, acting through apoptosis induction, glycolysis inhibition, ICD, and enhancement of combination therapies. While these findings highlight its translational promise, concerns regarding hepatotoxicity, limited bioavailability, and patient-dependent pharmacokinetics remain critical barriers. Only one outdated clinical trial has been reported, underscoring the urgent need for well-designed modern clinical studies. Future efforts should prioritise targeted delivery systems, toxicity mitigation, and biomarker-driven patient selection to establish shikonin as a viable adjuvant or stand-alone anticancer therapy.

## 6. Challenges and Future Perspectives

Despite compelling preclinical evidence supporting shikonin's anticancer potential across multiple tumour types and its promise as a combination therapy candidate, significant barriers continue to hinder its clinical translation. Pharmacokinetic limitations remain a primary challenge, as poor aqueous solubility, rapid metabolism, and variable first-pass effects lead to extremely low systemic bioavailability. These issues are compounded by dose-dependent hepatotoxicity, skin sensitisation, and non-specific accumulation in healthy tissues such as the liver, raising important safety concerns. Such risks underline the need for comprehensive toxicological assessments before advancing to large-scale human trials. Furthermore, while preliminary studies suggest synergistic potential in treatment combinations, supporting evidence remains limited, with few clinical investigations to date. This underscores a persistent gap between promising preclinical findings and real-world application.

Addressing these limitations will require coordinated strategies, including optimised formulations, a clearer understanding of shikonin's mechanisms, and well-designed clinical trials. One promising direction is tumour-targeted delivery systems, particularly nanoparticles, which can improve aqueous solubility, create prodrug analogues, and enhance selective accumulation at tumour sites. Advances in plant-derived nanomedicine also make it possible to co-encapsulate shikonin with complementary phytochemicals, maximising therapeutic efficacy while limiting off-target toxicity. A strong example is the MUC1@ACS



nanocomplex, which combines shikonin with chitosan-coated silver nanoparticles and an MUC1 aptamer for highly specific delivery to TNBC cells. This system achieved a 6.02-fold increase in tumour accumulation compared to free drugs, induced necroptotic ICD via the RIPK3/p-RIPK3/MLKL pathway and triggered potent systemic anti-tumour immunity. As a result, it produced a 68.5% reduction in primary tumour growth and a 91% decrease in metastatic lung and liver nodules, with complete distal tumour regression in some cases. Notably, it showed no significant organ toxicity, highlighting nanoparticles as a viable way to overcome shikonin's bioavailability and safety barriers [195].

In the longer term, shikonin's multifaceted mechanisms of action position it as a strong candidate for integration into precision oncology frameworks. Continued progress will depend on sustained multidisciplinary collaboration involving pharmacology and clinical oncology. With strategic optimisation and well-designed clinical trials, shikonin or its derivatives could become valuable therapeutic options for cancers that currently lack effective treatments, potentially transforming patient outcomes. Overall, bridging preclinical promise with clinical validation remains the critical step for establishing shikonin as a translational anticancer therapy.

## 7. Conclusions

In conclusion, although conventional cancer therapies have advanced, persistent challenges such as drug resistance and systemic toxicity continue to limit patient outcomes. Shikonin, a multifunctional dietary phytochemical, exerts potent anticancer activity through multiple forms of regulated cell death, suppression of EMT, and PKM2-driven metabolic disruption. Its ability to synergise with conventional therapeutic strategies presents a promising avenue to overcome therapeutic resistance. However, its clinical application is still restricted by poor bioavailability, dose-dependent hepatotoxicity, and the lack of well-designed clinical trials. Overcoming these obstacles through advanced drug delivery systems, thorough preclinical evaluation, and translational studies will be essential for establishing clinical feasibility. By bridging the principles of traditional medicine with modern oncology, shikonin and its derivatives stand out as compelling candidates for innovative, synergistic cancer therapies, particularly in treatment-resistant malignancies. This review highlights shikonin as a bridge between natural product pharmacology and precision oncology, underscoring its potential to transform future cancer therapy.

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## Abbreviations

The following abbreviations are used in this manuscript:

|       |                              |
|-------|------------------------------|
| 1-κBα | I kappaB alpha               |
| 4-OHT | 4-hydroxytamoxifen           |
| 5-FU  | 5-fluorouracil               |
| AMPK  | AMP-activated protein kinase |

|                  |                                           |
|------------------|-------------------------------------------|
| APCs             | Antigen-presenting cells                  |
| ATF4             | Activating transcription factor 4         |
| β-carotene       | Beta-carotene                             |
| Bax              | Bcl-2-associated X protein                |
| Bcl              | B-cell lymphoma                           |
| Bid              | BH3-interacting domain death agonist      |
| Ca <sup>2+</sup> | Calcium                                   |
| ccRCC            | Clear cell renal cell carcinoma           |
| CDK              | Cyclin-dependent kinase                   |
| CHOP             | C/EBP homologous protein                  |
| CML              | Chronic myeloid leukaemia                 |
| CRC              | Colorectal cancer                         |
| CRT              | Calreticulin                              |
| CSCs             | Cancer stem cells                         |
| CTL              | Cytotoxic T lymphocytes                   |
| DAMPs            | Damage-associated molecular patterns      |
| DC               | Dendritic cells                           |
| DISC             | Death-inducing signalling complex         |
| DNases           | Deoxyribonucleases                        |
| DR               | Death receptor                            |
| DX               | Doxorubicin                               |
| EGFR             | Epidermal growth factor receptor          |
| EMT              | Epithelial–mesenchymal transition         |
| ER               | Endoplasmic reticulum                     |
| ER <sup>+</sup>  | Oestrogen receptor–positive               |
| ER <sup>−</sup>  | Oestrogen receptor–negative               |
| ERK              | Extracellular signal-regulated kinase     |
| ESCC             | Oesophageal squamous cell carcinoma       |
| ESM1             | Endothelial cell specific molecule 1      |
| FADD             | Fas-associated death domain               |
| FASL             | Fas ligand                                |
| Fe <sup>2+</sup> | Ferrous iron                              |
| FPE              | First-pass effect                         |
| GC               | Gastric cancer                            |
| GOT-1            | Glutamic-oxaloacetic transaminase 1       |
| GPOR             | G protein-coupled oestrogen receptor      |
| GRP78            | Glucose-regulated protein 78              |
| GSDME            | Gasdermin E                               |
| GTPBP4           | Guanosine triphosphate binding protein 4  |
| HCC              | Hepatocellular carcinoma                  |
| HDAC             | Histone deacetylase                       |
| HIF-1α           | Hypoxia-inducible factor 1-alpha          |
| HMOX1            | Heme oxygenase 1                          |
| HNSCC            | Head and neck squamous cell carcinomas    |
| HPLC             | High-performance liquid chromatography    |
| HSCCC            | High-speed counter-current chromatography |
| ICD              | Immunogenic cell death                    |
| IHC              | Immunohistochemistry                      |
| IR               | Ionising radiation                        |
| IV               | Intravenous                               |
| JNK              | c-Jun N-terminal kinase                   |
| KM               | Kunming                                   |
| KRAS             | Kirsten rat sarcoma virus                 |
| lncRNAs          | Long non-coding RNAs                      |

|                    |                                                                      |
|--------------------|----------------------------------------------------------------------|
| MAE                | Microwave-assisted extraction                                        |
| MAPK               | Mitogen-activated protein kinase                                     |
| MitoROS            | Mitochondrial ROS                                                    |
| MLKL               | Mixed lineage kinase domain-like protein                             |
| MM                 | Multiple myeloma                                                     |
| MMP                | Mitochondrial membrane potential                                     |
| MMP-2              | Matrix metalloproteinase-2                                           |
| MMP-9              | Matrix metalloproteinase-9                                           |
| MS                 | Murashige and Skoog                                                  |
| NF-κB              | Nuclear factor kappa B                                               |
| NHE1               | Sodium–hydrogen exchanger 1                                          |
| NSCLC              | Non-small cell lung cancer                                           |
| OSCC               | Oral squamous cell carcinoma                                         |
| OXA                | Oxaliplatin                                                          |
| PAK1               | p21-activated kinase 1                                               |
| PAMPs              | Pathogen-associated molecular patterns                               |
| PARP               | Poly (ADP-ribose) polymerase                                         |
| PBMC               | Patient peripheral blood mononuclear cell                            |
| PCD                | Programmed cell death                                                |
| PDK1               | Pyruvate dehydrogenase kinase 1                                      |
| PFKFB2             | 6-phosphofructo-2-kinase/fructose-2,6-biphosphate                    |
| PHD3               | Prolyl hydroxylase 3                                                 |
| PKM2               | Pyruvate kinase isoform M2                                           |
| PTEN               | Phosphatase and tensin homologue                                     |
| PUMA               | p53 upregulated modulator of apoptosis                               |
| PYCR1              | Pyrroline-5-carboxylate reductase 1                                  |
| rhApo2L/TRAIL      | Apo2 ligand/tumour necrosis factor-related apoptosis-inducing ligand |
| RIPK1              | Receptor-interacting protein kinase1                                 |
| RIPK3              | Receptor-interacting protein kinase3                                 |
| ROS                | Reactive oxygen species                                              |
| RT-PCR             | Real-time polymerase chain reaction                                  |
| SC-CO <sub>2</sub> | Supercritical carbon dioxide                                         |
| SCLC               | Small cell lung cancer                                               |
| SD                 | Sprague Dawley                                                       |
| STP                | Sarcoma-targeting-peptide                                            |
| TAM                | Tamoxifen                                                            |
| TCM                | Traditional Chinese Medicine                                         |
| TGF-β              | Transforming growth factor β                                         |
| TIME               | Tumour immune microenvironment                                       |
| TLRs               | Toll-like receptors                                                  |
| TME                | Tumour microenvironment                                              |
| TNBC               | Triple negative breast cancer                                        |
| TNFα               | Tumour necrosis factor-α                                             |
| TRAP1              | Tumour necrosis factor receptor-associated protein 1                 |
| Tregs              | Regulatory T Cells                                                   |
| TrxR1              | Thioredoxin reductase 1                                              |
| UAE                | Ultrasonic-assisted extraction                                       |
| XBP-1              | X-box binding protein 1                                              |
| Zn-SHK-PEG         | Zinc-shikonin-polyethylene glycol                                    |

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## Review

# Recent Research on the Role of Phytochemicals from Ginseng in Management of Osteosarcoma, Osteoporosis, and Osteoarthritis

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**Abstract:** Ginseng phytochemicals have attracted considerable attention for their potential therapeutic applications in bone-related diseases including osteosarcoma, osteoporosis, and osteoarthritis. Recent research has highlighted the promising effects of ginsenosides and polysaccharides from ginseng by studying multi-target effects and combination therapies in osteosarcoma progression. Beyond osteosarcoma, ginseng phytochemicals have been explored for their effects on osteoporosis. Various ginsenosides and ginseng extract were shown to regulate signaling pathways involved in activating osteoblast and inhibiting osteoclast in vitro and in vivo models. Ginseng ginsenosides have also demonstrated potential anti-osteoarthritic properties. Recent studies discussed how ginsenoside reduced inflammation and cartilage degradation as a therapeutic candidate for osteoarthritis management. In this review, we examine the anti-osteosarcoma, anti-osteoporotic, and anti-osteoarthritic activities of ginseng-derived phytochemicals reported in studies published between 2014 and 2024. This review also provides a comprehensive overview of the working mechanisms of these compounds in various model systems. Furthermore, we address the limitations of current research approaches and outline future directions to maximize the therapeutic application of ginseng phytochemicals in the management of bone-related diseases.

**Keywords:** ginseng; ginsenoside; polysaccharide; extract; osteosarcoma; osteoporosis; osteoarthritis

## 1. Introduction

### 1.1. Phytochemicals from Ginseng

Ginseng, particularly *Panax ginseng* C.A. Meyer, has been a cornerstone of traditional medicine in Asia for thousands of years. As a member of the Araliaceae family, it is often referred to as an “all-healing” due to its extensive health benefits, largely attributed to active compounds such as ginsenosides [1–3]. Ginseng is primarily classified into two types: white ginseng and red ginseng. White ginseng is produced by air-drying fresh roots, whereas red ginseng is produced through a steaming process that modifies its chemical composition to enhance both its therapeutic properties and stability [4,5]. Due to these unique chemical transformations, red ginseng is particularly valued in traditional medicine and is regarded as having superior medicinal efficacy compared to white ginseng. A wide variety of phytochemicals in ginseng have been identified and extensively documented in the literature [6–8]. Of them, ginsenosides are the most significant, and over 100 types of ginsenosides have been documented [9,10]. Ginsenosides are broadly categorized into dammarane and oleanane types. The dammarane type is further divided into the protopanaxadiol and protopanaxatriol groups. Some of the well-known ginsenosides in these groups include Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg3. The oleanane type consists of

the oleanolic acid and ocotillol groups, with Ro being a prominent example. In addition to ginsenosides, ginseng contains polysaccharides, phenolic compounds, alkaloids, fatty acids, mineral oils, and amino acids [11–16]. Ginsenosides are the most extensively studied among all phytochemicals of ginseng, with Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg3 being the most abundant. Collectively, these phytochemicals play a pivotal role in mediating the pharmacological effects of ginseng.

### *1.2. Health Benefits and Pharmacological Activities of the Phytochemicals from Ginseng*

The primary bioactive components in ginseng are ginsenosides, which are steroidal saponins that contribute to various therapeutic effects. Research has demonstrated that ginsenosides possess anti-oxidative and anti-cancer properties, and can enhance immunity, energy levels, and sexual function, while also combating cardiovascular diseases, diabetes, and neurological disorders [17–20]. For example, ginsenoside Rg3 has shown promise in killing different types of cancer cells and protecting against DNA damage in normal human cells [21,22]. Ginseng polysaccharides are potential therapeutic agents for various health conditions, including cancer, immune disorders, and metabolic diseases, due to their multifaceted pharmacological activities [23]. A recent study reported that ginseng polysaccharides enhance the production of complement component 4, a core component of the complement system, by promoting its gene transcription via specific promoter regions [24]. In addition, the ginseng berry polysaccharide portion has been shown to suppress cancer growth by activating natural killer cells [25]. Phenolic compounds from ginseng offer numerous health benefits due to their potent anti-oxidant and anti-inflammatory properties [18]. These compounds, including phenolic acids and flavonoids, have been shown to protect against oxidative stress and reduce the risk of chronic diseases such as cancer, cardiovascular disease, and neurodegenerative disorders [1]. Research demonstrated that the total phenol content in ginseng fruit and leaves is higher than in ginseng roots, with chlorogenic acid, gentisic acid, p- and m-coumaric acid, and rutin being the major phenolic compounds found in ginseng [26]. Additionally, steaming ginseng can increase the content and bioavailability of phenolic compounds, potentially enhancing their health-promoting effects [1]. Overall, ginseng is a powerful herbal remedy with a wide range of health benefits, largely due to its diverse phytochemicals, which work synergistically to produce pharmacological effects. Various health benefits of the phytochemicals from ginseng properties could make it a valuable supplement for promoting the well-being of the human body.

### *1.3. Recent Research on Phytochemicals to Manage Osteosarcoma*

Osteosarcoma is the most common primary bone cancer, typically affecting children and young adults, with a peak incidence during adolescent growth spurts [27]. It usually develops in the metaphyseal regions of long bones, particularly around the knee, hip, and shoulder, and is characterized by the production of immature bone or osteoid tissue by malignant cells [28]. Common symptoms include localized pain, swelling, and limited range of motion, with diagnosis typically involving imaging studies (X-rays, MRI, CT scans) and biopsy [29]. Although amputation may be necessary in some cases, treatment usually involves a combination of chemotherapy and surgery, with limb-salvage procedures often possible [30]. The prognosis for osteosarcoma has improved significantly over the past few decades, with survival rates around 70% for localized disease, but outcomes are poorer for metastatic or recurrent cases [27]. Phytochemicals show promise as complementary approaches for managing various types of cancer, either alone or in combination with conventional therapies [31]. In fact, there are lots of recent studies on phytochemicals for managing osteosarcoma. A study identified 28 metabolites from soursop leaf extract

that showed potential anti-cancer effects against osteosarcoma [32]. Three compounds (2'-hydroxy-5'-methyl chalcone, linoleic acid, and annonacin) showed good binding affinity to platelet-derived growth factor receptor  $\alpha$ , a kind of osteosarcoma target protein. The leaf extract exhibited cytotoxicity against MG-63 osteosarcoma cells in vitro. Some phytochemicals can help restore sensitivity of cancer cells to chemotherapy drugs. A study found that combining capsaicin with cisplatin had synergistic inhibitory effects on osteosarcoma cells in culture and in xenografts [33]. The combination induced apoptosis, cell cycle arrest, and inhibited cell invasion more effectively than either compound alone. In addition, compounds such as sulforaphane, carvacrol, thymoquinone, and ursolic acid have shown anti-cancer effects through various mechanisms including cell cycle arrest, apoptosis induction, and inhibition of key signaling pathways [34–37]. There is continued interest in identifying and evaluating novel plant-derived compounds for their potential in osteosarcoma treatment. More studies are needed to fully elucidate mechanisms of action and optimal dose and combinations for clinical use.

#### 1.4. Recent Research on Phytochemicals to Manage Osteoporosis

Osteoporosis is a systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to increased bone fragility and susceptibility to fractures [38]. It is often called a “silent” disease because it typically doesn't cause symptoms until a fracture occurs, most commonly affecting the hip, spine, and wrist [39]. The condition develops when bone breakdown exceeds bone formation, resulting in a loss of bone mineral density and changes in bone structure [40]. Osteoporosis is more prevalent in women, especially after menopause, and in individuals over 50 years old [41]. Diagnosis is usually made through bone mineral density (BMD) scans using dual-energy X-ray absorptiometry which measures BMD at the hip and spine [41]. The standard therapy for osteoporosis typically aims to slow or stop bone loss through a combination of lifestyle changes, proper nutrition, and medications. Bisphosphonates are generally considered the first-line treatment for most patients with osteoporosis, especially those at high risk of fracture. These medications work by slowing bone breakdown and have been shown to reduce the risk of fractures by 30–70%, depending on the specific bone site [42]. Recent research has focused on specific plants such as green tea, rosehip berries, ginger root, turmeric root, and pomegranate peel and their potential in osteoporosis management [43]. For instance, a recent study examined the relationship between phytochemical-rich food intake and osteoporosis incidence in pre- and postmenopausal women. The research found that postmenopausal women in the highest phytochemical index quartile had a 16% lower risk of osteoporosis compared to those in the lowest quartile [44]. This suggests that consuming foods rich in phytochemicals may have a protective effect against osteoporosis in postmenopausal women. Also, several phytochemicals have shown promising results in maintaining bone health. Genistein from *Sophora japonica* has demonstrated anti-osteoporosis effects similar to soybean genistein [45,46]. Sophoricoside increased bone mechanical strength and osteogenic biochemical markers in ovariectomized rats [46]. Kurarinone, 8-prenylkaempferol, and (2S)-2'-methoxykurarinone have shown potential in preventing osteoclastic bone resorption and promoting bone health [47]. These phytochemicals work through various mechanisms, such as promoting mesenchymal stem cell differentiation, improving osteoblast proliferation, and suppressing osteoclast formation [43]. While these findings are promising, more research is needed to fully understand the efficacy of phytochemicals in osteoporosis management and to develop targeted interventions. Future studies should focus on validating these results using biochemical data and exploring the potential of combining phytochemicals with conventional treatments for enhanced efficacy in managing osteoporosis.



### 1.5. Recent Research on Phytochemicals to Manage Osteoarthritis

Osteoarthritis is a degenerative joint disease characterized by the breakdown of cartilage and other joint tissues [48]. It is the most common form of arthritis and primarily affects older adults, though it can also occur in younger individuals due to joint injuries or genetic factors [48]. Osteoarthritis typically affects weight-bearing joints such as the knees, hips, and spine, but it can also impact the hands, neck, and lower back [49]. The condition is marked by joint pain, stiffness (especially after periods of inactivity), swelling, and loss of flexibility [49]. Risk factors include age, obesity, previous joint injuries, repetitive stress on joints, genetic predisposition, and sex (with women being more susceptible) [50]. Various treatments can help manage symptoms and improve quality of life. For example, non-steroidal anti-inflammatory drugs (NSAIDs) may be used to control pain [51]. In severe cases, joint replacement surgery may be considered to alleviate pain and restore function [52]. Recent research has increasingly focused on the role of phytochemicals derived from traditional medicinal plants in managing osteoarthritis. These natural compounds found in fruits, vegetables, and herbs, have been recognized for their anti-inflammatory and anti-oxidant properties, making them potential alternatives to conventional pharmacological treatments [53]. The interest in phytochemicals stems from their accessibility, low cost, and the growing desire for complementary and alternative medicine options, particularly for individuals seeking to mitigate the side effects associated with long-term use of NSAIDs and other medications [54]. For instance, a recent study demonstrated that oral administration of *Arnica montana* flower methanol extract reduced clinical signals of osteoarthritis and improved the histological status of hind limb joints in a collagen-induced arthritis rat model [55]. Mechanistically, *Arnica montana* flower methanol extract-treated rats showed lower levels of inflammatory mediators such as nitric oxide and TNF- $\alpha$ . They analyzed the phenolic and flavonoid compounds in *Arnica montana* flower methanol extract by GC/MS and suggested that the therapeutic effects could be due to their combined actions. Another study investigated the protective effects of curcumin on monosodium iodoacetate-induced osteoarthritis rat model [56]. Curcumin treatment significantly reduced joint diameter, improved Mankin's score, and increased paw withdrawal threshold in osteoarthritis rats. The expression of inflammatory biomarkers MyD88, p-I $\kappa$ B $\alpha$ , NF- $\kappa$ B, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in synovial fluid was lower in the curcumin-treated group compared to osteoarthritis rat groups. While phytochemicals show promise in treating osteoarthritis, there are several limitations to their use such as lack of rigorous clinical trials and poor understanding of mechanisms. By addressing these limitations, researchers could aim to harness the full potential of phytochemicals as complementary and alternative agents for osteoarthritis treatment, potentially offering more effective and safer options for patients in the future.

### 1.6. Outline of Review

This review focuses on the current understanding and future directions in phytochemicals from ginseng in managing osteosarcoma, osteoporosis, and osteoarthritis. This review deals with main ginseng phytochemicals such as ginsenosides, polysaccharides, phenolic compounds, and alkaloids and emphasizes potential therapeutic targets at various stages of osteosarcoma, osteoporosis, and osteoarthritis progression. By understanding the key signaling pathways and molecules involved in the disease's onset and development, researchers aim to pinpoint new targets for drug development based on ginseng. This review aims to inform and influence the development of future treatment strategies for osteosarcoma, osteoporosis, and osteoarthritis, providing a comprehensive overview of recent knowledge and emerging trends and serving as a valuable reference for researchers and clinicians in the field.

## 2. Phytochemicals from Ginseng in Management of Osteosarcoma

### 2.1. Ginsenosides

Ginsenosides from *Panax ginseng* have shown promising potential in the management and treatment of various kinds of cancers including osteosarcoma through their pharmacological activities related to cancer cell signaling pathways [57].

Firstly, specific kinds of ginsenosides induce apoptosis in osteosarcoma. Research revealed that ginsenoside Rf triggered G2/M phase cell cycle arrest and promoted apoptosis in MG63 cells via the mitochondrial-mediated pathway [58]. MTT assay result showed the highest cell toxicity of Rf treatment in MG63 cells with 11  $\mu$ M for 24 h. Ginsenoside Rg3 was reported to induce DNA damage in human osteosarcoma cells, leading to cell cycle arrest and apoptosis [22]. MG63 cell was the most sensitive osteosarcoma cell line with about 150  $\mu$ M of IC50 value in MTT assay for 24 h incubation. This genotoxic effect was evident through increased DNA single-strand breaks and the formation of  $\gamma$ H2AX foci. Another study demonstrated that ginsenoside Rg3 suppressed the proliferation of osteosarcoma cells and induced apoptosis through various signaling pathways, including the PI3K/Akt/mTOR pathway [59]. Interestingly, they showed a relatively low IC50 value for MG3 cells (about 2.5  $\mu$ M) in CCK8 assay for 24 h incubation. Furthermore, Rg3 treatment was shown to inhibit osteosarcoma development in the MG63 cell xenograft mouse model [60]. Tumor volume and weight in the Rg3 group (20 mg/kg) were reduced up to about 50% for 21 days compared to the control group. They also suggested that the anti-osteosarcoma activity of Rg3 was associated with the circ\_0003074/miR-516b-5p/KPNA4 axis. Another study reported that Rg3 inhibited the migration and invasion of osteosarcoma cells via regulation of the Wnt/ $\beta$ -Catenin and EMT signaling pathway [61]. In addition to Rg3, Rg5 was reported to trigger apoptosis by regulating the PI3K/Akt/mTORC1 signaling pathway and activating the LC3-mediated autophagy pathway [62]. Rg5 treatment showed 0.64  $\mu$ M of the IC50 value for 24 h and a relatively stronger anti-proliferative effect against MG63 cells compared to Rg3. The PI3K/Akt signaling pathway was identified as one of the related signaling pathways that Rg5 might regulate in osteosarcoma treatment [63]. Also, they found PIK3CA, SRC, TP53, MAPK1, EGFR, and VEGFA as target proteins using network pharmacology and molecular docking technology. Anti-osteosarcoma activity of Rh2, another kind of ginsenosides, was reported to impede the growth and migration of U2OS cells [64]. MTT assay results showed that the IC50 value of Rh2 for 48 h was about 8  $\mu$ M that was slightly higher than that of cisplatin. Rh2 induced apoptosis by regulating MAPK, PI3K/Akt/mTOR, and NF- $\kappa$ B signaling pathways in U2OS. A bioinformatic approach explored how a treatment of Rh2 might work against osteosarcoma by showing the differences between primary and metastatic osteosarcoma at the genetic level [65]. They found that the MAPK and PI3K/Akt/mTOR pathways could play an important role in the metastasis of osteosarcoma, which was targeted by Rh2. Ginsenoside CK was also reported to inhibit the growth, migration and invasion, and survival of human osteosarcoma cells by triggering apoptosis and interfering with the PI3K/mTOR/p70S6K1 signaling pathway [66]. The IC50 value for 72 h was observed around 20  $\mu$ M in MG63 cells. Western blotting analysis showed that CK treatment regulated PI3K/mTOR/p70S6K1 signaling pathway in MG63 cells, which was enhanced synergistically by RAD001 treatment.

Secondly, ginsenoside stimulates the anti-osteosarcoma activity of other compounds or chemotherapies. A recent study demonstrated that Rg1 enhanced the anti-osteosarcoma activity of Timosaponin AIII, a kind of spirostanol saponin from *Anemarrhena asphodeloides* [67]. The single treatment of Rg1 (250  $\mu$ M) or Timosaponin AIII (6  $\mu$ M) did not induce cytotoxic effects in MG63 cells but the combination treatment of Rg1 (250  $\mu$ M) and Timosaponin AIII (6  $\mu$ M) reduced cell viability up to 87% compared to the control. Their combinatorial treatment of them suppressed the MAPK signaling pathway, caused apoptosis, and

inhibited the migration of MG63 cells. In another study, the same group reported that Rb1 (250  $\mu$ M) and Rc (250  $\mu$ M) had synergistic anti-osteosarcoma activities with Timosaponin AIII (6  $\mu$ M) in MG63 cells [68]. The synergistic anti-osteosarcoma activity of Rg3 and doxorubicin was also investigated in vitro and in vivo model of osteosarcoma by regulating mTOR/HIF-1 $\alpha$ /VEGF and EMT signaling pathways [69]. Colony formation assay result demonstrated that both treatments of Rg3 (80  $\mu$ g/mL) and doxorubicin (0.3125  $\mu$ g/mL) in 143B cells caused the synergistic reduction of colony number (almost 0), whereas the colony number of the control, Rg3 only, and doxorubicin only was 430, 287, and 189, respectively. In the 143B xenograft mouse model, the tumor volume of the Rg3 group (20 mg/kg) plus doxorubicin (2 mg/kg) was reduced up to about 30% for 21 days compared to the control group. Compound K (CK), another kind of ginsenoside, enhanced the susceptibility of 143B osteosarcoma cells to cisplatin through induction of apoptosis via mitochondria-mediated pathways such as cytochrome C release [70]. MTT assay results for 72 h showed that CK treatment (10  $\mu$ M) with cisplatin (5  $\mu$ M) inhibited 143B cell viability up to about 28% compared to the control. In the xenograft (143B cells) mouse model, tumor volume in the CK and cisplatin administration group (10 mg/kg and 10 mg/kg) were reduced up to about 30% for 28 days compared to the control group. (20S)-protopanaxatriol was reported to enhance MG63 and U2OS cell sensitivity to ferroptosis-inducing chemicals such as RSL3, ML162, and IKE by increasing ACSL4 expression, a key enzyme that promotes lipid peroxidation [71]. The combination of (20S)-protopanaxatriol (0.5  $\mu$ M) and RSL3 (1  $\mu$ M) significantly reduced MG63 cell viability up to about 50% compared to the control. Interestingly, (20S)-protopanaxatriol from 0.1 to 100  $\mu$ M did not affect cell viability. The combined treatment with (20S)-protopanaxatriol and IKE (20 mg/kg and 20 mg/kg) for 28 days suppressed osteosarcoma xenograft volume (74.4% reduction) in vivo compared to the control group.

## 2.2. Polysaccharides

Ginseng polysaccharides have shown promising anti-cancer effects in a lot of studies [11,23,72]. Between 2014 and 2024, there was only one report on the anti-osteosarcoma activity of ginseng polysaccharide [73]. According to the author, polysaccharides with approximately 8 kDa molecular weight from ginseng significantly suppressed the viability of MG63 cells in a dose- and time-dependent manner. IC<sub>50</sub> value of polysaccharide was observed around 20  $\mu$ M in MG63 cells for 24 h. Interestingly, polysaccharides with  $\gamma$ -ray radiation markedly suppressed the colony formation in MG63 cells compared to radiation alone treatment. Polysaccharides were found to induce apoptosis and autophagy, which was associated with a decrease in the phosphorylation of p38 MAPK and Akt in MG63 cells.

## 2.3. Summary

As shown in Table 1, recent studies indicate that ginsenosides could be used as single agents or complementary elements in the treatment of osteosarcoma by offering potential therapeutic options or enhancing the efficacy of conventional therapies. However, there was no specific information about the anti-osteosarcoma effects of different types of phytochemicals such as alkaloids or peptides from ginseng based on the searched publication from 2014 to 2024. The research focused mainly on ginsenosides and polysaccharides, in relation to osteosarcoma. We could find two specific studies dealing with the anti-osteosarcoma activities of ginsenosides. Lu et al. developed nanoparticles with thicknesses from 2 to 3 nm by combining graphene oxide with indocyanine green, folic acid, polyethylene glycol, and Rg3 [74]. They investigated the therapeutic potential of these nanoparticles in conjunction with photodynamic therapy for treating osteosarcoma in vitro and in vivo models.

**Table 1.** Phytochemicals from ginseng with anti-osteosarcoma activity.

| No. | Phytochemicals         | Cell Model | Viability Assay  | Dose <sup>a</sup> , Time                                 | Animal Model           | Dose, Time                                  | Mechanism                                                                                                                              | Year, Ref. |
|-----|------------------------|------------|------------------|----------------------------------------------------------|------------------------|---------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|------------|
| 1   | Ginsenoside Rf         | MG63       | MTT              | 11 $\mu$ M, 24 h                                         | N/A                    | N/A                                         | Induction of apoptosis                                                                                                                 | 2014, [58] |
| 2   | Ginsenoside Rg3        | MG63       | MTT              | 150 $\mu$ M, 24 h                                        | N/A                    | N/A                                         | Induction of double-strand DNA damage                                                                                                  | 2014, [22] |
| 3   | Ginsenoside Rg3        | MG63       | CCK8             | 2.5 $\mu$ M, 24 h                                        | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration, suppression of PI3K/Akt/mTOR pathway                                                  | 2018, [59] |
| 4   | Ginsenoside Rg3        | MG63       | EDU              | 80 $\mu$ M, 24 h                                         | Xenograft mouse (MG63) | 20 mg/kg, 21 days                           | Induction of apoptosis, inhibition of migration and invasion, regulation of circ_0003074/miR-516b-5p/KPNA4 axis                        | 2021, [60] |
| 5   | Ginsenoside Rg3        | MG63       | MTT              | 80 $\mu$ M, 24 h                                         | N/A                    | N/A                                         | Inhibition of migration, suppression of Wnt/ $\beta$ -Catenin and EMT pathway                                                          | 2020, [61] |
| 6   | Ginsenoside Rg5        | MG63       | MTT              | 0.64 $\mu$ M, 24 h                                       | N/A                    | N/A                                         | Induction of apoptosis and autophagy, suppression of PI3K/Akt/mTORC1 pathway                                                           | 2021, [62] |
| 7   | Ginsenoside Rh2        | U2OS       | MTT              | 8 $\mu$ M, 48 h                                          | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration, suppression of PI3K/Akt/mTOR and NF- $\kappa$ B pathway, activation of MAPK pathway   | 2020, [64] |
| 8   | Ginsenoside Rh2        | MG63       | MTT              | 50 $\mu$ M, 36 h                                         | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration and invasion, suppression of PI3K/Akt/mTOR and EMT pathway, activation of MAPK pathway | 2021, [65] |
| 9   | Ginsenoside CK         | MG63       | MTT              | 20 $\mu$ M, 72 h                                         | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration and invasion, suppression of PI3K/Akt/mTOR pathway                                     | 2020, [66] |
| 10  | Ginsenoside Rg1        | MG63       | CCK8             | 250 $\mu$ M, 24 h with 6 $\mu$ M Timosaponin AIII        | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration, suppression of MAPK pathway                                                           | 2020, [67] |
| 11  | Ginsenoside Rb1, Rc    | MG63       | CCK8             | 250 $\mu$ M, 24 h with 6 $\mu$ M Timosaponin AIII        | N/A                    | N/A                                         | Induction of apoptosis, inhibition of migration, suppression of MAPK pathway                                                           | 2019, [68] |
| 12  | Ginsenoside Rg3        | 143B       | Colony formation | 80 $\mu$ g/mL, 7 days with 0.3125 $\mu$ g/mL doxorubicin | Xenograft mouse (143B) | 20 mg/kg, 21 days with 2 mg/kg doxorubicin  | Inhibition of migration and invasion, suppression of Akt/mTOR, HIF1 $\alpha$ /VEGF, and EMT pathway                                    | 2023, [69] |
| 13  | Ginsenoside CK         | 143B       | MTT              | 10 $\mu$ M, 72 h with 5 $\mu$ M cisplatin                | Xenograft mouse (143B) | 10 mg/kg, 28 days with 10 mg/kg doxorubicin | Induction of apoptosis                                                                                                                 | 2018, [70] |
| 14  | (20S)-Protopanaxatriol | MG63       | MTT              | 0.5 $\mu$ M, 24 h with 1 $\mu$ M RSL3                    | Xenograft mouse (MG63) | 20 mg/kg, 28 days with 20 mg/kg IKE         | Increase in sensitivity to ferroptosis, increases in ACSL4 expression                                                                  | 2024, [71] |
| 15  | Polysaccharide         | MG63       | CCK8             | 20 $\mu$ M, 24 h                                         | N/A                    | N/A                                         | Induction of apoptosis and autophagy, suppression of Ak and p38                                                                        | 2017, [73] |

Dose <sup>a</sup>: IC50 value for single treatment of ginsenoside, combinatorial effect dose for double treatment of ginsenoside and other compounds. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. CCK8: Cell counting kit 8. EDU: 5-ethynyl-2'-deoxyuridine. N/A: Not available.

When used as a single treatment, the IC50 value of ginsenosides was arranged from 0.64 to 150  $\mu$ M, which was evaluated by cell viability approaches such as MTT, CCK8, EDU, and colony formation assay. The treatment time with ginsenosides varied among studies and most experiments were performed for 24 h. MG63, U2OS, and 143B osteosarcoma cells were adapted as in vitro model systems. The main target of ginsenosides and

polysaccharides was demonstrated to be the PI3K/Akt and MAPK signaling pathway. Also, ginsenosides and polysaccharides showed anti-osteosarcoma activities such as the promotion of apoptosis and inhibition of migration. Just one study evaluated the anti-osteosarcoma activities of Rg3 in a xenograft mouse model using MG63 cells. In the case of combination treatment, five studies showed that ginsenosides enhanced the anti-osteosarcoma activity of Timosaponin AIII, cisplatin, doxorubicin, and RSL3. While these findings are promising, it is important to note that most of the research has been conducted in vitro on cell lines. Further studies, especially clinical trials, are needed to fully establish the efficacy and safety of ginsenosides or polysaccharides from ginseng as a treatment for osteosarcoma in humans.

### 3. Phytochemicals from Ginseng in Management of Osteoporosis

#### 3.1. Ginsenosides

Various ginsenosides, including Rb1, Rb2, Rc, Re, Rg1, Rg3, Rg5, Rh1, Rk1, and CK, found in ginseng, have shown promising effects in the treatment of osteoporosis [75,76]. Ginsenosides have been reported to activate the differentiation of osteoblasts and inhibit the function of osteoclasts by regulating bone formation-related signaling pathways and markers in vitro and in vivo models [75].

Zhu et al. reported that ginsenoside Rb1 attenuated aluminum chloride (AlCl<sub>3</sub>)-induced impairment in osteoblasts isolated from the skull of the rats [77]. CCK8 assay showed that the viability of osteoblast was decreased by AlCl<sub>3</sub> (0.126 mg/mL) to 10% but increased by AlCl<sub>3</sub> (0.126 mg/mL) plus ginsenoside Rb1 (0.0145 mg/mL) to 145% compared to the control. In addition, Rb1 treatment alleviated the reduced mRNA expression level of TGF- $\beta$ 1, BMP-2, IGF-I, and Cbfa1 and the increased ROS concentration caused by AlCl<sub>3</sub> in osteoblast. Another study demonstrated the anti-osteoporosis activity of Rb1 in dexamethasone (DEX)-induced osteoporosis rat model by regulating aryl hydrocarbon receptor (AHR)/proline/arginine-rich end leucine-rich repeat protein (PRELP)/NF- $\kappa$ B signaling pathway [78]. Bone density (about 170 mg/cm<sup>3</sup>) in DEX-induced osteoporosis rats was lower than that of the control rats (300 mg/cm<sup>3</sup>). Remarkably increased bone density (280 mg/cm<sup>3</sup>) was observed in DEX-induced osteoporosis rats administrated with Rb1 (6 mg/kg) for 12 weeks, which was about the similar effect by 15  $\mu$ g/kg of 17  $\beta$ -estradiol (E2). They also suggested that the upregulation of AHR by Rb1 could activate the expression of PRELP leading to inhibition of NF- $\kappa$ B pathway, which underlays the anti-osteoporotic impact of Rb1. However, another study indicated that Rb1 did not prevent bone loss in the ovariectomized (OVX) osteoporosis rat model [79]. Authors demonstrated that Rb1 (10  $\mu$ M) with osteogenic medium increased alkaline phosphatase activity (ALP) up to about 140% in mesenchymal stem cells from rat bone marrow compared to osteogenic medium. Also, Rb1 increased mineralization and the expression of osteogenic proteins, such as osteopontin, osteoprotegerin, ALP, and Runt-related transcription factor 2 (Runx2). However, administration of Rb1 (6 mg/kg) for 12 weeks could not recover the bone loss of the lumbar vertebra and femur that was decreased significantly in OVX rats. Thus, it is still necessary to acknowledge that the results of in vitro experiments may not always align with those of in vivo experiments and there might be differences in experimental results based on in vivo models.

Huang et al. investigated the anti-osteoporosis activity of ginsenoside Rb2 in the MC3T3-E1 cell model and in the ovariectomized osteoporosis mouse model [80]. Rb2 (10  $\mu$ M) treatment for 48 h nearly restored the ALP activity in MC3T3-E1 cells damaged by H<sub>2</sub>O<sub>2</sub> treatment. The administration of Rb2 (18.5  $\mu$ mol/kg) for 12 weeks in the ovariectomized mouse almost recovered bone mass and structure of the distal femur. Mechanistically, Rb2 reduced H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and the expression of bone-resorbing



factors such as receptor activators of NF- $\kappa$ B ligand (RANKL) and IL-6. Another study demonstrated that Rb2 suppressed osteoclast differentiation of RAW264.7 cells by regulating NF- $\kappa$ B and signal transducer and activator of transcription protein 3 (STAT3) signaling pathway [81]. After promoting RAW264.7 cells with RANKL, Rb2 (10  $\mu$ M) treatment effectively reduced the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells by up to 30% compared to the control. Recently, Rb2 has been reported to show anti-osteoporosis activity in a ketogenic-diet-induced osteoporosis mouse model [82]. Microstructures of cancellous bone in the distal femur were degenerated in the ketogenic-diet-induced osteoporosis mouse, which was analyzed by micro-CT. The authors demonstrated that compared to the control group, bone mineral density (BMD) was increased by 70.7% in the ketogenic-diet-induced osteoporosis mouse administrated with Rb2 (20 mg/kg) in 12 weeks.

Ginsenoside Rc has been reported to display anti-osteoporosis activity and the underlying mechanisms in vitro and in vivo [83]. Authors showed that Rc (500  $\mu$ M) treatment for 14 days increased the ALP activity in MC3T3-E1 cells up to about 260% compared to the control. They also proved that administration of Rc (50 mg/mL) for 3 months recovered the reduced BMD parameter (14%) in the OVX mouse group, which had about the same effect as 1 mg/kg of E2. Rc enhanced MC3T3-E1 cell development by activating the Wnt/ $\beta$ -catenin signaling cascade. Wang et al. published the anti-osteoporosis result using Rc in the primary osteoblasts cells from human skulls and OVX rats [84]. ALP activity in the primary osteoblasts cell was increased up to 200% by Rc (10  $\mu$ M) treatment. The BMD parameter in OVX rats was apparently reduced to the control group, while Rc (20 mg/mL) or E2 (2  $\mu$ g/kg) administration for 4 weeks rescued the decreased BMD parameter of femurs up to 122 and 125% in the OVX rats. In addition, Western blotting results revealed that Rc administration upregulated the TGF- $\beta$ /Smad signaling pathway in femur tissues of OVX rats.

Ginsenoside Re promoted osteoblast differentiation in MC3T3-E1 cells and zebrafish [85]. According to the study, Re increased ALP activity in MC3T3-E1 cells in a dose-dependent manner. ALP activity was stimulated up to 200% and 350% by re-treatment at concentrations of 50 and 100  $\mu$ M for 14 days. The zebrafish scales exposed to ginsenoside Re (50  $\mu$ M) for 35 days exhibited elevated calcium levels, which were analyzed by the Alizarin Red S staining. The strength of Alizarin Red S staining was 2.4 times greater than that observed in the untreated control zebrafish scales. In another study, the same research group reported that ginsenoside Re inhibited osteoclast differentiation in macrophages from mouse bone marrow and zebrafish [86]. Re-treatment significantly decreased TRAP activity in macrophages from mouse bone marrow in a dose-dependent manner. At concentrations of 5  $\mu$ M, ginsenoside Re decreased TRAP activity by approximately 33% compared to the untreated control group. Re-treatment (5  $\mu$ M) for 35 days resulted in a 63% decrease in TRAP intensity compared to the control zebrafish scale. Western blotting analysis showed that re-treatment induced the downregulation of NFATc1, c-Fos, and pErk and suppression of NF- $\kappa$ B pathway in macrophage from mouse bone marrow.

Gu et al. reported that ginsenoside Rg1 induced osteogenic differentiation of mesenchymal stem cells from rat bone marrow and accelerated the healing of rat tibial fractures [87]. The ALP activity in Rg1 (1  $\mu$ M) treated mesenchymal stem cells was about 2-fold higher compared to the control cells. Micro-CT analysis revealed that BMD was significantly increased in the Rg1 treatment group in time time-dependent manner. Unfortunately, they did not perform a quantitative analysis of the micro-CT results. Mechanistically, Rg1 stimulated the BMP-2/Smad signaling pathway by regulating the translocation of the glucocorticoid receptor (GR) receptor into the nucleus. Chen et al. studied the anti-osteoporosis activity of Rg1 in osteoprogenitor cells from rat femur and high glucose-induced diabetic

osteoporosis (Goto-Kakizaki) rat model [88]. The analysis of ALP and Alizarin Red S staining assay showed that Rg1 treatment (164.8  $\mu$ M) for 7 days induced the differentiation of the isolated osteoprogenitor cells from rat femur into osteoblast. Although the treatment dose seemed relatively higher than those used in other studies, Rg1 increased the strength of ALP and Alizarin Red S staining up to 300 and 500-fold compared to the control, respectively. Quantitative micro-CT analysis also showed that Rg1 (10 mg/kg) administration for 12 weeks increased BMD by about 1.5-fold in diabetic osteoporosis (Goto-Kakizaki) rats compared with the control group. Authors suggested that Rg1 could facilitate the coupling between bone formation and blood vessel growth by stimulating osteoprogenitor cells exposed to high glucose conditions to release vascular endothelial growth factor (VEGF), thereby enhancing angiogenesis. Jiang et al. reported that Rg1 enhanced osteogenesis in MC3T3-E1 and DEX-induced osteoporosis zebrafish by modulating the G protein-coupled estrogen receptor (GPER)/PI3K/AKT pathway [89]. When compared to the DEX-treated MC3TC-E1 cells, the Rg1-treated MC3TC-E1 cells (50  $\mu$ M, 7 days) displayed an approximately 3-fold increase in ALP activity, which was a similar effect of E2 treatment (100 nM). The number of Alizarin Red S staining positive vertebrae of DEX-induced osteoporosis zebrafish was increased by about 2.5-fold with 50  $\mu$ M of Rg1 treatment for 10 days. Authors proposed that Rg1 might regulate osteogenesis by mediating GPER expression within the PI3K/AKT signaling pathway in glucocorticoid-induced osteoporosis.

Ginsenoside Rg3 inhibited osteoclast differentiation in RAW264.7 cells via suppression of NF- $\kappa$ B and MAPK signaling pathways [90]. Rg3 (10  $\mu$ M) treatment for 7 days reduced RANKL-induced activation of TRAP activity up to about 20% in RAW264.7 cells. Authors indicated that Rg3 inhibited the RANKL-induced activation of NF- $\kappa$ B and MAPK signaling pathways in RAW264.7 cells. Also, they showed that Rg3 modulated cathepsin K, an important enzyme in bone resorption signaling cascades by *in silico* molecular docking interaction study. The study combined *in silico* and *in vitro* approaches to elucidate the mechanisms of the working mechanism of Rg3. Zhang et al. reported that Rg3 effectively mitigated glucocorticoid-induced osteoporosis through modulation of the bone morphogenic protein-2 (BMP-2)/BMP receptor 1A (BMPR1A)/Runx2 signaling pathway [91]. In primary osteoblasts from rat skulls, DEX substantially reduced ALP activity, while treatment of Rg3 (10  $\mu$ M) for 14 days notably enhanced ALP activity by about 2-fold compared to the DEX-treated cells. Interestingly, the author showed that high doses (20 and 100  $\mu$ M) of Rg3 caused osteoblasts cell death. Using the DEX-induced osteoporosis rat model, the author demonstrated that Rg3 (20 mg/kg) administration for 5 weeks recovered DEX-induced BMD reduction by about 1.4-fold compared to the DEX-treated rat group. Song et al. reported that Rg3 had a protective effect against AlCl<sub>3</sub>-induced osteoporosis in rats by regulation of oxidative stress [92]. X-ray image analysis showed the BMD (about 210 mg/cm<sup>2</sup>) of femoral metaphysis was higher in the Rg3 administrated rat group (20 mg/kg for 1 month) than that (about 180 mg/cm<sup>2</sup>) of the AlCl<sub>3</sub> group. Rg3 reduced malondialdehyde and reactive oxygen species (ROS) levels but increased glutathione peroxidase and superoxide dismutase activity of femora in the Rg3 administrated rat group compared to the AlCl<sub>3</sub> group. Zhang et al. explored the anti-osteoporosis effect of Rg3 in MC3T3-E1 cells and ovariectomy-induced osteoporosis rats via promoting autophagy and the AMPK/mTOR signaling pathway [93]. Rg3 treatment (20  $\mu$ M) for 5 days was found to increase ALP activity in MC3T3-E1 cells by about 3-fold compared to the control. These effects were reversed by Compound C (an AMPK inhibitor). Rg3 administration (20 mg/kg) for 5 weeks recovered BMD declines by 2-fold in ovariectomy-induced osteoporosis rats. Song et al. demonstrated that Rg3 attenuated AlCl<sub>3</sub>-induced bone impairment both *in vivo* and *in vitro* by modulating the TGF- $\beta$ 1/Smad signaling pathway [94]. Rg3 treatment (10  $\mu$ M) for 24 h resulted in a significant increase (about 50%) in the ALP activity

of MC3T3-E1 cells compared to AlCl<sub>3</sub>-treated cells. The administration of Rg3 (20 mg/mL) for 1 month reduced the cell apoptosis and histologically impaired modifications caused by AlCl<sub>3</sub> in the femurs of rats. Unfortunately, authors did not provide quantitative analysis such as measurement of BMD by CT to monitor bone healing progress.

Siddiqi et al. reported the anti-osteoporotic properties of the mixture of ginsenosides Rg5 and Rk1 in MC3T3-E1 cells [95]. The mechanism of action of the Rg5 and Rk1 mixture appeared to involve the activation of BMP-2 and Runx2 pathways. The treatment of Rg5 and Rk1 mixture (20 µg/mL) increased ALP activity (1.8-fold) and Alizarin Red S staining density (2.5-fold) in MC3T3-E1 cells for 12 days. The same research group reported that Ginsenoside Rh1 promoted osteoblast differentiation in MC3T3-E1 cells [96]. Similar to the mechanism of action of the Rg5 and Rk1 mixture, the effects of Rh1 were mediated through the BMP-2 and Runx2 signaling pathways. Rh1 treatment (10 µM) for 12 days increased ALP activity by 1.5-fold in MC3T3-E1 cells.

Ginsenoside compound K (CK) could be a potential therapeutic agent for treating fractures by promoting osteogenesis [97]. Alizarin Red S staining analysis showed that CK treatment (10 µM) for 14 days increased the formation of calcium deposits in bone marrow mesenchymal stem cells from rats. In the rat femoral fracture model, micro-CT results showed that CK administration (500 µM) for 4 weeks increased BMD averagely by 1.5-fold. The mechanism of action involved the activation of β-catenin and Runx2 in fracture calluses. Interestingly, authors suggested that CK increased angiogenesis around the fracture callus by the formation of H-type vessels. In another study, CK attenuated RANKL-mediated osteoclast differentiation in RAW264.7 cells and OVX-induced osteoporosis in a mouse model [98]. TRAP staining analysis showed that CK treatment (10 µM) for 5 days reduced the number of multinucleated RAW264.7 cells by about 50% compared to the RANKL-treated control cells. Authors indicated that CK suppressed the phosphorylation of NF-κB p65 responsible for the activation of RAW264.7 cells. In vivo experiments confirmed that CK administration (10 mg/mL) for 8 weeks increased OVX-induced BMD loss in rats by about 2-fold compared to the OVX group through alleviating oxidative stress in bone tissue.

### 3.2. Extracts

Recent research findings have demonstrated that not only ginsenosides but also ginseng extract show potential in treating osteoporosis. In fact, recent studies highlight the multifaceted approach of ginseng extract in protecting bone health, addressing both the enhancement of bone formation and the suppression of bone resorption in various experimental models.

Kim et al. investigated the potential of Korean Red Ginseng extract as a natural therapeutic agent to prevent or treat DEX-induced osteoporosis both in vitro and in vivo [99]. MC3T3-E1 cells exposed to DEX exhibited reduced ALP activity. However, when cells were treated with both DEX and Korean Red Ginseng extract (125 µg/mL) for 7 days, ALP activity was increased by 1.8-fold compared to the DEX-treated cells. Interestingly, the treatment of a higher dose of Korean Red Ginseng extract (250 and 500 µg/mL) decreased the ALP activity compared to the Korean Red Ginseng extract (125 µg/mL) treated cells. Micro-CT image analysis showed that DEX mice exhibited a decrease in trabecular BMD within the femur. In contrast, mice administrated with Korean Red Ginseng treatment (500 mg/mL) for 5 weeks demonstrated a notably increased BMD. The authors did not provide the present quantitative analysis results of the micro-CT image. Lee et al. reported that ginseng extract and ginsenoside Re could be promising natural agents for preventing osteoporosis, especially in cases of estrogen deficiency [100]. The treatment of ginseng extract (50 µg/mL) for 5 days inhibited RANKL-induced TRAP activity by 30% in RAW264.7 cells compared to the RANKL-treated cells, which seemed to have a

similar effect of E2 (10 nM). HPLC analysis showed that the ginseng extract contained various ginsenosides. Among them, ginsenoside Re was shown to be a major component of the extract to suppress osteoclast differentiation in RAW264.7 cells. In vivo studies using OVX-induced osteoporosis rat models demonstrated administration of ginseng extract (300 mg/kg) for 8 weeks increased BMD by about 25% compared to the OVX rats. Another study demonstrated that ginseng leaf extract attenuated RANKL-induced osteoclast differentiation in vitro by downregulation of high mobility group box-1 (HMGB1) and upregulation of (nuclear factor-erythroid 2-related factor 2) Nrf2 and heme oxygenase-1 (HO-1) [101]. Although the authors did not provide the quantitative results, TRAP staining analysis showed that the treatment of ginseng leaf extract (1 mg/mL) for 5 days with RANKL decreased TRAP-positive RAW264.7 cell number as much as the vehicle control. Seo et al. discussed the effects of ginseng-derived extracellular nanovesicles on osteoclast differentiation [102]. Ginseng-derived extracellular nanovesicles were isolated using sucrose gradient ultracentrifugation and showed about 71.42 nm of diameter. Ginseng-derived extracellular nanovesicles contained high levels of ginsenoside Rb1 and Rg1. The treatment of ginseng-derived extracellular nanovesicles (1 µg/mL) for 4 days exhibited about 20% fewer TRAP-positive macrophages from mouse bone marrow than the RANKL control group. In vivo, studies using lipopolysaccharide-induced osteoporosis mouse models demonstrated ginseng-derived extracellular nanovesicles administration (1 mg/mL, 7 days) recovered almost the reduced BMD in lipopolysaccharide-induced osteoporosis mouse model. Mechanistically, ginseng-derived extracellular nanovesicles caused attenuation of the expression level of pIκBα, pJNK, pp38, and pERK activated by RANKL in mouse bone marrow-derived macrophage.

### 3.3. Summary

As shown in Table 2, recent studies reveal that various ginsenosides and ginseng extracts were explored to have anti-osteoporosis activity by regulating signaling pathways involved in activating osteoblast or inhibiting osteoclast using several kinds of in vitro and in vivo models. Similar to the research results on the anti-osteosarcoma activity of phytochemicals from ginseng, there was no report about the anti-osteoporosis activity of alkaloids or peptides from ginseng based on the search results from 2014 to 2024. There were five publications on the anti-osteoporosis activity of Rg3. Rg3 is the most extensively studied compound in the field of osteoporosis, which makes it a compelling subject for continued investigation in the field of bone health and osteoporosis treatment. For example, Siddiqi et al. reported that fermented ginseng extraction to enrich Rg3 displayed pro-osteoblastic activity in MC3T3-E1 cells [103]. Fermented ginseng extraction treatment increased ALP activity and the expression of type-1 collagen. The authors showed that after fermentation, the levels of Rh1 (11%) and Rg2 (16%) slightly increased, while Rg3 (33%) significantly increased. The in vitro effects of ginsenosides may not directly correlate with their in vivo efficacy in treating osteoporosis. For instance, Rb1 showed promise in promoting MSC differentiation into osteoblasts in vitro but it did not translate to significant bone preservation in the OVX rat model [79]. There is an interesting study about the identification of novel targets involved in treating osteoporosis by ginsenoside CK using a network pharmacology approach [104]. Authors showed that CK interacted with c-Fms protein, stabilized by hydrogen bonds and hydrophobic interactions. Authors suggested that CK could interfere with osteoporosis progression through the c-Fms-mediated MAPK and PI3K signaling axis that regulates osteoclast differentiation.

**Table 2.** Phytochemicals from ginseng with anti-osteoporosis activity.

| No. | Phytochemicals  | Cell Model                                  | Assay <sup>a</sup> | Dose <sup>b</sup> , Time | Animal Model                                    | Dose, Time                  | Mechanism                                                                                                                             | Year, Ref. |
|-----|-----------------|---------------------------------------------|--------------------|--------------------------|-------------------------------------------------|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------|------------|
| 1   | Ginsenoside Rb1 | Osteoblasts from rat skull                  | CCK8               | 0.0145 mg/mL, 24 h       | N/A                                             | N/A                         | Increase in osteoblasts viability, upregulation of TGF- $\beta$ 1, BMP-2, IGF-I, and Cbf $\alpha$ 1, inhibition of oxidative stress   | 2016, [77] |
| 2   | Ginsenoside Rb1 | Osteoblasts from rat skull                  | ALP                | 0.0145 mg/mL, N/A        | Glucocorticoid-induced osteoporosis rats        | 6 mg/kg, 12 weeks           | Induction of osteoblasts differentiation, regulation of AHR/PRELP/NF- $\kappa$ B pathway                                              | 2022, [78] |
| 3   | Ginsenoside Rb1 | Mesenchymal stem cells from rat bone marrow | ALP                | 10 $\mu$ M, 7 days       | Ovariectomized osteoporosis rats                | 6 mg/kg, 12 weeks           | Induction of osteoblast differentiation, inhibition of oxidative stress                                                               | 2018, [79] |
| 4   | Ginsenoside Rb2 | MC3T3-E1                                    | ALP                | 10 $\mu$ M, 48 h         | Ovariectomized osteoporosis mice                | 18.5 $\mu$ mol/kg, 12 weeks | Increase in osteoblasts viability, induction of osteoblasts differentiation, inhibition of oxidative stress                           | 2014, [80] |
| 5   | Ginsenoside Rb2 | RAW264.7                                    | TRAP               | 10 $\mu$ M, 72 h         | N/A                                             | N/A                         | Suppression of osteoclast differentiation, suppression of NF- $\kappa$ B and STAT3 pathways                                           | 2017, [81] |
| 6   | Ginsenoside Rb2 | N/A                                         | N/A                | N/A                      | Ketogenic-diet-induced osteoporosis mice        | 20 mg/kg, 12 weeks          | Increase in osteoblast activity, reduction in osteoclast activity                                                                     | 2021, [82] |
| 7   | Ginsenoside Rc  | MC3T3-E1                                    | ALP                | 200 $\mu$ M, 14 days     | Ovariectomized osteoporosis mice                | 50 mg/kg, 3 months          | Induction of osteoblasts differentiation, activation of Wnt/ $\beta$ -catenin                                                         | 2022, [83] |
| 8   | Ginsenoside Rc  | Osteoblasts from human skull                | ALP                | 10 $\mu$ M, N/A          | Ovariectomized osteoporosis rats                | 20 mg/kg, 4 weeks           | Induction of osteoblasts differentiation, activation of TGF- $\beta$ /Smad pathway                                                    | 2024, [84] |
| 9   | Ginsenoside Re  | MC3T3-E1                                    | ALP                | 50 $\mu$ M, 14 days      | Zebrafish                                       | 50 $\mu$ M, 5 weeks         | Induction of osteoblasts differentiation                                                                                              | 2016, [85] |
| 10  | Ginsenoside Re  | Macrophages from mouse bone marrow          | TRAP               | 5 $\mu$ M, 72 h          | Zebrafish                                       | 5 $\mu$ M, 5 weeks          | Suppression of osteoclast differentiation, downregulation of NFATc1, c-Fos, and pErk, suppression of NF- $\kappa$ B pathway           | 2016, [86] |
| 11  | Ginsenoside Rg1 | Mesenchymal stem cells from rat bone marrow | ALP                | 1 $\mu$ M, 14 days       | Tibial fractured rats                           | 20 mg/kg, 3 weeks           | Induction of osteoblasts differentiation, activation of BMP-2/Smad pathway                                                            | 2016, [87] |
| 12  | Ginsenoside Rg1 | Osteoprogenitors from rat femur             | ALP                | 164.8 $\mu$ M, 7 days    | Goto-Kakizaki type 2 diabete rats               | 10 mg/kg, 12 weeks          | Induction of osteoblasts differentiation, activation of angiogenesis                                                                  | 2022, [88] |
| 13  | Ginsenoside Rg1 | MC3T3-E1                                    | ALP                | 50 $\mu$ M, 7 days       | Glucocorticoid-induced osteoporosis Zebrafishes | 50 $\mu$ M, 10 days         | Induction of osteoblasts differentiation, activation of GPER/PI3K/Akt pathway                                                         | 2024, [89] |
| 14  | Ginsenoside Rg3 | RAW264.7                                    | TRAP               | 10 $\mu$ M, 7 days       | N/A                                             | N/A                         | Suppression of osteoclast differentiation, suppression of NF- $\kappa$ B and MAPK pathways                                            | 2015, [90] |
| 15  | Ginsenoside Rg3 | Osteoblasts from rat skull                  | ALP                | 10 $\mu$ M, 14 days      | Glucocorticoid-induced osteoporosis rats        | 20 mg/kg, 5 weeks           | Induction of osteoblasts differentiation, activation of BMP-2/BMPRII/Runx2 pathway                                                    | 2016, [91] |
| 16  | Ginsenoside Rg3 | N/A                                         | N/A                | N/A                      | AlCl3-induced osteoporosis rats                 | 20 mg/kg, 1 month           | Increase in osteoblast growth factors, inhibition of oxidative stress                                                                 | 2020, [92] |
| 17  | Ginsenoside Rg3 | MC3T3-E1                                    | ALP                | 20 $\mu$ M, 5 days       | Ovariectomized osteoporosis rats                | 20 mg/kg, 5 weeks           | Induction of osteoblasts differentiation, activation of osteoblast autophagy, activation of AMPK pathway, suppression of mTOR pathway | 2020, [93] |
| 18  | Ginsenoside Rg3 | MC3T3-E1                                    | ALP                | 10 $\mu$ M, 24 h         | AlCl3-induced osteoporosis rats                 | 20 mg/kg, 1 month           | Induction of osteoblasts differentiation, inhibition of osteoblast apoptosis, activation of TGF- $\beta$ 1/Smad pathway               | 2021, [94] |



Table 2. Cont.

| No. | Phytochemicals                                       | Cell Model                                  | Assay <sup>a</sup> | Dose <sup>b</sup> , Time | Animal Model                                 | Dose, Time         | Mechanism                                                                                                                         | Year, Ref.  |
|-----|------------------------------------------------------|---------------------------------------------|--------------------|--------------------------|----------------------------------------------|--------------------|-----------------------------------------------------------------------------------------------------------------------------------|-------------|
| 19  | Ginsenosides Rg5 + Ginsenosides Rk1                  | MC3T3-E1                                    | ALP                | 20 µg/mL, 12 days        | N/A                                          | N/A                | Induction of osteoblasts differentiation, activation of BMP-2/Runx2 pathway                                                       | 2014, [95]  |
| 20  | Ginsenoside Rh1                                      | MC3T3-E1                                    | ALP                | 10 µM, 12 days           | N/A                                          | N/A                | Induction of osteoblasts differentiation, inhibition of oxidative stress, activation of BMP-2/Runx2 pathway                       | 2014, [96]  |
| 21  | Ginsenoside CK                                       | Mesenchymal stem cells from rat bone marrow | Alizarin Red S     | 10 µM, 14 days           | Femoral fractured rats                       | 500 µM, 4 weeks    | Induction of osteoblasts differentiation, upregulation of $\beta$ -catenin and Runx2                                              | 2022, [97]  |
| 22  | Ginsenoside CK                                       | RAW264.7                                    | TRAP               | 10 µM, 5 days            | Ovariectomized osteoporosis mice             | 10 mg/kg, 8 weeks  | Suppression of osteoclast differentiation, inhibition of oxidative stress, suppression of NF- $\kappa$ B pathway                  | 2023, [98]  |
| 23  | Red ginseng extract                                  | MC3T3-E1                                    | ALP                | 125 µg/mL, 7 days        | Glucocorticoid-induced osteoporosis mice     | 500 mg/kg, 5 weeks | Induction of osteoblasts differentiation, upregulation of pAkt, downregulation of pJNK                                            | 2015, [99]  |
| 24  | Ginseng extract                                      | RAW264.7                                    | TRAP               | 50 µg/mL, 5 days         | Ovariectomized osteoporosis rats             | 300 mg/kg, 8 weeks | Suppression of osteoclast differentiation                                                                                         | 2015, [100] |
| 25  | Ginseng leaf extract                                 | RAW264.7                                    | TRAP               | 1 mg/mL, 5 days          | N/A                                          | N/A                | Suppression of osteoclast differentiation, inhibition of oxidative stress, downregulation of HMGB1, upregulation of Nrf2 and HO-1 | 2024, [101] |
| 26  | Ginseng-derived ginsenosides containing nanovesicles | Macrophages from mouse bone marrow          | TRAP               | 1 µg/mL, 4 days          | Lipopolysaccharide-induced osteoporosis mice | 1 mg/kg, 1 week    | Suppression of osteoclast differentiation, suppression of NF- $\kappa$ B and MAPK pathways                                        | 2023, [102] |

Assay <sup>a</sup>: Specific kinds of basic assay for the anti-osteoporosis ability. Dose <sup>b</sup>: Most effective dose of each ginsenoside and extract in cell model assay. ALP: Alkaline phosphatase. TRAP: Tartrate-resistant acid phosphatase. CCK8: Cell counting kit 8. N/A: Not available.

In vitro assay model, the most effective dose of ginsenosides ranged from 1 to 200 µM, which was evaluated by either ALP or TRAP assays. ALP and TRAP assays are two different kinds of techniques used in bone research, each serving a distinct purpose. ALP targets osteoblast activity (bone formation), while TRAP targets osteoclast activity (bone resorption) [105]. In the case of ginseng extract, the most effective dose was from 50 µg/mL to 1 mg/mL. The treatment time with ginsenosides varied among studies and most experiments were performed for 7 or 14 days. Both ALP and TRAP assays were used to assess the rate of differentiation to osteoblast or osteoclast cells and it requires several days to weeks to accumulate sufficient levels of enzymatic products necessary to induce cell differentiation. Many studies adapted MC3T3-E1 osteoblast or RAW264.7 osteoclast cells as an in vitro model system as shown in Table 2. The main target signaling pathways of ginsenosides and ginseng extract involved Wnt/ $\beta$ -catenin, TGF- $\beta$ /Smad, BMP-2/Smad, BMP-2/Runx2, PI3K/Akt for induction of osteoblasts differentiation and NF- $\kappa$ B, STAT3, and MAPK for suppression of osteoclast differentiation. Also, ginsenosides and ginseng extract showed anti-osteoporosis activities through regulation of osteoblast or osteoclast and suppression of oxidative stress. There were several kinds of in vivo models such as glucocorticoid-induced osteoporosis animals (mice, rats, and zebrafish), ovariectomized osteoporosis animals (mice and rats), AlCl<sub>3</sub>-induced osteoporosis rats, lipopolysaccharide-induced osteoporosis mice, ketogenic-diet-induced osteoporosis mice, tibial fractured rats, and femoral fractured rats. After inducing osteoporosis primarily through compounds or physical damage, the anti-osteoporotic effects were examined by orally administering specific ginsenosides or ginseng extracts and applying various osteoporosis recovery methods. Although it varied among studies, researchers primarily administered ginsenosides at a dose of 20 mg/kg

for up to 12 weeks. In most vivo studies, BMD (Bone Mineral Density) was presented and analyzed as the most fundamental anti-osteoporosis indicator. Additionally, some studies also showed results using other indicators such as BV/TV (Bone Volume/Total Volume), Tb.N (Trabecular Number), Tb.Sp (Trabecular Separation), and SMI (Structure Model Index). While these findings are promising, it is still important to note that most of the research has been conducted in vitro on cell lines and in vivo models. Further studies, especially clinical trials, are needed to fully establish the efficacy and safety of ginsenosides or ginseng extract as a treatment for osteoporosis in humans.

## 4. Phytochemicals from Ginseng in Management of Osteoarthritis

### 4.1. Ginsenosides

Phytochemicals from ginseng have demonstrated significant effects on rheumatic diseases including osteoarthritis, offering promising potential as therapeutic agents [75,106,107]. Especially, recent studies discussed how ginsenoside Rb1, Rg1, Rg3, Rg5, Ro, and CK reduced inflammation and exhibited potential as therapeutic candidates for osteoarthritis management.

Ginsenoside Rb1 was demonstrated to show potential anti-inflammatory effects in osteoarthritis treatment [108]. According to the study, Rb1 treatment (80  $\mu$ M) for 24 h decreased IL-1 $\beta$ -induced NO production by 46% compared to the IL-1 $\beta$  treated SW1353 cells, a kind of human chondrosarcoma cell lines. Rb1 inhibited the protein expression of inducible nitric oxide synthase (iNOS), a key enzyme in NO production. Rb1 suppressed NF- $\kappa$ B activation through increased I $\kappa$ B $\alpha$  protein content, reduced nuclear translocation of p65 protein, and decreased DNA binding activity of NF- $\kappa$ B. In another study, Rb1 showed promising effects in inhibiting MMP-13 expression and promoting type II collagen expression through regulation of the Notch signaling pathway in osteoarthritis [109]. Treatment of SW1353 cells with Rb1 (80  $\mu$ M) for 24 h reduced IL-1 $\beta$ -induced protein expression of MMP-13 up to about 70% compared to the cells only treated with IL-1 $\beta$ . In the anterior cruciate ligament transection (ACLT) osteoarthritis rat model, Rb1 treatment (80  $\mu$ M) for 6 weeks ameliorated cartilage degradation and improved histological scores by about 50%. Similarly, Chen et al. reported that Rb1 treatment reduced IL-1 $\beta$  levels, attenuated cartilage degeneration, and lowered histologic damage scores by suppressing MMP-13 and type X collagen expression in C5.18 cells (rat chondrocyte) and rat osteoarthritis model combining ACLT and medial cruciate ligament transection (MCLT) [110]. Aravinthan et al. reported that Rb1 displayed anti-inflammatory and chondro-protective effects in monoiodoacetate (MIA)-induced and ovariectomized osteoarthritis rat model [111]. X-ray radiography analysis revealed that Rb1 (10  $\mu$ g/kg) administration for 4 weeks decreased knee joint thickness by about 70% compared to the control osteoarthritis rat groups. The authors showed that the mRNA expression of MMP-13 in chondrocytes from the Rb1 (10  $\mu$ g/kg) administration group was about half of the control osteoarthritis group. Luan et al. reported the therapeutic potential of Rb1 in attenuating MIA-induced osteoarthritis by modulating the miR-21-5p/FGF18 axis [112]. Rb1 (10  $\mu$ M) treatment for 24 h reduced miR-21-5p-mediated inhibition of FGF18, which was associated with activation of matrix synthesis and cell proliferation in chondrocytes from healthy rats. Similarly, in MIA-induced osteoarthritis rat models, Rb1 (10 mg/kg) administration for 2 weeks improved joint histology, suppressed pro-inflammatory cytokines (e.g., IL-1 $\beta$  and IL-6), and enhanced chondrocyte viability. Mohammad et al. reported a protective effect of Rb1 against osteoarthritis pathogenesis-induced hollow trephine on the femur trochlea of rabbits by down-regulation of p-Akt, p-P38, and p-P65, which were associated with inflammatory processes and cartilage degradation in osteoarthritis [113]. The study utilized a rabbit knee osteoarthritis model, where hydrogel-based Rb1 sheets were inserted into the knee joint. Hydrogel-based

Rb1 (100 µg/kg) administration exhibited dose-dependent chondroprotective effects, as evidenced by macroscopic and micro-CT investigations.

Another ginsenoside Rg1 was reported to show protective effects against IL-1 $\beta$ -induced chondrocyte apoptosis by modulating the PI3K/Akt signaling pathway [114]. Rg1 treatment (10 µg/mL) for 72 h increased the cell viability of chondrocytes from healthy rats by about two-fold compared to the IL-1 $\beta$  treated group. Rg1 suppressed chondrocyte apoptosis by upregulation of pAkt, Bcl2, and TIMP-1 and downregulation of Bax and MMP-13. Wendan et al. reported that Rg1 prevented osteoarthritis pathogenesis by targeting inflammatory, apoptotic, and ECM-degrading pathways in human chondrocytes and rat ACLT models [115]. Rg1 (10 µg/mL) treatment for 24 h suppressed IL-1 $\beta$ -induced mRNA expression of MMP-13 and COX-2 by 7.5- and 2.2-fold, respectively, in human chondrocytes from osteoarthritis patients with knee replacement. Oral administration of Rg1 (60 mg/kg for 12 weeks) reduced cartilage erosion, collagen II loss, and MMP-13 levels. Zhiqiang et al. reported that Rg1 exerted protective effects against IL-1 $\beta$ -induced apoptosis in primary human chondrocytes [116]. Rg1 (100 µg/mL) treatment for 72 h restored the proliferation of primary human chondrocytes inhibited by IL-1 $\beta$  by about 9-fold. Rg1 treatment reduced ROS and MDA production. Rg1 treatment also downregulated Bax expression but upregulated Bcl2 expression. Huaisen et al. reported the therapeutic potential of Rg1 in countering thiram-induced osteoarthritis in chickens by modulating apoptosis and angiogenesis [117]. The cell viability of primary chicken chondrocytes was decreased to 75% by thiram exposure. Rg1 treatment (40 µM) for 24 h recovered the cell viability by about 95% compared to the control group without thiram and Rg1. Rg1 administration (40 mg/kg) for 18 days reduced tibial dyschondroplasia severity, improved growth plate structure, and restored cartilage matrix integrity in thiram-induced osteoarthritis chickens. Yanwei et al. reported that the combined therapy of Rg1 and adipose-derived stem cells embedded in a hyaluronic acid matrix displayed the anti-osteoarthritic efficacy in papain-induced osteoarthritis rabbits model [118]. Rg1 treatment (40 µM) for 5 days increased the proliferation by about 10% in chondrocytes differentiated from healthy rabbit adipose stem cells. The administration 1:1 (V:V) mixture of hyaluronic acid and adipose stem cells ( $2 \times 10^7$ /mL) suspension in 40 µM of Rg1 for 9 days reduced papain-induced osteoarthritis severity of rabbit model, which was evidenced by histological (scanning electron microscopy) improvements in cartilage thickness. Rg1 upregulated the expression of type II collagen and TIMP-1 but downregulated the expression of MMP-13.

Ginsenosides Rg3 treatment (20 µM) for 24 h reduced MMP-13 expression in IL-1 $\beta$ -stimulated SW1353 human chondrocytes by 58% compared to the IL-1 $\beta$  treated control [119]. In addition, Rg3 (2 µM) treatment for 3 days inhibited glycosaminoglycan release in IL-1 $\beta$ -stimulated rabbit cartilage cultures by 12.6% compared to the IL-1 $\beta$  treated control. In another study, Ching-Hou et al. reported a more detailed working mechanism of Rg3 in chondrocyte protection [120]. In TNF- $\alpha$ -stimulated human chondrocyte TC28a2, Rg3 (20 µM) treatment for 24 h upregulated SIRT1 expression by about 2-fold, which activates the SIRT1/PGC-1 $\alpha$ /SIRT3 signaling axis. This pathway mitigated mitochondrial dysfunction by reducing ROS accumulation. The resultant decrease in ROS attenuated caspase-dependent apoptosis and suppresses pro-inflammatory mediators such as IL-8 and MMP-9. Additionally, Rg3 inhibited TNF- $\alpha$ -induced NF- $\kappa$ B nuclear translocation by down-regulating p38 MAPK phosphorylation.

Ginsenoside Rg5 showed the chondroprotective effects in chondrocytes from healthy rats and the MCLT osteoarthritis rat model by inhibiting chondrocyte apoptosis and preserving cartilage matrix integrity through modulation of inflammatory pathways [121]. Rg5 (15 µM) treatment for 48 h reduced apoptosis of chondrocytes from healthy rats by 98% compared to the IL-1 $\beta$  treated control cells. Rg5 (15 mg/kg) administration for 1 month de-

creased apoptosis of chondrocytes in MCLT osteoarthritis rats by 98% compared to control rats, leading to prevention of synovial disintegration and preserving joint functionality.

Ginsenoside CK was demonstrated to exert cartilage-protective effects in chondrocytes from osteoarthritis patients through the regulation of IL-1 $\beta$ -induced MAPK pathways [122]. CK (5  $\mu$ M) treatment for 24 h suppressed IL-1 $\beta$ -driven NO production by 95.8% compared to IL-1 $\beta$ -treated control chondrocytes. CK treatment reduced IL-1 $\beta$ -induced iNOS, MMP-13, pJNK, pp38, and pErk expression and inhibited glycosaminoglycan release in chondrocytes from osteoarthritis patients. Yuguo et al. reported the therapeutic potential of CK in osteoarthritis by targeting NLRP3 inflammasome-mediated pyroptosis [123]. CCK8 assay using primary mouse chondrocytes revealed that TNF- $\alpha$  reduced cell viability by up to 50% compared to the control cell but CK (5  $\mu$ M) treatment for 48 h restored cell viability by about 75%. Mechanistically, CK suppressed NLRP3 inflammasome activation by reducing TNF- $\alpha$ -induced upregulation of NLRP3. In the MCLT osteoarthritis mouse model, CK administration (40 mg/kg) for 8 weeks decreased Osteoarthritis Research Society International (OARSI) scores from 20 to 15. CK also reduced cartilage degradation and normalized collagen II/MMP13 expression ratios in MCLT osteoarthritis mouse. Similarly with this study, Yicheng et al. reported that CK exhibited anti-osteoarthritis efficacy by regulating pyroptosis through targeting endoplasmic reticulum stress-mediated activation of the IRE1 $\alpha$ -TXNIP-NLRP3 axis [124]. In IL-1 $\beta$ -stimulated chondrocytes, CK (30 nM) treatment for 24 h reduced the protein expression of MMP-13 by about 50%. In monoiodoacetate-induced rat osteoarthritis models, CK administration (80 mg/kg) for 7 weeks decreased the OARSI and Mankin scores by about 45%. CK inhibited IRE1 $\alpha$  phosphorylation, reducing thioredoxin-interacting protein (TXNIP) translocation to NLRP3 and subsequent inflammasome assembly. This suppression was reversed by the endoplasmic reticulum stress inducer tunicamycin, confirming the dependency of CK on endoplasmic reticulum stress modulation.

#### 4.2. Summary

As shown in Table 3, recent studies demonstrated the anti-osteoarthritic activity of various kinds of ginsenosides through modulation of inflammatory pathways and cartilage protection in both in vitro and in vivo models. There was no report about the anti-osteoarthritic activity of alkaloids or peptides from ginseng based on the search results from 2014 to 2024. During this period, researchers reported 17 publications. Rb1 and Rg3 are the most extensively studied ginsenosides in the field of osteoporosis. There were six and five publications on the anti-osteoporosis activity of Rb1 and Rg1, respectively. Ginsenosides Rb1 and Rg3 are structurally related ginsenosides derived from ginseng but they exhibit distinct features in their glycosylation patterns. Both share a dammarane-type tetracyclic triterpenoid backbone (protopanaxadiol). Both have two glucose units attached at C3 via a  $\beta$ -(1 $\rightarrow$ 2) glycosidic bond. Rb1 possesses two glucose units linked via  $\beta$ -(1 $\rightarrow$ 6) but Rg3 is bonded with a free hydroxyl group at C20 instead of glucose. Rb1 and Rg3 are the most thoroughly researched ginsenoside in the field of osteoarthritis, making it an especially promising candidate for ongoing studies related to the treatment of osteoarthritis. For example, Tingting et al. reported that a novel biodegradable silk fibroin-gelatin porous scaffold co-loaded with ginsenoside Rb1 and TGF- $\beta$ 1 was developed to address inflammation and promote cartilage regeneration [125]. The scaffold had a porous microstructure designed for suitable mechanical strength, controlled degradation, and sustained release of both Rb1 and TGF- $\beta$ 1. Rat bone marrow-derived mesenchymal stem cells seeded onto the scaffold exhibited enhanced proliferation and chondrogenic differentiation compared to non-loaded controls. The scaffold significantly suppressed inflammatory gene expression and reduced inflammation in C5.18, rat chondrocyte cell

lines under IL-1 $\beta$  stimulation. In a rat osteochondral defect model, the implantation of the scaffold led to effective regeneration of hyaline cartilage after 12 weeks compared to other groups. In another study, Yongsheng et al. demonstrated that Rg1 incorporated poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) fibrous scaffolds mitigated IL-1 $\beta$ -induced dysregulation in rabbit articular chondrocytes [126]. This engineered scaffold maintained chondrocyte morphology and phenotypic stability under IL-1 $\beta$  stimulation conditions compared to the unmodified PHBV. The scaffold also displayed chondrocyte protective effects by down-regulating the expression of MMP-1 and upregulating expression of SOX9, type II collagen, and aggrecan. In research similar to the two studies above, Jun-Ho et al. explored the development of CK-loaded adhesive patches for cartilage tissue regeneration [127]. Authors functionalized hydrocaffeic acid-conjugated chitosan patches with CK, demonstrating stable adhesion to cartilage defects and sustained CK release. These patches inhibited apoptosis of chondrocytes, MMP-13 activity, and NF- $\kappa$ B signaling pathway in osteoarthritis mice model induced by destabilization of the medial meniscus surgery.

**Table 3.** Phytochemicals from ginseng with anti-osteoarthritis activity.

| No. | Phytochemicals  | Cell Model                                | Assay <sup>a</sup>   | Dose <sup>b</sup> , Time | Animal Model                                       | Dose, Time              | Mechanism                                                                                                                                                                                     | Year, Ref.  |
|-----|-----------------|-------------------------------------------|----------------------|--------------------------|----------------------------------------------------|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 1   | Ginsenoside Rb1 | SW1353                                    | Quantification of NO | 80 $\mu$ M, 24 h         | N/A                                                | N/A                     | Reduction in NO production, downregulation of iNOS, and nuclear p65                                                                                                                           | 2014, [108] |
| 2   | Ginsenoside Rb1 | SW1353                                    | WB of MMP-13         | 80 $\mu$ M, 24 h         | ACLT osteoarthritis rats                           | 80 $\mu$ M, 6 weeks     | Upregulation of type II collagen, downregulation of MMP-13, Notch1, and JAG1                                                                                                                  | 2015, [109] |
| 3   | Ginsenoside Rb1 | C5.18                                     | RT-PCR of MMP-13     | 100 $\mu$ g/mL, 24 h     | ACLT + MCLT osteoarthritis rats                    | 300 $\mu$ M, 4 weeks    | Downregulation of MMP-13 and type X collagen                                                                                                                                                  | 2016, [110] |
| 4   | Ginsenoside Rb1 | Chondrocytes from each group              | RT-PCR of MMP-13     | 10 $\mu$ g/kg, 4 weeks   | MIA-induced and Ovariectomized osteoarthritis rats | 10 $\mu$ g/kg, 4 weeks  | Downregulation of IFN- $\gamma$ , CCL-2/MCP1, IL-6, IL-1 $\beta$ , MMP-13, Cox-2, TGF- $\beta$ , CTX-1, and PGE2, upregulation of BMP-2                                                       | 2021, [111] |
| 5   | Ginsenoside Rb1 | Chondrocytes from healthy rats            | CCK8                 | 10 $\mu$ M, 24 h         | MIA-induced osteoarthritis rats                    | 10 mg/kg, 2 weeks       | Downregulation of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and miR-21-5p, upregulation of FGF18                                                                                                   | 2022, [112] |
| 6   | Ginsenoside Rb1 | N/A                                       | N/A                  | N/A                      | Hollow trephine-damaged osteoarthritis rabbits     | 100 $\mu$ g/kg, 10 days | Suppression of chondrocyte apoptosis, reduction in ROS production, upregulation of TIMP-1, downregulation of PGE2, MMP-1, MMP-3, MMP-13, TNF- $\alpha$ , Caspase-3, Bax, pAkt, pP65, and pp38 | 2022, [113] |
| 7   | Ginsenoside Rg1 | Chondrocytes from healthy rats            | MTT                  | 10 $\mu$ g/mL, 72 h      | N/A                                                | N/A                     | Suppression of chondrocyte apoptosis, upregulation of pAkt, Bcl2, and TIMP-1, downregulation of Bax and MMP-13                                                                                | 2014, [114] |
| 8   | Ginsenoside Rg1 | Chondrocytes from osteoarthritis patients | RT-PCR of MMP-13     | 10 $\mu$ g/mL, 24 h      | ACLT osteoarthritis rats                           | 60 mg/kg, 12 weeks      | Upregulation of type II collagen and aggrecan, downregulation of MMP-13 and COX-2                                                                                                             | 2017, [115] |
| 9   | Ginsenoside Rg1 | Chondrocytes from healthy humans          | CCK8                 | 100 $\mu$ g/mL, 72 h     | N/A                                                | N/A                     | Suppression of chondrocyte apoptosis, reduction in ROS and MDA production, upregulation of Bcl2, downregulation of Bax, Caspase-3, Caspase-8, Caspase-9, FasL, and AIF                        | 2022, [116] |
| 10  | Ginsenoside Rg1 | Chondrocytes from healthy chickens        | CCK8                 | 40 $\mu$ M, 24 h         | Thiram-induced osteoarthritis chickens             | 40 mg/kg, 18 days       | Suppression of chondrocyte apoptosis, upregulation of Bcl2, HIF-1 $\alpha$ , VEGFA, and VEGFR2, downregulation of Bax and Caspase-3                                                           | 2023, [117] |



Table 3. Cont.

| No. | Phytochemicals   | Cell Model                                                         | Assay <sup>a</sup>   | Dose <sup>b</sup> , Time            | Animal Model                          | Dose, Time                                                                                                              | Mechanism                                                                                                                                                                                                                        | Year, Ref.  |
|-----|------------------|--------------------------------------------------------------------|----------------------|-------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 11  | Ginsenoside Rg1  | Chondrocytes differentiated from healthy rabbit adipose stem cells | MTT                  | 40 $\mu$ M, 5 days                  | Papain-induced osteoarthritis rabbits | 1:1 (V:V) mixture of hyaluronic acid and adipose stem cells ( $2 \times 10^7$ /mL) suspension in 40 $\mu$ M Rg1, 9 days | Upregulation of TIMP-1, downregulation of MMP-13                                                                                                                                                                                 | 2024, [118] |
| 12  | Ginsenoside Rg3  | SW1353, chondrocytes from healthy rabbit                           | WB of MMP-13, DMMB   | 20 $\mu$ M, 24 h, 2 $\mu$ M, 3 days | N/A                                   | N/A                                                                                                                     | Downregulation of MMP-13, inhibition of glycosaminoglycan release                                                                                                                                                                | 2014, [119] |
| 13  | Ginsenoside Rg3  | TC28a2                                                             | WB of SIRT1          | 20 $\mu$ M, 24 h                    | N/A                                   | N/A                                                                                                                     | Suppression of chondrocyte apoptosis, reduction in ROS production, upregulation of Bcl2, downregulation of IL-8, MMP-13, Bax, and pp38, activation of SIRT1/PGC-1 $\alpha$ /SIRT3 pathway, suppression of NF- $\kappa$ B pathway | 2021, [120] |
| 14  | Ginsenosides Rg5 | Chondrocytes from healthy rats                                     | TUNEL                | 15 $\mu$ M, 48 h                    | MCLT osteoarthritis rats              | 15 mg/kg, 1 month                                                                                                       | Suppression of chondrocyte apoptosis, reduction in NO production, upregulation of type II collagen and TIMP-1, downregulation of MMP-13, IL-1 $\beta$ , iNOS, and TNF- $\alpha$                                                  | 2017, [121] |
| 15  | Ginsenoside CK   | Chondrocytes from osteoarthritis patients                          | Quantification of NO | 5 $\mu$ M, 24 h                     | N/A                                   | N/A                                                                                                                     | Reduction in NO production, downregulation of iNOS, MMP-13, pJNK, pp38, and pErk, inhibition of glycosaminoglycan release                                                                                                        | 2018, [122] |
| 16  | Ginsenoside CK   | Chondrocytes from healthy mouse                                    | CCK8                 | 50 $\mu$ M, 48 h                    | MCLT osteoarthritis mouse             | 40 mg/kg, 8 weeks                                                                                                       | Suppression of chondrocyte pyroptosis, upregulation of type II collagen, downregulation of IL-6, MMP-3, MMP-13, ADAMTS5, NLRP3, GSDMD, cleaved caspase-1, and IL-1 $\beta$ , inhibition of glycosaminoglycan release             | 2023, [123] |
| 17  | Ginsenoside CK   | Chondrocytes from healthy rats                                     | WB of MMP-13         | 30 nM, 24 h                         | MIA-induced osteoarthritis rats       | 80 mg/kg, 7 weeks                                                                                                       | Suppression of chondrocyte pyroptosis, upregulation of type II collagen, downregulation of MMP-3, MMP-13, ADAMTS4, ADAMTS5, NLRP3, GSDMD, cleaved caspase-1, and IL-1 $\beta$ ,                                                  | 2023, [124] |

Assay <sup>a</sup>: Specific kinds of basic assay for the anti-osteoarthritis ability. Dose <sup>b</sup>: Most effective dose of each ginsenoside in cell model assay. NO: Nitric oxide. ACLT: Anterior cruciate ligament transection. MCLT: Medial cruciate ligament transection. MIA: Monoiodoacetate. CCK8: Cell counting kit 8. DMMB: Dimethylmethyle blue assay. TUNEL: TdT-mediated dUTP-biotin nick end labeling. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. N/A: Not available.

As shown in Table 3, a variety of in vitro and in vivo models were used to study the anti-osteoarthritis activity of ginsenosides, which was chosen based on research goals and disease mechanisms. In vitro models, the most effective dose of each ginsenoside ranged from 2 to 125  $\mu$ M and was tested by various kinds of elementary assay. These assays included quantification of nitric oxide (NO), Western blot analysis of MMP-13, RT-PCR analysis of MMP-13, CCK8 assay, MTT assay, and TUNEL assay in chondrocyte cell line or primary chondrocyte extracted from knee tissue of human or animal. The chondrocyte cells were treated by each ginsenoside for 24 h in the most in vitro assays. In vivo models can be grouped by animal species and by the method of osteoarthritis induction. Common animal species included small animals such as mice and rats. These animals were widely used for genetic, molecular, and mechanistic studies since they allow for genetic modification and are cost-effective. Researchers used chemically induced

animal models by injection of chemicals such as papain and monosodium iodoacetate into the joint to induce osteoarthritis. In addition, surgically induced models were adapted through procedures such as anterior cruciate ligament transection, medial cruciate ligament transection, or destabilization of the medial meniscus.

Chondrocyte viability is a critical focus in anti-osteoarthritis therapy development because these cells are the sole producers and maintainers of cartilage extracellular matrix in articular cartilage [128–130]. In osteoarthritis, excessive apoptosis of chondrocytes disrupts this balance, leading to extracellular matrix breakdown and irreversible cartilage loss. Thus, an assay system targeting chondrocyte viability and apoptosis could address the core pathological mechanism of osteoarthritis by preserving cartilage integrity. MMP-13, also known as collagenase-3, is a primary therapeutic target for osteoarthritis due to its critical role in cartilage degradation and disease progression [131–133]. MMP-13 cleaves type II collagen, the main structural component of articular cartilage, faster than other collagenases. Except for type II collagen, MMP-13 is known to degrade other matrix components such as proteoglycans and osteonectin, exacerbating joint damage. Type II collagen is the primary structural protein in cartilage, constituting 85–90% of articular cartilage collagen and 50% of all cartilage protein. Type II collagen plays an important role in cartilage structure and modulates osteoarthritis progression through immune tolerance and cartilage protection [134–136]. For example, James et al. reported that undenatured type II collagen supplementation alleviated knee osteoarthritis symptoms across multiple randomized controlled trials [137]. Targeting inflammation in the development of anti-osteoarthritis therapies is recognized as a rational and promising strategy [138–140]. In fact, recently reported ginsenosides showed anti-osteoarthritis activities through regulation of inflammation which drives the production of enzymes such as MMPs to break down the extracellular matrix of cartilage via regulation of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . Two clinical studies have investigated the anti-osteoarthritis effect of ginseng. Hye In et al. reported that red ginseng supplementation improved pain scores and anti-oxidative activity in postmenopausal women with hand osteoarthritis [141]. After 12 weeks, the red ginseng supplementation (3 g/day) group showed significant improvement in pain scores and DASH scores compared to the placebo group. Anti-oxidant enzyme (superoxide dismutase) activity increased and oxidative stress marker (malondialdehyde) levels decreased in the red ginseng supplementation group compared to the placebo group. Su-jin et al. reported that the group receiving *Panax ginseng* extracts showed significant enhancements in WOMAC osteoarthritis index scores [142]. Among women with osteopenia, daily supplementation with 3 g of *Panax ginseng* extracts for 12 weeks led to marked improvements in knee arthritis symptoms such as knee pains and stiffness of joints.

## 5. Current Limitations and Future Direction

Ginseng and its bioactive compounds show therapeutic potential in bone-related diseases. However, there are several limitations to using them as therapeutic agents or functional food ingredients to treat or prevent bone-related diseases. First, the specific composition and concentration of phytochemicals from ginseng can vary depending on the ginseng species, plant part used, and processing methods [143–145]. This variability in ginsenoside content can lead to inconsistent therapeutic effects. Thus, advanced techniques such as two-dimensional liquid chromatography and hybrid mass spectrometry are required to identify and quantify ginsenoside metabolites. Also, a proper extract method needs to be considered for specific ginsenoside production. Secondly, it is hard to extract large quantities of phytochemicals from ginseng for industrial production [146,147]. It could be one of the options to consider biotransformation using synthetic biology tools [148].

For example, protopanaxatriol can also be produced by enzymatic conversion of other ginsenosides or precursors in engineered microorganisms [149]. In addition, investigating the synergistic effects of ginsenosides with other phytochemicals or conventional drugs could lead to more effective therapeutic strategies [150]. Thirdly, the safety and side effects of phytochemicals from ginseng should be carefully considered for dosage, duration, and individual susceptibility [151,152]. Potential concerns include gastrointestinal disturbances and the risk of immune suppression, particularly in patients receiving concurrent chemotherapy for osteosarcoma [153]. For example, some studies have raised alarms about the possibility of ginsenosides having tumor-promoting effects under certain conditions, highlighting the need for further investigation to clarify their role in cancer treatment and to establish appropriate clinical guidelines [154]. Thus, continued exploration of targeted delivery systems and combination therapies could significantly improve the treatment efficacy of phytochemicals from ginseng addressing safety concerns associated with conventional approaches. Fourthly, the poor bioavailability of phytochemicals from ginseng due to their hydrophilicity hampers their absorption in the gastrointestinal tract [155]. The degradation of ginsenosides by intestinal flora can affect their pharmacokinetics and pharmacological efficacy, necessitating studies on their metabolic pathways [156]. In humans, the half-lives of ginsenoside are usually less than 24 h, limiting their duration of action [157]. Some ginsenosides can be degraded or transformed during processing, which may affect their intended therapeutic effects. To solve the lower bioavailability of phytochemicals from ginseng, it is necessary to design the appropriate dosing regimens and sustained administration approach to maintain effective plasma concentrations. Fifthly, in-depth studies on the specific molecular mechanisms of phytochemicals from ginseng are crucial for understanding their therapeutic potential in bone-related disease to avoid unintended effects and optimize targeted therapy [75,158]. For example, ginsenosides modulate diverse pathways such as NF- $\kappa$ B, Wnt/ $\beta$ -catenin, and Nrf2, which complicates targeted therapy and increases the risk of unintended effects in hormone-sensitive conditions [159–161]. To minimize unintended effects, personalized approaches such as pathway-specific delivery systems and biomarker-guided dosing, could mitigate risks while preserving the benefits of phytochemicals from ginseng. Sixthly, several other plants contain other phytochemicals that can offer similar health benefits and biological activities, making them valuable alternatives or complements in therapeutic and nutritional contexts [162,163]. *Astragalus membranaceus*, commonly used in traditional Chinese medicine, contains triterpenoid saponins known as astragalosides. These compounds have been shown to have immunomodulatory, anti-inflammatory, and anti-oxidant effects, similar to some of the biological activities of ginsenosides [164,165]. *Eleutherococcus senticosus* (Siberian ginseng) contains eleutherosides, a class of compounds structurally different from ginsenosides but with analogous adaptogenic, immunomodulatory, and anti-fatigue properties. It is widely used as a ginseng alternative in traditional medicine [166]. Seventhly, more clinical trials with high quality are essential to validate the efficacy and safety of phytochemicals from ginseng in treating bone-related diseases. For this issue, several critical factors must be addressed based on existing research gaps and methodological insights. For example, variability in ginseng composition requires rigorous chemical characterization to ensure consistency across trials [167]. Long-term studies are needed to assess sustained benefits, since bone remodeling is a slow process [168]. High-risk groups such as elderly or postmenopausal individuals are carefully selected to evaluate efficacy in preventing osteoporosis progression [142]. Thus, clinical studies should also address issues of variability, bioavailability, and effectiveness in humans in reference to most current evidence derived from in vitro and in vivo results. Eighthly, most research on bone health benefits of ginseng was performed by Asian countries such as China, Korea, and Japan. Clinical trials in multiple countries could assess efficacy across

genetic and lifestyle backgrounds. Although there seems no reference to clinical trials on ginseng for bone-related diseases, emerging studies are paving the way for broader global adoption to expand the health benefits of ginseng [169–171]. For instance, a comprehensive review summarized evidence from 19 meta-analyses, confirming that ginseng intake is associated with improvements in fatigue, physical function, sexual function, menopausal symptoms, metabolic indicators, inflammatory markers, and cardiovascular health. These ongoing studies could facilitate broader acceptance of the health benefits of ginseng in a global population. These limitations highlight the need for further research to improve the bioavailability, stability, and clinical meaning of phytochemicals from ginseng for their effective use as therapeutic agents or functional food ingredients. By addressing these limitations and pursuing these future directions, researchers can unlock the full therapeutic potential of ginseng-derived phytochemicals in treating bone-related diseases.

## 6. Conclusions

Ginseng has been utilized in traditional medicine for centuries, celebrated for its diverse health benefits. Recent studies suggest that phytochemicals from ginseng, particularly ginsenosides, offer new avenues for treating bone-related diseases such as osteosarcoma, osteoporosis, and osteoarthritis. These phytochemicals show promise in modulating critical signaling pathways related to specific bone-related diseases in vitro and in vivo models. Limitations of phytochemicals from ginseng included variability, bioavailability, and insufficient clinical data highlighting the need for standardized formulations, rigorous clinical validation, and advanced delivery systems to optimize its role in bone health therapeutics. Overall, the ongoing research into the applications of ginseng-derived phytochemicals presents exciting possibilities for enhancing the management of osteosarcoma, osteoporosis, and osteoarthritis.

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## Review

# Gnetin C in Cancer and Other Diseases: What Do We Know So Far?

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**Abstract:** Stilbenes are a class of natural polyphenols with multiple positive pharmacologic assets such as antioxidant, anti-inflammatory and anticancer effects. While monomeric stilbenes, represented mostly by resveratrol and pterostilbene, have been studied intensely in the last two decades, oligomeric compounds, which may have better prospects of becoming potent nutraceuticals, are much less studied. The goal of this review is to compile all available literature to date on the beneficial pharmacologic effects of Gnetin C, a resveratrol dimer, in cancer and other diseases. While studies have shown the beneficial effects of Gnetin C, as a single compound or a component of melinjo seed extract, through cellular models, in vivo preclinical studies are still lacking. This is except for prostate cancer, where various animal models, including xenografts and transgenic mice, have been used to evaluate Gnetin C's more potent anti-inflammatory and anticancer effects compared to resveratrol and its monomeric analogs. Since Gnetin C's safety has already been demonstrated in healthy volunteers, it is now logical to evaluate its efficacy for prostate cancer chemoprevention, interception and therapy in clinical trials.

**Keywords:** Gnetin C; natural products; beneficial effects; anticancer effects; prostate cancer

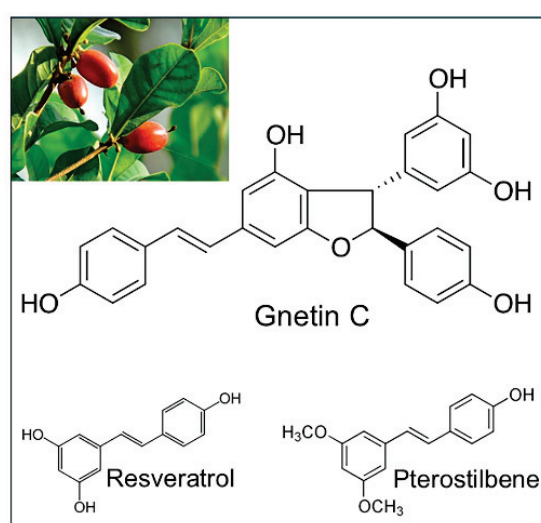
## 1. Introduction

Natural polyphenols are plant-derived chemicals with beneficial effects on human health. Polyphenols are divided into different classes depending on their basic chemical structure. Stilbenes or stilbenoids contain two aromatic rings connected by a central ethylene double bond. Most stilbenes in plants are produced in response to environmental stress including ultraviolet radiation, injury or infection. One of the most well-known naturally occurring stilbenes is resveratrol (trans-3,4',5-trihydroxystilbene), found in grapes and red wine. Resveratrol and pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene), found mainly in blueberries, are by far the two most extensively studied stilbenes in terms of their beneficial bioactivity in different diseases. Both compounds have been shown to exert a range of biological activities including antifungal, antibacterial, antioxidative, anti-inflammatory, anticancer and cardio- and neuroprotective effects [1–4].

Plentiful information is available on the cellular and molecular mechanisms by which resveratrol and its monomeric analogs interfere with carcinogenesis, cancer progression, cancer resistance and metastasis. These stilbenes have been shown to affect multiple targets in signaling pathways associated with cell proliferation, inflammation, angiogenesis and metastatic potential. However, despite a large amount of in vitro and in vivo experimental data, neither resveratrol nor pterostilbene is used therapeutically because clinical trials have been inconclusive [5]. Among the reasons for resveratrol's lack of success is its poor

bioavailability due to rapid and extensive conjugation in the intestinal tract, resulting in metabolites that are less biologically active than the parent compound [6,7]. On the other hand, resveratrol's methoxylated analog pterostilbene has greater anticancer activity due to its favorable pharmacokinetic parameters, with a bioavailability greater than ten times that of resveratrol [8].

In more recent years, various resveratrol oligomer derivatives have been described. These can occur either naturally or synthetically from the endogenous polymerization of two to eight resveratrol units to form dimers or more complex oligomers [9,10]. In many instances, these oligomers were found to exhibit superior beneficial activities compared to monomer derivatives [11–14]. The resveratrol dimer consisting of two *trans*-resveratrol units linked via a benzofuran ring, known as Gnetin C, is the focus of the current review (Figure 1). We will summarize what is known so far about the biological activities and molecular effects of Gnetin C in cancer and other human diseases.



**Figure 1.** Image of melinjo plant (*Gnetum gnemon*). Chemical structures of Gnetin C and stilbene monomers: resveratrol and pterostilbene.

## 2. Gnetin C, a Resveratrol Dimer

Gnetin C was isolated from the roots of the melinjo plant (*Gnetum gnemon*) belonging to the Gnetaceae family [15]. The seeds of melinjo also contain various resveratrol dimers including Gnetin C, gnemonoside A and gnemonoside D, as well as minor amounts of resveratrol [16]. Many of the health-promoting benefits of melinjo seed extract (MSE) are attributed to Gnetin C and its related substances.

Plants from the Gnetaceae family have long been used in traditional medicine for the treatment of bronchitis, arthritis, diabetes, heart problems and other diseases. The melinjo plant is native to rainforests in Malaysia, the Philippines, Singapore, northern India and French Polynesia. Its fruit, leaves and seeds are used in Indonesian cuisine. Crackers made from the seeds are the most commercially popular, while fruits and leaves are used in making soup or consumed as vegetables. Scientifically, Gnetin C, in either the purified form or in ethanolic extract mixtures, showed no toxicity in normal cells in culture [17–19] and in rodent *in vivo* studies [17,20]. Importantly, both MSE and Gnetin C appear to be safe in humans according to recent clinical trials [21–25].

Until recently, access to pure compounds was limited to their isolation from natural sources, which hampered their biological evaluation. Furthermore, the fraction of Gnetin C present in ethanolic extracts from melinjo plant can vary depending on the part of the plant and method of isolation used. While evaluation of such mixtures provides useful information, the specific effects contributed by Gnetin C alone are unclear. In the

current review, we primarily discuss studies with purified Gnetin C used as a single agent compared to either ethanolic extracts from the melinjo plant or other purified stilbenes (monomers and dimers).

### 3. Gnetin C and Cancer

Cancer is a leading cause of morbidity and mortality. The safety of standard chemotherapeutic drugs is a concern, and consequently, natural polyphenols, among them stilbenes, have become the subject of increasing scientific interest for cancer prevention, interception and treatment.

Naturally occurring resveratrol oligomers, including Gnetin C, have been proposed as potential cancer chemopreventive compounds [26]. Summarized in Table 1 are the reported *in vitro* and *in vivo* studies evaluating the efficacy of Gnetin C compared to other stilbenes or MSE in preventing the development and progression of cancer through different signaling pathways.

**Table 1.** Anticancer activity of Gnetin C.

| Compound                                            | Model    | Dose                                         | Cell/Animal                                                                                                                                                                          | Mechanism of Action                                                                                                            | Ref  |
|-----------------------------------------------------|----------|----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|------|
| Gnetin C                                            | In vitro | 0–100 $\mu$ M                                | Acute myelogenous leukemia (AML) cells: MV4, THP1, U937, HL60<br>Chronic myeloid leukemia (CML) cells: K562, Oun1, KH88                                                              | Inhibition of ERK 1/2 and AKT/mTOR pathways; cell cycle arrest                                                                 | [18] |
|                                                     | In vivo  | 5 mg/kg/day, 5 weeks                         | AML-MT xenograft mice                                                                                                                                                                | Inhibition of leukemia; antitumor effects (blood, spleen, bone marrow); extended survival of mice                              |      |
| Gnetin C<br>MSE<br>Resveratrol<br>Gnetoside A, C, D | In vitro | 0.5–10 $\mu$ M<br>40 $\mu$ g/mL<br>5 $\mu$ M | Human umbilical vein endothelial cells (HUVECs)                                                                                                                                      | Inhibition of tube formation stimulated with VEGF and bFGF; reduction of cell viability and migration; ERK1/2 inactivation     | [19] |
|                                                     | In vivo  | 5% MSE                                       | Mouse dorsal air sac assay                                                                                                                                                           | Inhibition of tumor angiogenesis                                                                                               |      |
| Gnetin C<br>Resveratrol<br>MSE                      | In vitro | 0–100 $\mu$ M<br>0–400 $\mu$ g/mL            | LNCaP, PC3, Murine CaP8 prostate cancer cells; MCF7 breast cancer cells; HT-29, colon-26 colon cancer cells; PANC-1, AsPC1, Pan-02 pancreatic cancer cells; PWPE1 and HEK-293T cells | Inhibitory effects on cancer cells without affecting normal cells; induction of apoptosis via caspase 3/7-dependent mechanisms | [17] |
| MSE                                                 | In vivo  | 50 and 100 mg/kg/day, oral                   | Colon-26 tumor-bearing mouse model                                                                                                                                                   | Inhibition of tumor growth, angiogenesis and liver metastasis                                                                  |      |
| Gnetin C<br>Resveratrol                             | In vitro | 2–16 $\mu$ M                                 | Murine melanoma B16 cells                                                                                                                                                            | Inhibitory activity against tyrosinase and melanin biosynthesis                                                                | [27] |
| Gnetin C<br>Melinjo fruit extract                   | Ex vivo  | 50% extract at 100 mg/kg/day                 | Cultured murine Peyer's patch cells                                                                                                                                                  | Enhanced T-cell-dependent immune responses, IL2 $\uparrow$ , IFN $\gamma$ $\uparrow$                                           | [28] |

$\uparrow$  upregulation; MSE, melinjo seed extract. LNCaP (AR-positive); PC3 (AR-negative); MCF7 (ER-positive).

In an acute myeloid leukemia (AML)-MT xenograft model, Gnetin C significantly lowered the development of leukemia and tumor incidence in the blood, bone marrow and spleen. Moreover, mechanistic studies using several AML and chronic myeloid leukemia (CML) cell lines showed the induction of cell cycle arrest and inhibition of ERK1/2 and AKT/mTOR pathways [18] (Table 1). It was also demonstrated that Gnetin C inhibited the growth of human leukemia HL60 cells with an IC<sub>50</sub> value of 13  $\mu$ M [29]. Inhibition of tumor angiogenesis was also demonstrated after treatment with 5% MSE. Furthermore, Gnetin C in comparison with resveratrol, other dimers and MSE in human umbilical vein endothelial

cells (HUVECs) was found to have a superior inhibitory effect on VEGF- and BFGF-stimulated tube formation and ERK1/2-mediated cell proliferation and migration [19] (Table 1). Narayanan et al. evaluated Gnetin C anticancer effects in comparison with resveratrol and MSE against various cancer cell lines in vitro and demonstrated that Gnetin C was more effective in causing kinase 3/7-mediated apoptosis in these cells. In addition, in a colon-26 tumor-bearing mouse model, MSE reduced tumor growth, improved histology and inhibited angiogenesis and liver metastasis [17] (Table 1). Superior biological activity of Gnetin C compared to resveratrol was also shown in melanoma cells in vitro [27] (Table 1). Interestingly, in a separate study, pterostilbene-induced inhibition of VCAM-1 and Bcl-2 in B16-F10 metastatic melanoma cells reduced metastatic growth in the liver by decreasing the colonization ability of these cells [30]. Lastly, Gnetin C in comparison with resveratrol and MSE showed more potent immunomodulatory activity in cultured murine Peyer's patch cells [28] (Table 1).

#### 4. Gnetin C and Prostate Cancer

Due to its long latency period, prostate cancer is considered an ideal type of cancer for nutritional chemoprevention. Some studies have demonstrated that stilbenes, like other polyphenols, exhibit multitargeted effects in prostate cancer by causing cell cycle arrest/apoptosis and obstructing cell survival and angiogenesis through various mechanisms [31,32]. Our current knowledge about the anticancer activities of stilbenes, including Gnetin C, is largely based on a series of in vitro and preclinical studies in prostate cancer performed by Dr. Anait S. Levenson's group. These studies were focused on two essential pathways that were regulated by stilbenes: AR-dependent and -independent pathways represented by MTA1-mediated cell survival and metastasis. The results from these studies gave the most compelling evidence for cancer prevention, interception and therapy by stilbenes, showing that both oral and parenteral administration of these compounds was linked to a reduced risk of prostate cancer.

Regarding anti-androgenic mechanisms of action, numerous studies showed that resveratrol and other monomeric stilbenes exert antiproliferative and pro-apoptotic effects in prostate cancer by inhibiting the expression or hindering the function of AR [33–41]. Importantly, monomeric stilbenes inhibit not only full-length but also truncated AR, a hallmark of castrate-resistant prostate cancer (CRPC), apparently through different mechanisms compared to traditional non-steroidal antiandrogens, suggesting that combination strategies may result in synergistic effects [37,42,43]. A recently published paper showed that Gnetin C alone and in combination with enzalutamide (Enz) effectively inhibited both full-length and AR splice variant AR-V7 in vitro and in vivo [44] (see more details below).

Metastasis-associated protein is part of the nucleosome remodeling and deacetylation (NuRD) complex, which is involved in gene-specific transcriptional regulation controlling inflammation-driven prostate tumorigenesis and prostate cancer survival pathways and metastasis [45,46]. Earlier experiments with resveratrol demonstrated that it targets MTA1 for degradation in prostate cancer cells and rescues p53 acetylation, resulting in the induction of Bax- and p21-mediated apoptosis [47]. Further, pterostilbene was seven-fold more effective than resveratrol in inhibiting MTA1. It also showed an improved reduction in tumor growth and site metastases in an orthotopic DU145-xenograft mouse model. DU145-MTA1 knockdown-sensitized tumors responded to stilbene treatment with an additional reduction in size, confirming the pleotropic effects of stilbenes [48]. Based on the above findings, subsequent studies with pterostilbene were designed to investigate its effects in prostate-specific Pten-heterozygous ( $Pten^{+/-}; Cre^{+}$ ) and Pten-null ( $Pten^{-/-}; Cre^{+}$ ) transgenic mouse models, which express high prostate levels of MTA1. In the chemoprevention modality ( $Pten^{+/-}; Cre^{+}$ ), mice were fed a diet supplemented with pterostilbene (100 mg/kg



diet), while in the intervention modality (*Pten<sup>f/f</sup>; Cre<sup>+</sup>*), mice were treated with daily pterostilbene (10 mg/kg bw) i.p. injections. Data showed that pterostilbene treatment inhibited the conversion of high-grade prostate intraepithelial neoplasia (PIN) lesions to cancer by acting through MTA1-mediated signaling to modulate PTEN acetylation, p-Akt/Akt, AR, CyclinD1, NFκB, TGFβ and IL-1β [41]. In combination with histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA), pterostilbene showed more potent antitumor effects in *Pten<sup>f/f</sup>; Cre<sup>+</sup>* mice, mediated through reduced angiogenesis (MTA1-associated pro-angiogenic factors HIF-1α, VEGF and IL-1β) [49]. Finally, pterostilbene dietary supplementation provided to a prostate-specific MTA1-overexpressing transgenic mouse model mimicking high-risk premalignant prostate cancer (*R26<sup>MTA1</sup>; Pten<sup>+/f</sup>; Cre<sup>+</sup>*) resulted in an MTA1-mediated (Cyclin D1, Notch2, IL-6, IL-1β, miR-22, miR-34a) reduction in tumor growth and progression, favorable histopathology and reduced angiogenesis, highlighting the potential for oral pterostilbene in prostate cancer [50].

Overall, pterostilbene is recognized as a lead monomeric analog of resveratrol with the most potent anticancer activity in prostate cancer. We observed superior anti-inflammatory (TGFβ, NFκB, IL-1β, IL-2) and anticancer effects of pterostilbene through MTA1 downregulation-associated increase in p53 and PTEN acetylation, inhibition of survival pathways (pAkt/Akt, Notch2, ETS2, CyclinD1, AR), less angiogenesis (VEGF, HIF1α, IL-1β) and a higher apoptotic index (p53, Bax, Bak, cleaved caspase 3).

What about Gnetin C? Gnetin C comparative in vitro experiments with resveratrol and pterostilbene as reference stilbenes showed more potent MTA1 inhibition (in both mRNA and protein levels) and greater MTA1-mediated cytotoxicity in various prostate cancer cells such as DU145, PC3M, 22RV1 and VCAP. Gnetin C displayed substantial inhibitory effects in prostate cancer cells expressing MTA1, while in MTA1 knockdown cells, Gnetin C showed partial MTA1-independent mechanisms that inhibit cell metastatic potential and induce apoptosis. Superior MTA1-mediated anticancer effects of Gnetin C also included a downregulation of oncogenic/tumor-promoting ETS2, pAkt/Akt, Cyclin D1, p-mTOR/pS6K/p4EBP1 and AR signaling [44,51–54] (Table 2).

Importantly, preclinical animal models, both xenografts and transgenic mice, were used for evaluating Gnetin C's MTA1-targeted efficacy. First, in vivo effects of Gnetin C in prostate cancer were shown in PC3M-Luc subcutaneous xenografts with i.p. injections [52]. A significant reduction in MTA1-mediated tumor growth and angiogenesis with an induction of apoptosis were observed with Gnetin C (50 mg/kg bw, i.p.). Moreover, the effects of Gnetin C at half the concentration (25 mg/kg bw) were comparable with the antitumor effects of resveratrol and pterostilbene at 50 mg/kg bw, indicating a more potent bioactivity of Gnetin C (Table 2). As confirmation, we found an accumulation of Gnetin C in tumor tissues, while resveratrol and pterostilbene did not reach detectable limits [52]. Further, Gnetin C alone (40 mg/kg bw, i.p.) and combined with Enz (7 mg/kg bw, i.p.) effectively inhibited AR- and MTA1-promoted tumor progression in castrate-resistant 22Rv1-Luc xenografts, resulting in a lower proliferative index (Ki67) and angiogenesis (CD31), and higher apoptosis (CC3) in tumor tissues. Once again, Gnetin C was the most potent inhibitor of AR-V7 and a sensitizer for the Enz response in 22Rv1-resistant cells. It seems that targeting two major pathways in prostate cancer maximizes the efficacy of Gnetin C without compromising its safety profile [44] (Table 2). Finally, two carefully designed preclinical experimental approaches for evaluating Gnetin C's potential in chemoprevention, interception and therapy in prostate cancer involved transgenic mouse models: one depicting a high-risk premalignant prostate tissues overexpressing MTA1 on the background of *Pten* heterozygosity (*R26<sup>MTA1</sup>; Pten<sup>+/f</sup>; Cre<sup>+</sup>*) [54] and another representing MTA1-overexpressing advanced prostate cancer (*R26<sup>MTA1</sup>; Pten<sup>f/f</sup>; Cre<sup>+</sup>*) [53]. A Gnetin C-supplemented diet reduced the progression of prostate cancer in high-risk, early-stage prostate tumors by reducing cell

proliferation, inflammation and formation of blood vessels. The data also showed a more potent MTA1/PTEN/Akt response to Gnetin C-supplemented diets (35 and 70 mg/kg diet) compared to the reference pterostilbene-supplemented diet (70 mg/kg diet) [54]. Similarly, in an advanced prostate cancer model, Gnetin C (7 mg/kg/bw, i.p.) significantly blocked tumor progression through MTA1/Akt/mTOR signaling [53], suggesting that Gnetin C could be effective not only in cancer chemoprevention and interception but also as an active therapeutic strategy (Table 2).

**Table 2.** Gnetin C and prostate cancer.

|             | Compound        | Model                                    | Dose                                                                   | Mechanism of Action                                                | Ref                                                                                                                                                 |      |
|-------------|-----------------|------------------------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Mechanistic | In vitro        | Gnetin C<br>Resveratrol                  | DU145, PC3M,<br>PC3M-shMTA1,<br>DU145-shMTA1                           | 0–50 μM                                                            | MTA1-mediated inhibitory effects on cell viability, colony formation, migration, induction of apoptosis; MTA1↓ (protein and RNA), ETS2↓             | [51] |
|             |                 | Gnetin C<br>Resveratrol<br>Pterostilbene | DU145, PC3M                                                            | 0–100 μM                                                           | Cytotoxicity, reduction of clonogenic survival and motility                                                                                         | [52] |
|             |                 | Gnetin C<br>Enzalutamide<br>combination  | 22Rv1, VCaP                                                            | 0–100 μM/<br>0–50 μM                                               | Inhibition of cell viability, clonogenic survival and migration, synergism at certain doses; MTA1↓, AR-FL↓, AR-V7↓, PSA↓                            | [44] |
|             |                 | Gnetin C<br>Pterostilbene                | 22Rv1                                                                  | 25 μM                                                              | MTA1↓, pAkt/Akt↓, PTEN↑                                                                                                                             | [54] |
|             |                 | Gnetin C                                 | PC3M,<br>PC3M-shMTA1                                                   | 25, 50 μM                                                          | MTA1↓, Cyclin D1↓, pAkt/Akt↓, p-mTOR/pS6K/p4EBP1↓                                                                                                   | [53] |
| Preclinical | Xenografts      | Gnetin C<br>Resveratrol<br>Pterostilbene | PC3M-Luc                                                               | 25 and<br>50 mg/kg/day, i.p.                                       | Tumor growth reduction, inhibition of angiogenesis and induction of apoptosis; MTA1↓, CyclinD1↓, Notch2↓                                            | [52] |
|             |                 | Gnetin C<br>Enzalutamide<br>combination  | 22Rv1-Luc                                                              | GnC<br>40 mg/kg/day + Enz<br>7 or<br>10 mg/kg/day, i.p.            | Inhibition of tumor growth and angiogenesis, induction of apoptosis; MTA1↓, AR-FL↓, AR-V7↓                                                          | [44] |
|             | Transgenic mice | Gnetin C<br>Pterostilbene                | R26 <sup>MTA1</sup> ;<br>Pten <sup>+/<i>f</i></sup> ; Cre <sup>+</sup> | GnC-Diet 35 and<br>70 mg/kg diet, or<br>Pter-Diet<br>70 mg/kg diet | Reduction of cell proliferation, angiogenesis and inflammation; MTA1↓, pAkt/Akt↓, PTEN↑, IL2↓ in serum                                              | [54] |
|             |                 | Gnetin C                                 | R26 <sup>MTA1</sup> ;<br>Pten <sup><i>f/f</i></sup> ; Cre <sup>+</sup> | 7 mg/kg bw, i.p.,<br>12 weeks                                      | Inhibition of cell cycle progression, proliferation and angiogenesis, induction of apoptosis; MTA1↓, Cyclin D1↓, p-mTOR/pS6K/p4EBP1↓, IL2↓ in serum | [53] |

↑ upregulation; ↓ downregulation; GnC, Gnetin C; Enz, enzalutamide. DU145 (AR-negative); PC3M (AR-negative); 22Rv1 (AR-positive, AR-V7); VCaP (AR-positive, AR-V7).

Studies have shown that stilbenes exhibit cytokine-mediated anti-inflammatory effects in cancer [28,55–58]. In prostate cancer, the inhibitory effects of pterostilbene on circulating levels of IL-6 and IL-1β were reported in prostate cancer xenografts and transgenic mouse models [49,50]. A low-dose Gnetin C-supplemented diet significantly suppressed levels of pro-inflammatory IL-2 [53,54], and, to a lesser extent, IL-6, in an early-stage prostate cancer model. Interestingly, a high-dose Gnetin C diet had the opposite effect on IL-6 levels [54]. This phenomenon of low-dose natural polyphenols having better efficacy than high doses, when used alone or in combination with other natural agents or chemotherapeutic drugs, has been reported and discussed [17,59–62]. Further studies are necessary to clarify the dose-and-efficacy outcome of Gnetin C alone and in combination with other agents as anti-inflammatory and anticancer drugs.

To summarize, there are currently only five publications demonstrating the anticancer activity of Gnetin C in different types of cancers, shown mostly in vitro (Table 1). However,

comprehensive mechanistic and preclinical studies in prostate cancer support AR- and MTA1-targeted chemopreventive, interceptive and therapeutic effects of Gnetin C.

## 5. Gnetin C and Other Effects

There are few reports on Gnetin C as a single agent in diseases other than cancer; therefore, studies using MSE, which contains Gnetin C, will also be discussed in this section. Representative studies are outlined in Table 3.

**Table 3.** Gnetin C and other effects.

|                              | Compound                                                    | Model    | Dose                                                                          | Cell/Animal                                                                    | Mechanism of Action                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Ref.    |
|------------------------------|-------------------------------------------------------------|----------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|
| Cardioprotective             | Gnetin C<br>Resveratrol<br>Other stilbenes                  | Ex vivo  | 500 $\mu$ M                                                                   | Human platelet-collagen adhesion assay                                         | Res inhibits arachidonic acid- and thrombin-induced platelet aggregation; Gnetin C more potently inhibits platelet-collagen adhesion compared to Res                                                                                                                                                                                                                                                                                                                                                                                                               | [63]    |
|                              | Gnetin C<br>MSE<br>Resveratrol<br>Grape extract             | In vitro | 0.05 nM angiotensin II + GnC 30 $\mu$ M, MSE 300 $\mu$ g/mL or Res 30 $\mu$ M | Radioligand binding assay in transfected HEK-293 cells transfected COS-1 cells | GnC and MSE: inhibit ATII-type 1 receptor binding; GnC, MSE, grape extract, Res: mild agonists at PPAR $\alpha$ and PPAR $\gamma$                                                                                                                                                                                                                                                                                                                                                                                                                                  | [21]    |
| Neuroprotective              | Gnetin C<br>Resveratrol<br>$\epsilon$ -viniferin            | In vitro | 0–20 $\mu$ M                                                                  | SH-SY5Y cells                                                                  | Gnetin C more potently A $\beta$ 42 $\downarrow$ secretion, BACE1 $\downarrow$ , A $\beta$ oligomers $\downarrow$ , A $\beta$ monomers $\uparrow$ , MMP-14 $\uparrow$ , mitigated A $\beta$ 42-induced cytotoxicity                                                                                                                                                                                                                                                                                                                                                | [64]    |
|                              | Gnetin C                                                    | In vitro | 0–10 $\mu$ M                                                                  | U373MG and SH-SY5Y cells: poly-IC-induced TLR3-mediated inflammation           | IFN- $\beta$ $\downarrow$ , STAT1 phosphorylation $\downarrow$ , CCL2 $\downarrow$ , CCL5 $\downarrow$                                                                                                                                                                                                                                                                                                                                                                                                                                                             | [65]    |
| Metabolic                    |                                                             | In vivo  | HFCD diet supplemented with 0.5% MSE, 12 wk                                   | HFCD diet-induced NAFLD mouse model                                            | MSE: body weight $\downarrow$ , liver weight $\downarrow$ , Tg $\downarrow$ , NEFAs $\downarrow$ , ALT $\downarrow$ , liver steatosis $\downarrow$ , hepatic fibrosis $\downarrow$                                                                                                                                                                                                                                                                                                                                                                                 |         |
|                              | Gnetin C<br>MSE<br>Resveratrol                              | In vivo  | HFCD diet supplemented with GnC or Resv 150 mg/kgbw/day, 12 wk                | HFCD diet-induced NAFLD mouse model                                            | GnC and Res: body weight $\downarrow$ , liver weight $\downarrow$ , IL-1b $\downarrow$ , adiponectin $\uparrow$ , liver steatosis $\downarrow$ , hepatic fibrosis $\downarrow$ , collagen deposition $\downarrow$ , COL1A1 $\downarrow$ , TGF $\beta$ 1 $\downarrow$ ; GnC: glucose $\downarrow$ , insulin sensitivity $\uparrow$ , lipids $\downarrow$ , ACC1 $\downarrow$ , chREBP $\downarrow$ , DGAT1 $\downarrow$ , DGAT2 $\downarrow$ , MTP $\downarrow$ , LDLR $\downarrow$ , PPAR $\alpha$ $\downarrow$ , PGC-1 $\alpha$ $\downarrow$ , SIRT1 $\downarrow$ | [66]    |
|                              | Gnetin C<br>MSE                                             | In vivo  | 2% MSE-supplemented diet, 21 days                                             | Streptozotocin-induced diabetic mice (model for endothelial senescence)        | SA- $\beta$ -gal-positive cells $\downarrow$ , aortic SIRT1 $\uparrow$ ; Plasma Gnetin C component 6-fold higher than resveratrol                                                                                                                                                                                                                                                                                                                                                                                                                                  | [16]    |
|                              | Resveratrol<br>Gnemonosides A, D                            | In vitro | 100 $\mu$ mol/L                                                               | HUVEC H <sub>2</sub> O <sub>2</sub> -induced endothelial senescence            | Only resveratrol component able to SA- $\beta$ -gal-positive cells $\downarrow$ , SIRT1 $\uparrow$ , eNOS $\uparrow$ , PAI1 $\downarrow$                                                                                                                                                                                                                                                                                                                                                                                                                           |         |
|                              | Gnetin C<br>Resveratrol<br>Gnetin L<br>Gnemonosides A, C, D | In vitro | Constituents extracted from dried melinjo endosperm and purified              | DPPH radical scavenging activity<br>Pancreatic digestive enzymes<br>Food       | Gnetin C ED50: 10.7 $\mu$ M<br>Resveratrol ED50: 13.2 $\mu$ M<br>Gnetin C has greater lipase and $\alpha$ -amylase inhibition than resveratrol<br>Moderate antimicrobial activity                                                                                                                                                                                                                                                                                                                                                                                  | [15]    |
| Anti-inflammatory—Anti-aging | Gnetin C<br>Resveratrol                                     | In vitro | 2–8 $\mu$ M                                                                   | Murine B16 cells                                                               | GnC and Res: Similar inhibitory potency of tyrosine activity and melanin biosynthesis; neither is cytotoxic                                                                                                                                                                                                                                                                                                                                                                                                                                                        | [27]    |
|                              |                                                             | In vitro | 2–16 $\mu$ M                                                                  | Cell-free inhibition of tyrosinase enzyme                                      | GnC has less direct inhibition of tyrosine activity                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |         |
|                              | Gnetin C<br>Resveratrol                                     | In vivo  | 10 mg/kg i.p. daily for 7–8 days                                              | Ligature-induced periodontitis mouse model                                     | GnC and Res: 8-OHdG $\downarrow$ ; GnC: bone healing $\uparrow$ and IL-1 $\beta$ $\downarrow$                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | [67,68] |
|                              |                                                             | In vivo  | As above                                                                      | Nrf2 <sup>−/−</sup> transgenic mice                                            | Neither treatment able to induce bone healing                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |         |

$\uparrow$  upregulation;  $\downarrow$  downregulation; Res, resveratrol; MSE, melinjo seed extract; GnC, Gnetin C.

**Cardioprotective Effects:** Resveratrol was first noted for its cardiovascular benefits from the moderate consumption of red wine, known as “the French paradox”, back in 1992 [69]. Since then, countless studies have confirmed resveratrol’s beneficial effect

on atherosclerosis, hypertension, thrombosis, myocardial infarction, heart failure and stroke [70–72]. Gnetin C treatment of COS-1 cells resulted in a mild agonistic effect on PPAR $\alpha$  and PPAR $\gamma$ , which are involved in HDL-C upregulation. In addition, AT1 receptor binding was significantly inhibited by Gnetin C and MSE [21] (Table 3). Gnetin C was also found to more potently inhibit platelet–collagen adhesion compared to resveratrol [63]. Importantly, two randomized, double-blind, placebo-controlled clinical trials demonstrated the safety and cardioprotective effects of Gnetin C and MSE [21,24] (Table 4).

**Table 4.** Human clinical trials with Gnetin C or MSE.

| Trial Type                                   | Treatment                                                                 | Population                             | Number of Participants and Duration                           | Markers                                                                                                                                                                                      | Outcome                                                                                          | Ref  |
|----------------------------------------------|---------------------------------------------------------------------------|----------------------------------------|---------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|------|
| Randomized, double-blind, placebo-controlled | Gnetin C 150 mg/day, orally                                               | Healthy Japanese subjects              | N = 12<br>Days: 14                                            | No change in CRP, Tg, 8-OHdG or pentosidine; LDL-C $\downarrow$ , HDL-C $\downarrow$ , adiponectin $\downarrow$ , NK cells $\uparrow$                                                        | Safety; Cardioprotective effect                                                                  | [24] |
| Randomized, double-blind, placebo-controlled | MSE 750 mg daily, orally                                                  | Nonobese Japanese males, 35–70 yrs old | N = 30<br>Weeks: 8                                            | Serum uric acid $\downarrow$ , LDL-C no change, HDL-C $\uparrow$                                                                                                                             | Cardioprotective                                                                                 | [21] |
| No placebo                                   | Single-dose study: Res 6.80 mg/day; MSE 1000 mg/day, orally               | Healthy volunteers                     | N = 10 (6 men, 4 women)<br>23–34 yrs old<br>Days: 28          |                                                                                                                                                                                              | Safety; Pharmacokinetics                                                                         | [22] |
| Placebo-controlled                           | Repeated doses MSE 1000 mg, 2000 mg or 5000 mg                            | Healthy volunteers                     | N = 44 (22 men, 22 women)<br>32–49 yrs old<br>Days: 14 and 28 | Blood pressure, pulse, body mass index; biochemical parameters in blood, urine                                                                                                               |                                                                                                  |      |
| No placebo                                   | MSE tablets (38.5% MSE powder equivalent to 262 mg Gnetin C): 20 tabs/day | Healthy volunteers                     | N = 5 (3 males, 2 females)<br>34–46 yrs old<br>Days: 28       | Blood, circulating: Immune cells<br>Surface immune receptors<br>Treg cells<br>CTL (GZMB)<br>NK (NKG2D receptor)<br>Inflammatory cytokines IFN $\gamma$ ; TNF $\alpha$<br>8-OHdG $\downarrow$ | Safety<br>Antioxidant effects<br>Effects on circulating lymphocytes<br>Chemopreventive potential | [23] |
| Randomized, double-blind, placebo-controlled | MSE 150 mg or 300 mg daily vs. placebo                                    | Healthy young volunteers               | N = 42<br>Days: 14                                            | HMW/total APN $\uparrow$<br>LDL-C $\downarrow$ , ALT $\downarrow$                                                                                                                            | Anti-inflammatory<br>Insulin sensitivity<br>Cardioprotective                                     | [25] |

$\uparrow$  upregulation;  $\downarrow$  downregulation; MSE, melinjo seed extract; Res, resveratrol.

**Neuroprotective Effects:** Accumulation of beta-amyloid (A $\beta$ ) oligomers is an important factor in neurodegenerative diseases, such as Alzheimer’s disease and other cognitive disorders. Although resveratrol is well-known for its neuroprotective effects, we are only beginning to understand the role that resveratrol dimers can play in neuronal damage. Seino et al. have demonstrated that by reducing A $\beta$ 42 secretion, downregulating BACE1 and upregulating MMP-14, Gnetin C may be particularly beneficial in diseases characterized by A $\beta$  accumulation [64] (Table 3). Further, Riviere et al. demonstrated 39% inhibition of A $\beta$  fibril formation by Gnetin C and 63% inhibition by resveratrol [73], while four glucosides of Gnetin C were found to be active inhibitors of A $\beta$  [74]. However, regarding potential neuroprotective effects of stilbenes in Parkinson’s disease and anxiety disorders related to MAO-A- and MAO-B-mediated dopamine and serotonin breakdown, it has been shown that stilbenes act as MAO-A and MAO-B inhibitors. Interestingly, while resveratrol showed preferential MAO-A inhibitory activity [75,76], pterostilbene was the most potent MAO-B inhibitor among 13 tested dietary phenolics, including Gnetin C, which did not show appreciable inhibition of either enzyme [75]. Another study indicated that by negatively regulating IFN $\beta$  expression in astrocytoma cells and STAT1 phosphorylation in neuroblastoma cells, Gnetin C could control TLR3-mediated brain inflammation and its neurodegenerative consequences [65]. While there are numerous studies with resveratrol, pterostilbene and other stilbenes in Alzheimer’s disease in vitro and in vivo [77–79], to the best of our knowledge, no in vivo studies involving Gnetin C in Alzheimer’s disease or any other neurodegenerative disease are publicly available.

*Metabolic Effects:* Beneficial effects of MSE in preventing obesity-related disorders and promoting longevity have been shown in mice fed with high-fat diets. Authors found that MSE dose-dependently reduced body weight gain, visceral fat weight and insulin resistance [80].

Non-alcoholic fatty liver diseases (NAFLDs) include a range of conditions such as fatty liver, steatohepatitis, cirrhosis and hepatocellular carcinoma. Advanced cases are often characterized by lipid deposition, inflammation and fibrosis. In their study using a high-fat choline-deficient (HFCD) diet-induced NAFLD mouse model, Kabir et al. found that a diet supplemented with MSE, Gnetin C or resveratrol could significantly reduce body and liver weight, lower plasma triglycerides and non-esterified fatty acids (NEFA) and reduce steatosis and hepatic fibrosis [66] (Table 3). Furthermore, Gnetin C was found to improve blood glucose levels and insulin insensitivity and had a greater lipid-lowering effect compared to resveratrol. In addition, Gnetin C significantly reduced the expression of proteins involved in fatty acid synthesis, transport and lipid metabolism as well as markers in the TGF- $\beta$ 1 signaling pathway (Table 3). Another study revealed the inhibitory effect of MSE on endothelial senescence in streptozotocin-induced (STZ)-diabetic mice. Curiously, despite higher Gnetin C plasma concentrations, in vitro experiments using HUVECs revealed that resveratrol had a greater positive effect on key markers of vascular senescence [16]. Finally, Gnetin C in addition to its antioxidant property showed higher potential to reduce fat absorption and control blood glucose levels compared to resveratrol [15] (Table 3).

*Anti-Inflammatory and Anti-Aging Effects:* Dermatological age-related changes are often caused by an accumulation of reactive oxygen species (ROS) that promote oxidative damage to cells. The cytoplasmic SOD1 enzyme is an important antioxidant that protects cells by catalyzing superoxide radicals and restoring a healthy redox balance. Watanabe et al. reported that administration of resveratrol- and MSE-supplemented diets in SOD1-deficient mice provide protective effects against skin age-related diseases by upregulating Col1a1 and Sirt1 levels and reducing p53, a known accelerator of skin aging [81]. In addition, these diets reduce other markers of oxidative stress, namely ROS levels in bone marrow and 8-isoprostone in plasma in SOD1-deficient mice [82]. Antioxidant and anti-inflammatory effects of Gnetin C in comparison with resveratrol were demonstrated in a mouse model of periodontitis. Gnetin C induced greater bone healing compared to resveratrol at each time point during eight days of treatment. Furthermore, Gnetin C was superior to resveratrol in inhibiting IL-1 $\beta$  and oxidative stress markers 8-hydroxy-2'-deoxyguanosine and ROS expression. The antioxidative effect of Gnetin C is likely mediated through Nrf2, an important molecule of the oxidative stress response [67,68] (Table 3). Furthermore, Gnetin C was also suggested as a potential safe and effective skin-whitening agent due to its effective inhibition of melanogenesis [27].

## 6. Gnetin C and Human Clinical Trials

Unfortunately, many clinical trials with natural products (single or combinations) either fail or show conflicting results [59]. Clinical trials with stilbenoids, mainly resveratrol, produced inconsistent data due to differences in trial design, concentrations and formulations used and heterogeneity of the cohort enrolled. The pleiotropic effects of these compounds also influence the interpretation of trial results, confounded further by a lack of solid information regarding mechanistic data on pathways and targets (reviewed in [83–85]). Considering that a major obstacle of resveratrol is its extensive metabolism and low bioavailability, it would make sense to pursue clinical trials with Gnetin C instead, which shows biologically active concentrations in mice and humans [16,17,22,24]. In addition to its improved bioactivity, Gnetin C is characterized by reduced clearance, a longer mean residence time (MRT) and overall greater systemic exposure compared to resveratrol



and pterostilbene [8,22]. This superior pharmacokinetic profile makes Gnetin C a more suitable candidate for clinical trials.

So far, five clinical trials (four with MSE and one with Gnetin C) have been performed in healthy volunteers with the goal of establishing pharmacological safety and the chemopreventive potential [21–25] (Table 4). Randomized, double-blind, placebo-controlled trials were well-tolerated and showed anti-inflammatory, antioxidant and cardioprotective effects of MSE and Gnetin C.

We are only beginning to appreciate the benefits of Gnetin C in humans. Many more well-designed clinical trials are needed to evaluate Gnetin C's preventive and therapeutic activity in different diseases including obesity, diabetes, cardiovascular disease and cancer. Strategies such as developing more potent Gnetin C derivatives or suitable nanocarriers that may enhance Gnetin C's activity should be considered.

## 7. Conclusions

Based on the published scientific data currently available, we believe that there is great promise for Gnetin C as a lead stilbene compound to be considered for therapeutic goals in cancers and for a range of other diseases. Although there is much work yet to be done to reveal the full potential of Gnetin C, substantial information already exists to justify the evaluation of Gnetin C in chemoprevention/interception prostate cancer clinical trials, in which only those patients under active surveillance with MTA1 overexpression are recruited. These patients could benefit considerably from dietary interventions containing Gnetin C. Furthermore, trials where Gnetin C is used in combination with endocrine or chemotherapeutic drugs to sensitize and potentiate anticancer efficacy could be considered for patients with advanced prostate cancer.

With the exception of prostate cancer, there is currently a deficiency in comprehensive preclinical evidence for Gnetin C which delays clinical trials in other diseases and cancer types. In addition, further investigation is needed to explore the pharmacokinetics and pharmacodynamics of Gnetin C used alone or in combination with other agents to maximize chemopreventive and therapeutic efficacy.

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## Abbreviations

|              |                                                        |
|--------------|--------------------------------------------------------|
| A $\beta$    | Amyloid beta                                           |
| A $\beta$ 42 | Amyloid beta 42                                        |
| ACC1         | Acetyl coenzyme A carboxylase 1                        |
| AKT          | V-akt murine thymoma viral oncogene (protein kinase B) |
| ALT          | Alanine aminotransferase                               |
| AML          | Acute myeloid leukemia                                 |
| AML-MT       | Acute myeloid leukemia xenograft                       |
| APN          | Adiponectin                                            |
| AR           | Androgen receptor                                      |
| AR-FL        | Androgen receptor-full length                          |
| AR-V7        | Androgen receptor-variant 7                            |

|                |                                                       |
|----------------|-------------------------------------------------------|
| AsPC1          | Human pancreatic cancer cells                         |
| AT1            | Angiotensin II type 1 receptor                        |
| BACE1          | Beta-site amyloid precursor protein cleaving enzyme 1 |
| Bak            | Homolog of Bax                                        |
| Bax            | Bcl-2-associated X protein                            |
| Bcl-2          | B-cell leukemia/lymphoma 2                            |
| B16-F10        | Mouse melanoma cells                                  |
| BFGF           | Basic fibroblast growth factor                        |
| bw             | Body weight                                           |
| CaP8           | Mouse prostate cancer cells                           |
| chREBP         | Carbohydrate response element binding protein         |
| CC3            | Cleaved caspase 3                                     |
| CCL2           | Chemokine (C-C motif) ligand 2                        |
| CCL5           | Chemokine (C-C motif) ligand 5                        |
| CD31           | Cluster of differentiation 31                         |
| CML            | Chronic myeloid leukemia                              |
| Col1a1         | Collagen type I alpha 1 chain                         |
| COS-1          | Monkey kidney cells                                   |
| Cre            | Cre recombinase                                       |
| CRP            | C-reactive protein                                    |
| CRPC           | Castrate-resistant prostate cancer                    |
| CTL            | Cytotoxic T lymphocytes                               |
| DGAT1          | Diacylglycerol acyltransferase 1                      |
| DGAT2          | Diacylglycerol acyltransferase 2                      |
| DU145          | Human prostate cancer cells                           |
| eNOS           | Endothelial nitric oxide synthase                     |
| Enz            | Enzalutamide                                          |
| ERK1/2         | Mitogen-activated protein kinase 1 and 2              |
| ETS2           | ETS Proto-oncogene 2, transcription factor            |
| GnC            | Gnetin C                                              |
| HDAC           | Histone deacetylase                                   |
| HDL-C          | High-density lipoprotein cholesterol                  |
| HEK-293T       | Human kidney cells                                    |
| HFCD           | High-fat choline deficient                            |
| HIF-1 $\alpha$ | Hypoxia-inducible factor 1- alpha                     |
| HL60           | Human leukemia cells                                  |
| HT-29          | Human colon cancer cells                              |
| HUVEC          | Human umbilical vein endothelial cells                |
| IFN- $\beta$   | Interferon beta                                       |
| IFN- $\gamma$  | Interferon gamma                                      |
| IL-1 $\beta$   | Interleukin-1 beta                                    |
| IL-2           | Interleukin 2                                         |
| IL-6           | Interleukin 6                                         |
| i.p            | Intraperitoneal                                       |
| K562           | Human CML cells                                       |
| KH88           | Human CML cells                                       |
| Ki67           | Cellular protein marker of proliferation              |
| LDH            | Lactate dehydrogenase                                 |
| LDL-C          | Low-density lipoprotein-cholesterol                   |
| LDLR           | Low-density lipoprotein receptor                      |
| LNCaP          | Human prostate cancer cells                           |
| Luc            | Luciferase                                            |
| MAO-A          | Monoamine oxidase A                                   |
| MAO-B          | Monoamine oxidase B                                   |

|                  |                                                                      |
|------------------|----------------------------------------------------------------------|
| MCF7             | Human breast cancer cells                                            |
| miR-22           | MicroRNA-22                                                          |
| miR-34a          | MicroRNA-34a                                                         |
| MMP-14           | Matrix metalloproteinase 14                                          |
| MRT              | Mean residence time                                                  |
| MSE              | Melinjo seed extract                                                 |
| MTA1             | Metastasis-associated protein 1                                      |
| MTP              | Microsomal triglyceride transfer protein                             |
| mTOR             | Mammalian target of rapamycin                                        |
| MV4              | Human AML cells                                                      |
| NAFLD            | Nonalcoholic fatty liver disease                                     |
| NEFA             | Nonesterified fatty acids                                            |
| NF- $\kappa$ B   | Nuclear factor kappa-light-chain-enhancer of activated B cells       |
| NK               | Natural killer cells                                                 |
| Nrf2             | Nuclear factor erythroid 2-related factor 2                          |
| NuRD             | Nucleosome remodeling deacetylation complex                          |
| Oun1             | Human CML cells                                                      |
| p21              | Cyclin-dependent kinase inhibitor 1A                                 |
| p4EBP1           | Eukaryotic translation initiation factor 4E-binding protein 1        |
| p53              | Tumor protein p53                                                    |
| PAI1             | Plasminogen activator inhibitor-1                                    |
| p-AKT            | Phosphorylated AKT                                                   |
| Pan-02           | Mouse pancreatic cancer cells                                        |
| PANC-1           | Human pancreatic cancer cells                                        |
| PC3              | Human prostate cancer cells                                          |
| PC3M             | Human prostate cancer cells                                          |
| PGC-1 $\alpha$   | peroxisome proliferator-activated receptor gamma coactivator 1 alpha |
| PIN              | Prostatic intraepithelial neoplasia                                  |
| PPAR $\alpha$    | Peroxisome proliferator-activated receptor alpha                     |
| PPAR $\gamma$    | Peroxisome proliferator-activated receptor gamma                     |
| pS6K             | p70-S6 Kinase                                                        |
| PSA              | Prostate specific antigen                                            |
| PTEN             | Phosphatase and tensin homolog                                       |
| PWPE1            | normal prostate epithelial cells                                     |
| R26              | Rosa26                                                               |
| Res              | Resveratrol                                                          |
| ROS              | Reactive oxygen species                                              |
| 22Rv1            | Human prostate cancer cells                                          |
| SA- $\beta$ -gal | Senescence-associated beta-galactosidase                             |
| SAHA             | Suberoylanilide hydroxamic acid                                      |
| shMTA1           | Short-hairpin MTA1                                                   |
| Sirt1            | Sirtuin 1                                                            |
| SOD1             | Superoxide dismutase 1                                               |
| STAT1            | Signal transducer and activator of transcription 1                   |
| STZ              | Streptozotocin                                                       |
| Tg               | Triglyceride                                                         |
| TGF $\beta$      | Transforming growth factor beta                                      |
| THP1             | Human leukemia cells                                                 |
| TLR3             | Toll-like receptor 3                                                 |
| U937             | Human lymphoma cells                                                 |
| VCAM-1           | Vascular cell adhesion molecule-1                                    |
| VCaP             | Human prostate cancer cells                                          |
| VEGF             | Vascular epidermal growth factor                                     |
| WT               | Wild type                                                            |

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