Evidence-Based Complementary and Alternative Medicine

# Natural Products as a Source for New Leads in Cancer Research and Treatment

Lead Guest Editor: Célia Cabral
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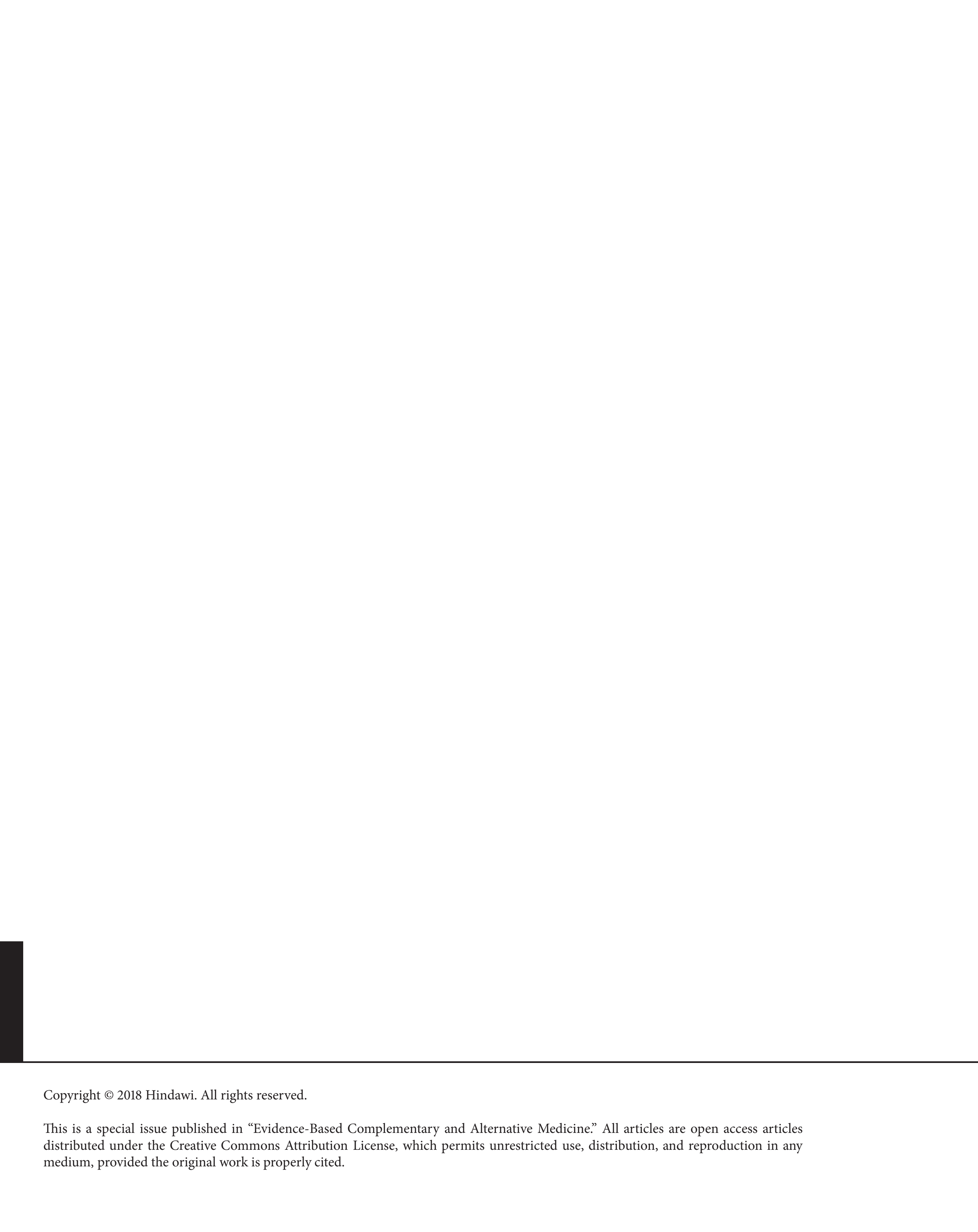

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*Editorial*

# Natural Products as a Source for New Leads in Cancer Research and Treatment

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Cancer is a general term that refers to over 100 distinct pathologies affecting many different tissues and cell types. However, all forms of cancer are characterised by abnormal cell growth resulting from inherited or environmentally induced genomic instability and mutations. Cancer involves cellular transformation, dysregulation of apoptosis and other types of cell death, increased proliferation, angiogenesis, immune evasion, inflammatory responses, and, ultimately, metastatic spread.

The development of a new class of anticancer drugs that lacks the toxicity of conventional chemotherapeutic agents unaffected by common mechanisms of chemoresistance would be a major advance in cancer therapeutics. Several important chemotherapy agents, such as taxanes, certain topoisomerase inhibitor, and *Vinca* alkaloids, were originally identified from natural sources. Therefore, with a myriad of organisms yet to be explored, and new technologies employed, biologically active compounds obtained from natural products will certainly continue to offer vast opportunities as sources of new anticancer therapeutic leads.

For this special issue, focusing on natural products as source for new leads in cancer research and treatment, we have invited authors to contribute with research and review articles illustrating and stimulating the continuing effort in drug discovery and drug development using natural products as source of bioactive molecules in cancer research and treatment. Studies relevant to unveiling of potential targets in cancer biology and the development of innovative therapeutic strategies were also welcome.

From submissions, nine high-quality manuscripts were selected covering the above-mentioned topics.

With the aim of providing a good coverage of the state of the art of this field, this special issue starts with three review papers, followed by six research papers. The first paper (by P. A. Ayeka) is a critical review on mushroom immune modulating potential in cancer. This is followed by another review regarding anticancer properties of natural products, especially essential oils (by K. Blowman et al.). The review third paper dealt with the main characteristics of the studies using flavonoids in the treatment of hepatocellular carcinoma in murine models (by E. R. García et al.).

The remaining papers described original research studies focused on a wide range of topics regarding novel anticancer actions of natural products from different sources, J.-T. Pai et al. revealed that suppression lung cancer cells migration and invasion with propolin C treatment occurred through EMT regulation. The authors also showed how downregulation EGFR-mediated PI3K/Akt and ERK signaling pathways

involved in propolin C-regulated EMT in EGFR-mutated lung cancer cells. L. Yanwei et al. proposed that the traditional herbal formula NPC01 could exert its antitumor effect by suppressing the PI3K/Akt/mTOR signaling pathway. The study performed by M. Valenzuela et al. showed novel research concerning anticancer properties of selected grape juice extracts (GJE) by revealing selective cytotoxicity and the ability to reduce invasiveness of colon cancer cells. H. Li et al. evaluated tumor growth inhibition of human hepatocellular carcinoma HepG2 cells in a nude mouse xenograft model by the total flavonoids from *Arachniodes exilis*. Next, the MMP inhibitory effects of flavonoid glycosides from edible medicinal halophyte *Limonium tetragonum* were evaluated in a study by Bae and colleagues. Finally, the last study in this special issue, conducted by Y. Zhu and S. Bu, evaluated the effect of curcumin in human pancreatic cancer cells, revealing that it induces autophagy, apoptosis, and cell cycle arrest.

Overall, the current special issue on natural products as leads for the development of novel anticancer drugs clearly demonstrates that natural resources are still very attractive to find and develop novel tools for the treatment of cancer patients. Taking the phytochemical isolation of novel compounds and their cellular and molecular investigation in *in vitro* cancer cells as a basis, *in vivo* experimentation and clinical trials in human cancer patients have to follow to pave the way for new drugs with better pharmacological features concerning efficacy to kill tumor cells and safety to spare side effects towards normal tissues.

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*Review Article*

# Potential of Mushroom Compounds as Immunomodulators in Cancer Immunotherapy: A Review

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Since time immemorial, plants and their compounds have been used in the treatment and management of various ailments. Currently, most of conventional drugs used for treatment of diseases are either directly or indirectly obtained from plant sources. The fungal group of plants is of significance, which not only provides food directly to man but also has been source of important drugs. For instance, commonly used antibiotics are derived from fungi. Fungi have also been utilized in the food industry, baking, and alcohol production. Apart from the economic importance of the microfungi, macrofungi have been utilized directly as food, which is usually got from their fruiting bodies, commonly known as mushrooms. Due to their richness in proteins, minerals, and other nutrients, mushrooms have also been associated with boosting the immune system. This makes mushrooms an important food source, especially for vegetarians and immunosuppressed individuals including the HIV/AIDS persons. In complementary and alternative medicines (CAMs), mushrooms are increasingly being accepted for treatment of various diseases. Mushrooms have been shown to have the ability to stimulate the immune system, modulate humoral and cellular immunity, and potentiate antimutagenic and antitumorigenic activity, as well as rejuvenating the immune system weakened by radiotherapy and chemotherapy in cancer treatment. This potential of mushrooms, therefore, qualifies them as candidates for immunomodulation and immunotherapy in cancer and other diseases' treatment. However, a critical review on mushroom's immune modulating potential in cancer has not been sufficiently addressed. This review puts forward insights into the immune activities of mushroom associated with anticancer activities.

## 1. Introduction

Humanity continues to suffer the scourge of cancer, a disease that is associated with uncontrolled cell growth. In 2013, it was reported to be among the leading causes of death, second to cardiovascular diseases. It is estimated that death due to cancer will rise to thirteen million in 2030 [1–3]. The fight against cancer has intensified in the past decades with multidirectional approach including behavioral and dietary change, chemotherapy, radiotherapy, surgery, and recently immunotherapy. Unfortunately, these approaches are not void of serious side effects spanning from recurrence and weakened immune system to reduced quality of life (QoL) of patients. This has raffled scientists, leading to concerted efforts of finding better therapies that, apart from managing the cancerous cells, boost the immune system to fight cancer and other diseases [4]. Among these therapies, complementary and alternative medicine (CAM) has been fronted as an alternative due to its potential of holistic treatment including augmenting the immune system. Many CAMs are plant-derived, including algae and mushrooms that have been used widely in many parts of the world, where they are regarded as biological response modifiers (BRMs) and immunoceuticals [5]. Mushrooms are the spore-producing reproductive structures of fungi. Ancient classification placed fungi in plant kingdom, but current classification recognizes fungi as an independent group of organisms under the kingdom Mycota, basically due to possession of chitin within their cell walls. Mushrooms are the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its substrate, mainly by the Basidiomycota and Ascomycota group. Although in wild mushrooms are seasonal and can

Table 1: Bioactive compounds from mushrooms with anticancer activity.

| Mushroom | Cancer | Common name | Compound/extract |
|---|---|---|---|
| *Agaricus bisporus* | Breast, colorectal | White button | Polysaccharides, lectin |
| *Ganoderma lucidum* | Breast, colorectal, cervical, prostate, liver, and lung | Lingzhi/reishi | *Ganoderma* polysaccharides, polysaccharide-peptides |
| *Coriolus versicolor* | Breast, colorectal, and skin | Yun Zhi | Krestin, PSK, PSP |
| *Lentinus edodes* | Cervical/ovarian, gastric, and skin | Shiitake | Lentinan |
| *Grifola frondosa* | Breast and bladder | Maitake | Grifolan, Maitake D fraction |
| *Agaricus blazei* | Leukemia, hematological, stomach, and lung | Brazilian | *Agaricus* polysaccharides |
| *P. tuber-regium* | Liver | King tuber | Pleuran |
| *Flammulina velutipes* | Skin | Winter | Flammulin |

Modified from Roupas et al. (2012).

be collected and used, they can be domesticated through spore or tissue culture in the laboratories. There are over 14,000 mushroom species but only about 3000 are edible, with approximately 700 exhibiting medicinal properties and 1% being poisonous [6]. For many years, mushrooms have been associated with nutritional and medicinal properties including immune modulation and antitumor properties [6–11]. Edible mushrooms, according to research, are believed to strengthen the immune system by exerting their effects on cellular activities, secondary production of chemical compounds that boost the immune system, and helping treat diseases and restore cell immunity destroyed by radiation and chemotherapy, and this is linked majorly to $\beta$-glucans [12, 13].

A key, frequently reported protective mechanism exerted by mushrooms against cancer is the capacity to stimulate the immune system response, where beta-glucan, a water-soluble polysaccharide, activates immune cells and proteins and macrophages, T cells, natural killer cells, and cytokines that attack tumor cells [10]. White button mushroom *Agaricus bisporus* is an example of dietary mushrooms; apart from having bioactive antioxidants and anticarcinogenic substances, these bioactive compounds also alter aromatase enzyme activity. This enzyme is involved in the conversion of androgens to proliferative estrogenic intermediates which are closely linked to breast cancer development [14, 15]. Furthermore, nonpolysaccharide constituents in species like Shiitake and Oyster mushrooms have biological activity against murine skin cancer and human prostate carcinoma cells [16]. The antitumor and immunomodulation activity of mushroom is exhibited by both crude fungal extracts and pure compounds. The polysaccharide fraction that is mainly composed of $\beta$-glucans present in the cell walls is responsible for immune modulating effects in a number of ways including activating phagocytic activity and production of reactive oxygen intermediates, inflammatory mediators, and cytokines production [7, 10, 17].

## 2. Selected Medicinal Mushrooms and Their Anticancer Activity

Mushrooms can either be edible, medicinal, or poisonous. Many mushroom species, either edible or poisonous, contain bioactive compounds that are of significance to human health.

Mushroom cell walls contain two important compounds, chitin and $\beta$-glucans. Of these two, $\beta$-glucans $\beta(1\to3)$, $\beta(1\to4)$, and $\beta(1\to6)$ make mushroom of significance in health and treatment of various diseases [18–20]. In addition to these compounds, there are other important components in mushrooms. They include polysaccharides, polysaccharide-protein complexes, agaritine, ergosterol, selenium, polyphenols, and terpenoids. Apart from therapeutic properties associated with these compounds, they are generally regarded as biological response modifiers (BRMs). Both *in vitro* and *in vivo* experiments support the therapeutic activities of mushroom compounds. These compounds modulate the immune system to fight tumors and other diseases. These include augmenting the immune system through stimulating lymphocytes, NK cells, and macrophages, enhancing cytokine production, inhibiting proliferation of cancer cells, promoting apoptosis, and blocking angiogenesis, in addition to being cytotoxic to cancer cells [21, 22]. These compounds come in contact with intestinal cells, the frontline of intestinal immune system which interacts with the antigens, thereby playing a role in intestinal immune response and inducing inflammatory response if necessary [23]. Mushroom-derived polysaccharides and polysaccharide-protein complexes are considered as one of the major sources of therapeutic agents for immunomodulatory and antitumor properties [21, 24]. More than 50 mushroom species have yielded potential immunoceuticals with immunomodulatory and antitumor effects *in vitro* and *in vivo* and also in human cancers. They include lectins, polysaccharides, polysaccharides-peptides, polysaccharide-protein complexes like lentinan, schizophyllan, polysaccharide-K, polysaccharide P, active hexose correlated compounds (AHCC), and Maitake D fraction. These compounds are derived from *Ganoderma lucidum, G. tsugae, Schizophyllum commune, Sparassis crispa, Pleurotus tuber-regium, P. rhinoceros, Trametes robiniophila* Murill, *Coriolus versicolor, Lentinus edodes, Grifola frondosa,* and *Flammulina velutipes*, among others [17]. These mushrooms are associated with the treatment of various cancers including breast, colorectal, cervical, skin, liver, ovarian, bladder, prostate, gastric, skin, lung, leukemia, and stomach cancers (Table 1).

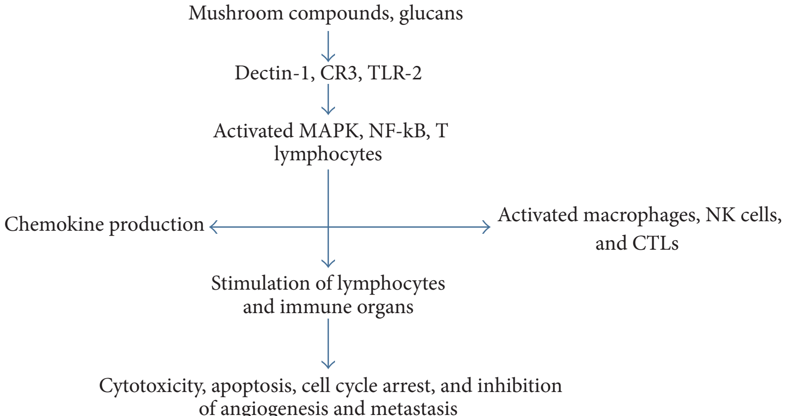


Figure 1: Probable immunomodulation mechanism of action of mushroom glucans. They utilize Dectin-1, CR3, and TLR-2 leading to activation and signal transduction of T lymphocytes, MAPK, and NF-kB, in turn leading to chemokine production and activation and stimulation of lymphocytes, macrophages, and NK cells, which results in inhibition of cancer proliferation through either direct toxicity, apoptosis, and cancer cell cycle arrest or hindering angiogenesis and metastasis of cancer cells.

Mushroom compounds utilize different mechanisms to modulate immunity system in cancer treatment. For instance, water extracts of *Agaricus blazei* Murill (AbM) fruiting bodies induce production of TNF-alpha, IL-8, and NO− [25]; it is low molecular weight polysaccharides that suppress tumor growth and angiogenesis *in vivo* [26], and they contain agaritine and ergosterol which are capable of inducing apoptosis in leukemia cells and inhibit tumor-induced angiogenesis [27, 28]. *Ganoderma lucidum* polysaccharides and triterpenoids are potent inhibitors of tumor growth *in vitro* and *in vivo* [14]. Furthermore, extracts of *G. lucidum* and *G. tsugae* are able to inhibit growth of colorectal cancer cells *in vitro* [29]. Schizophyllan, from *Schizophyllan commune*, a $\beta(1\text{–}3)$ and $\beta(1\text{–}6)$ D-glucan, is less effective against gastric cancer but increases survival of patients with head and neck cancer. In cervical cancer, it prolongs survival and time to recurrence for stage II, and it is more effective when injected directly to the cancer mass [30], suggesting a direct cytotoxicity effect to tumor cells. There is also a remarked increase in monocytes and granulocytes in blood and spleen, leading to production of IL- 6 and IL- 8 after use of Cauliflower mushroom *(Sparassis crispa)*, suggesting that it has immunomodulatory properties [31]. Other mushrooms like *P. tuber-regium* and *P. rhinoceros* polysaccharides have antitumor effects, where they are able to induce expression and proliferation of NK cells, macrophages, and T helper cells in mice [32–34] and *Trametes robiniophila* Murrill (Huaier), an officinal fungus in China, has been applied in TCM for approximately 1600 years [35] and its proteoglycans display apoptosis, antiangiogenesis, drug resistance reversal, antimetastasis, and system immune activation. Table 1 highlights selected mushrooms studied in various cancers.

## 3. Mechanism of Modulating the Immune System by Anticancer Mushroom Compounds

Mushroom compounds are known to fight cancers through modulating both innate (nonspecific) and adaptive (specific) immune systems. The response of an immune system after invasion by antigens heavily relies on interaction between host pattern recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs). PRRs initiate innate immunity through pathogen recognition, while toll-like receptors (TLRs) initiate signaling pathways that coordinate innate immunity and trigger adaptive immunity against various pathogens [56]. Mushroom cell walls have compounds, especially $\beta$-glucans, which are thought to be a major PAMP involved in initiating an immune response. The receptors of $\beta$-glucans, Dectin-1, are expressed on dendritic cells, macrophages, neutrophils, and monocytes [57, 58]. Binding of Dectin-1 and $\beta$-glucans leads to signal transduction which in turn activates T cells, mitogen activated protein kinases (MAPK), and nuclear factor kappa B (NF-kB), resulting in cytokine production [59, 60]. More so, mushroom compounds are recognized by the PRR, by utilizing the Dectin-1, toll-like receptor 2 (TLR-2), and the complement receptor 3 (CR3). PAMP binds to TLR2 initiating the adaptive immunity and PAMP-PRR on monocytes, dendritic cells, granulocytes, and NK cells of the innate immune system [61–65] leading to activation of immune cells, cytokine production, and expression of adhesion molecules [66, 67], as illustrated in Figure 1.

In addition, glucans, which are pharmacologically important compounds of mushrooms, are resistant to acid and therefore they are able to pass through the stomach to the duodenum, where they interact with receptors, activating them to produce lysozyme, reactive oxygen radicals, and nitrogen oxides. These in turn stimulate the production of cytokines that activate phagocytes and leukocytes, leading to local or systemic immunity [68–70].

The efficiency of beta-glucans to activate leukocytes is dependent not only on their conformation but also on solubility in water, molecular weight, and degree of substitution and branching. Their pharmacological activity can be linked to interaction with specific $\beta$-glucopyranose receptors on leukocytes. This interaction activates leukocytes, which in

turn stimulate phagocytosis, cytotoxicity, and production of cytokines by leukocytes [71, 72].

## 4. Effects of Mushroom Compounds on Cytokine Production

Mushroom compounds exert their immune modulating properties through a variety of molecular mechanisms. Some upregulate genes which leads to production of anti-inflammatory and anticancer cytokines. Studies with mushroom compounds have shown that a number of genes and cytokines are variously affected following *in vitro* and *in vivo* treatments. Cytokines are the messengers of the immune system and are either proteins or glycoproteins, secreted by immune cells, to regulate innate and adaptive immune system [6]. Following an oral uptake of mushrooms/mushroom compounds, intestinal immune factors are activated, that is, dendritic cells and macrophages that secrete cytokines that initiate local or systemic immunity. Intestinal epithelial cells are also stimulated to secrete IL-7, an important cytokine in cancer immunotherapy [73, 74].

Incubation of promonocytic THP-1 cells with *Agaricus blazei* Murill extract upregulates many genes that are associated with anticancer chemokines, leading to secretion of a number of cytokines such as IL-23$\alpha$ subunit in the IL-12 family, IL-1$\beta$, monocyte chemoattractant protein-1 (MCP-1), granulocyte colony stimulating factor (G-CSF), and tumor necrosis factor-alpha (TNF-$\alpha$) [27, 36]. Furthermore, Volman et al. [75] showed that *Agaricus bisporus* fruit bodies, caps, and stipe increase TNF-$\alpha$ production by bone marrow derived macrophages (BMM).

*Ganoderma lucidum*, on the other hand, is longevity-promoting tonic herb and the biological activities, especially antitumor and immunomodulatory properties, include stimulating T cells and inflammatory response by expression and production of chemokines including IL-1, IL-2, IL-6, TNF-$\alpha$, and interferon-gamma (IFN-$\gamma$) [4, 41, 42]. Grifolan from *Grifola frondosa* promotes macrophage activities by increasing IL-1, IL-6, and IL-8 production, ultimately activating and increasing the number of leukocytes [45–47, 76]. Other compounds from mushrooms such as polysaccharide peptide (PSP), polysaccharide (PSK), and lentinan provoke *in vitro* secretion of varied cytokines, namely, IL-1, IL-2, IL-6, IL-8, TNF, and interferons [49].

In addition, Bittencourt et al. [52] demonstrated that $\alpha$-glucan from *Pseudallescheria boydii* stimulates *in vitro* secretion of TNF-$\alpha$ and IL-12. The increased secretion of IL-12 indicates a polarization of naïve T cells into T helper (Th) type 1 skewed responses which are important in fighting cancer cells [37, 77]. The extract from *Sparassis crispa* stimulates splenocytes to secrete cytokines in mice and this is triggered by granulocyte macrophage colony stimulating factor (GM-CSF) and Dectin-1, which is $\beta$-glucan receptor [48].

## 5. Effect of Mushroom Compounds on Immune Cells

Mushroom compounds injected directly into tumor cells or taken orally activate the immune cells to initiate a cell mediated or direct cytotoxicity on tumor cells after being recognized by pathogen recognition receptors. Compounds like lentinan increase the production of cytotoxic T lymphocytes and macrophages and also induce nonspecific immune responses [49]. *Pleurotus tuber-regium* and *P. rhinoceros* extracts confer antitumor effects by promoting maturation of lymphocytes and NK cells and increasing macrophages proliferation, T helper cells, and CD4/CD8 ratio and population, which is accompanied by increase in weight and size of spleen, and this increase is attributed to the higher numbers of monocytes and granulocytes among other immune cells [32–34, 78]. Therefore, consumption of mushroom compounds initiates innate and adaptive immunity by enhancing immune-surveillance against cancer by involving monocytes, macrophages, NK cells, and B cells, CTLs secretion antitumor related cytokines and activation of immune organs, getting rid of cancers, and strengthening the weakened immune system [64, 65]. These actions by mushroom compounds lead to cancer cell apoptosis, cell cycle arrest, and prevention of angiogenesis and metastasis.

## 6. Inhibition of Proliferation and Cell Cycle Arrest by Mushroom Compounds

Various cancers, including hematological cancers in mouse and leukemia in humans, among other tumors are inhibited by mushrooms [27, 79]. Their mechanism of action is varied and is believed to include induction of apoptosis and upregulation of apoptosis inducing genes as well as arrest of cell division *in vitro and in vivo* [38, 39].

Mushroom compounds injected into tumor mass lead to apoptosis of the cells at different stages of cell cycle to curb tumor cell proliferation. For instance, lentinan and lectins from Shiitake are directly cytotoxic and cytostatic to MCF-7 breast cancer cells [53, 54]. They also show anti-inflammatory effect by reducing levels of neoangiogenic and granulocyte-chemoattractant factor IL-8 and increase infiltration of cytotoxic T cells by reducing intratumor formation of reactive oxygen and nitrogen species and ameliorating the skewed Th1/Th2 balance in late cancers [37, 40, 80–82]. This ability of phagocytes to infiltrate makes them important in eliminating advanced tumors by phagocytosis and secretion of cytokines for direct or indirect antitumor activities and antibody dependent cell mediated cytotoxicity (ADCC) [83]. Suppression of cell motility and blocking vasculature in tumor microenvironment is a good indicator for inhibition of cancer metastasis and proliferation. *Ganoderma lucidum* has the potential of suppressing cell motility, inhibiting cell proliferation, inducing apoptosis, and suppressing angiogenesis of highly invasive human breast and prostate cancer cells [43, 44]. PSK, on the other hand, when injected directly into human stomach tumors prior to surgery is quickly taken up by dendritic cells in and around the tumors, improving the survival and QoL of stomach cancer patients [50]. Thus, PSK has a direct cytotoxic effect on cancer cells. According to Hsu et al. [29], methanol extracts of *G. lucidum* and *G. tsugae* inhibit the growth of colorectal cancer cells within 72 hrs by downregulating cyclin A and B1 and upregulating p21 and p27, thereby arresting the cell cycle in G2/M, and thus they

Table 2: Summary of studies on the mechanism of action of mushrooms compounds.

| Mushroom | Biological activity | Study | Reference |
|---|---|---|---|
| *Agaricus blazei* Murill (AbM) | Secretion of IL-8, TNF-α, and NO production by macrophages, inhibition of cancer cell growth, upregulation of expression and secretion of anticancer gene and cytokines IL-23, IL-12, IL-1, MCP-1, G-CSF, and TNF-α, apoptosis, and NK activation | *In vitro* | [25, 27, 36–40] |
| | Suppress tumor growth and inhibit angiogenesis, stimulate cytokine and leukocyte growth factors production, amelioration of skewed Th1/Th2 balance | *In vivo* | [26] |
| *Agaricus bisporus* | Induce apoptosis, inhibit angiogenesis, stimulate TNF-α production by BMM | *In vitro* | [27] |
| *Ganoderma lucidum* | Cytotoxic to cancer cells, inhibit cancer cell growth, stimulate T cells, upregulate expression and secretion of IL-1, IL-2, IL-6, TNF-α, and IFNγ, suppress cell motility and angiogenesis, inhibit proliferation and induce apoptosis, downregulate cyclins A and B and upregulate p21 and p27, arrest cell cycle | *In vitro* | [4, 14, 29, 41–44] |
| *Ganoderma tsugae* | Inhibit cancer cell growth, downregulate cyclins A and B and upregulate p21 and p27, arrest cell cycle | *In vitro* | [29] |
| *Grifola frondosa* | Activate macrophages, stimulate production of IL-1, IL-6, and IL-8, stimulate leukocytes | *In vitro* | [45–47] |
| *Sparassis crispa* | Augment immune system, enhance IL-8 synthesis, activate leukocytes | *In vitro* | [31] |
| | Stimulate splenocytes to secrete cytokines | *In vivo* | [48] |
| *Pleurotus tuber-regium* | Stimulate proliferation of NK cells, macrophages, and T cells | *In vitro* | [32–34] |
| | Maturation of lymphocytes, NK cells, and macrophages, increase weight and size of spleen | *In vivo* | [32–34] |
| *Polyporus rhinoceros* | Stimulate proliferation of NK cells, macrophages, and T cells | *In vitro* | [32–34] |
| *Schizophyllum commune* | Prolong life of head/neck/cervical cancer patients | Clinical | [30] |
| *Trametes robiniophila* | Apoptosis, antiangiogenesis, antimetastasis, drug resistance reversal, activation of immune system | clinical | [35] |
| | Apoptosis, G0/G1 cell cycle arrest, and Cell damage | *In vitro* | [22] |
| *Coriolus versicolor* | Invoke secretion of cytokines IL-1, IL-2, IL-6, IL-8, TNF-α, and TNF | *In vitro* | [49] |
| | Improve survival of stomach cancer patients | Clinical | [50] |
| *Coprinus comatus* | Inhibit cancer cell proliferation | *In vitro* | [51] |
| *Pseudallescheria boydii* | Stimulate secretion of IL-12 and TNFα | *In vivo* | [52] |
| *Coprinellus* sp. | Inhibit cancer cell proliferation | *In vitro* | [51] |
| *Lentinula edodes* | Stimulate secretion of IL-1, IL-2, IL-6, IL-8, TNF-α, and TNF, cytotoxic and cytostatic to breast cancer cells, inhibit proliferation cancer cells, inhibit DNA synthesis | *In vitro* | [49, 53–55] |
| *Flammulina velutipes* | Inhibit cancer cell proliferation | *In vitro* | [51] |

are able to suppress tumor growth, induce cell death, and inhibit cell proliferation in human colorectal cancer cells *in vivo*. Volman et al. [75] confirmed that there is modulation of the immune response of enterocytes, where extracts from mushrooms lower the transactivation of NF-kB in Caco-2 cells, with *A. blazei* Murill and *Coprinus comatus* having the pronounced decrease in NF-kB transactivation, which can cause tumor cells to stop proliferating, die, or become sensitive to the action of antitumor agents. In addition, *L. edodes* fruit body water extracts exhibit inhibitory effects on the proliferation of MCF-7 cells and DNA synthesis, indicating that the cytostatic effect of this mushroom extract is much potent on cell cycle of cancer cells [55]. MCF-7 cells treated with Huaier *(Trametes robiniophila)* extract show G0/G1 arrest leading to cell damage and apoptosis [22] and hot water extracts of *Coprinellus* sp., *C. comatus*, and *Flammulina velutipes* have also shown inhibition of cellular proliferation of MCF-7, MDA-MB-231, and BT-20 cells [51].

Suffice it to say, researches have proven that mushrooms compounds exhibit anticancer potential in *in vitro*, *in vivo*, and clinical studies as summarized in Table 2. Therefore, critical research on anticancer mushroom compounds is important in the search for new drug discovery.

## 7. Conclusion and Future Perspective

Bioactive compounds from mushrooms have been shown to activate or modulate the immune system, thereby inhibiting cancer cell metastasis and growth. These compounds work by affecting the maturation, differentiation, and proliferation of immune cells. The major compounds of immune and cancer importance target the gut system, especially intestines as their site of contact and primary action, thereby affecting intestinal immunity and ultimately systemic immunity. These compounds are PAMP and act by interacting with receptors on leucocytes, upregulate genes associated with immunity, increase production of T lymphocytes and cytokines, activate activity of macrophages and cytokines, induce apoptosis, affect cell cycle, and increase infiltration of cytotoxic T cells in tumors. Critical studies on the mechanism of action and development of anticancer agents from mushrooms are very important so as to reduce the burden of cancer and improve quality of life of cancer patients.

Research, therefore, which targets modulation of the immune system to fight cancer, especially from mushroom compounds, is important. Future perspective should therefore be directed towards finding out the molecular mechanisms of different mushroom compounds in cancer immunotherapy and encouraging consumption of mushroom and other natural plant materials due to their holistic treatment. Further studies should be carried out on conservation of biodiversity of mushrooms, and critical analysis should be done to evaluate and compare the pharmacological importance and mushrooms of different regions.

## Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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*Review Article*

# Anticancer Properties of Essential Oils and Other Natural Products

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Essential oils are secondary metabolites with a key-role in plants protection, consisting primarily of terpenes with a volatile nature and a diverse array of chemical structures. Essential oils exhibit a wide range of bioactivities, especially antimicrobial activity, and have long been utilized for treating various human ailments and diseases. Cancer cell prevention and cytotoxicity are exhibited through a wide range of mechanisms of action, with more recent research focusing on synergistic and antagonistic activity between specific essential oils major and minor components. Essential oils have been shown to possess cancer cell targeting activity and are able to increase the efficacy of commonly used chemotherapy drugs including paclitaxel and docetaxel, having also shown proimmune functions when administered to the cancer patient. The present review represents a state-of-the-art review of the research behind the application of EOs as anticancer agents both *in vitro* and *in vivo*. Cancer cell target specificity and the use of EOs in combination with conventional chemotherapeutic strategies are also explored.

## 1. Introduction

Whilst some synthetic compounds unequivocally have an important role in disease prevention and therapy, there is also an extensive collection of naturally existing compounds that have been exploited for their unique medicinal purposes [1]. The use and demand of natural compounds have been increasing worldwide, showing their importance, which can be attributed to relevant medicinal properties [2]. Essential Oils (EOs) and other phytoproducts are examples of natural products that have gained interest, mainly due to their suitable chemical characteristics and biological activities [3].

As stationary organisms, plants have evolved a diverse range of protective mechanisms to lessen their vulnerability against external threats. These mechanisms can be classified as physical and chemical defenses. Physical deterrents include protective structural characteristics, which include waxy barriers, spikes, and "hair-like" trichomes, which release chemical compounds [4]. Chemical defense mechanisms include, for example, the production of a range of defensive metabolites bioactive compounds with the capability to repulse herbivores or even to target their endocrine and nervous system [5, 6]. These include EOs, enzymes, tannins, and flavonoids, amongst others. Importantly, these compounds are also of pharmacological interest.

EOs are complex and multifunctional substances with plant origin, which have been used for thousands of years for their role in the prevention and treatment of various ailments [3, 7, 8]. Chemically, EOs are aromatic plants secondary metabolites with several roles: defense against herbivores, insects, and microorganisms; communication with plants of the same species; and signaling within the plant in response to environmental stimuli [5]. As each plant species or subspecies has evolved to protect itself from a particular predator or group of predators, each plant produces its own specific "signature" mixture of EO chemical constituents [5, 7]. This can contain from 20 to 60 constituents at varying concentrations, with two or three primary constituents (20–70%) [9, 10].

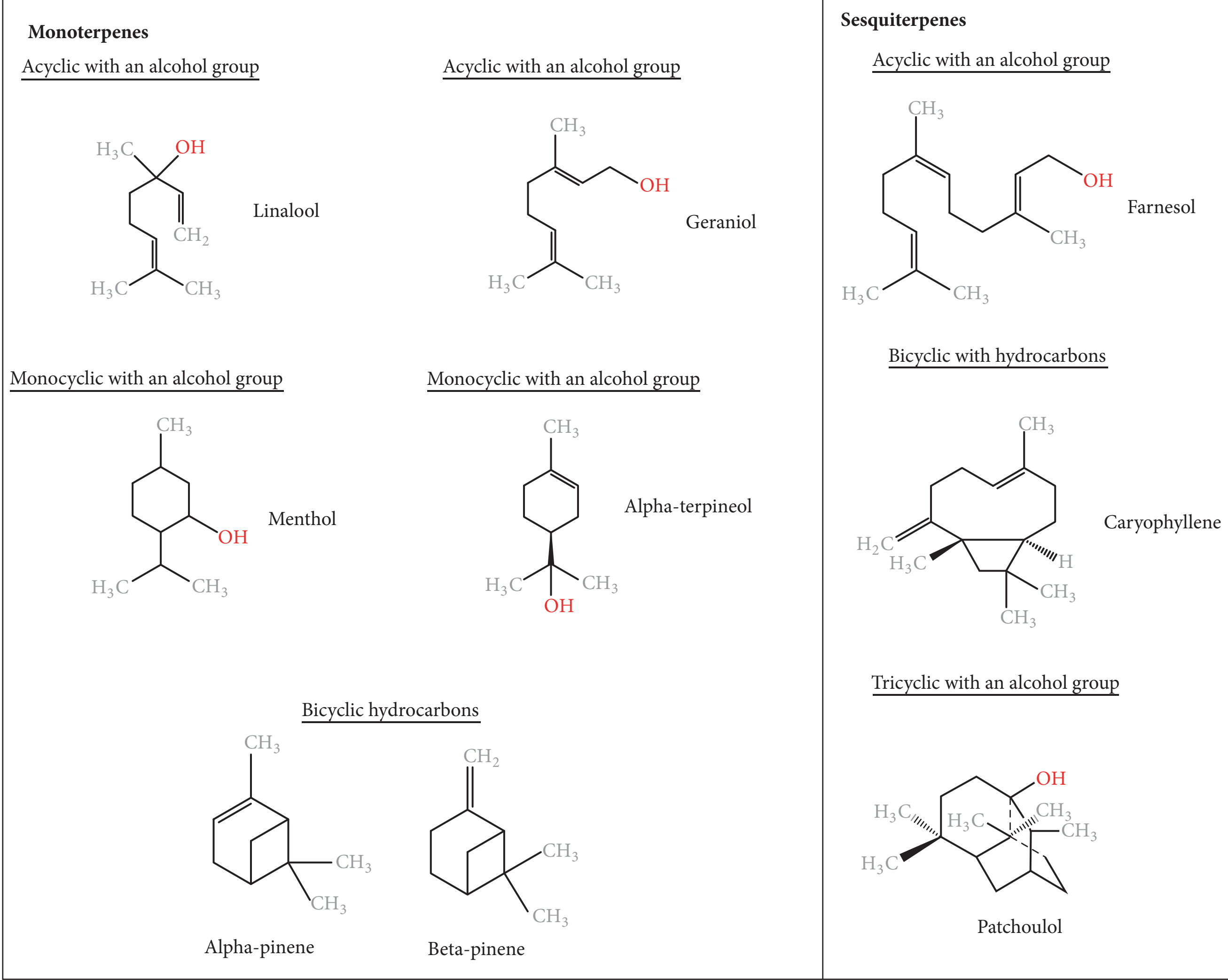


Figure 1: Chemical structures of essential oil constituents.

*1.1. Chemical Composition of EOs.* There are approximately 3000 EOs, from over 2000 different plants, with around 300 EOs possessing known biomedical features [2, 10, 11]. Together with the plant species, the developmental stage (flowering, fruiting) and aromatic compound extraction methods have a direct influence on the composition of EOs, which explains the variability of components in the reported EOs [12].

Based on their chemical compositions, EOs are broadly categorized into oxygenated compounds and hydrocarbons [9]. Oxygenated compounds include esters, aldehydes, ketones, alcohols, phenols, and oxides. Other active groups include aromatics and sulfur-containing components [9–12, 24]. Hydrocarbon compounds are composed of one specific chemical group called terpenes (Figure 1) [9]. These are composed of varying numbers of isoprene units ($C_5$). Monoterpenes ($C_{10}$) and sesquiterpenes ($C_{15}$) are the main terpenes, although the isoprene chains may also include diterpenes ($C_{20}$). Monoterpenes contribute to 90% of EO overall constituents [9]. Both monoterpenes and sesquiterpenes offer a large variety of structures through adjoining with other biologically active functional groups (monoterpenoids), and chemical rearrangement and addition of oxygenated groups (sesquiterpenoids) [9]. Terpenes may also be acyclic, monocyclic, or bicyclic and may contain an aromatic group [9]. The longer the isoprene chain, the more the chemical variations possible [9, 24]. The structures of several medicinally important terpenes are illustrated in Figure 1.

Due to the large range and complex blend of EOs constituents, as well as their many functional groups, it is thought that EOs do not possess a specific single cellular target, with each complex mixture initiating different cellular effects through their major constituents [9, 10]. However, it is important to consider the minor constituents of an EO, and the different cellular effects exhibited when the constituents are combined in the EO blend versus the isolated constituents. A study performed by Santana-Rios and coworkers (2001) isolated the main constituents of both white and green tea and created an artificial "mixed" tea with a total of 9 main constituents [25]. The artificial tea exhibited a

lesser antimutagenic effect than the whole tea extracts in the *Salmonella* assay in the presence of *N*-hydroxy-IQ, a potent mutagen. Furthermore, it has been shown that EOs extracted from the tea tree, eucalyptus, and thyme plants reduced *Herpes simplex* virus- (HSV-) 1 viral infectivity by more than 96% in an *in vitro* study through inactivation of virus-free particles, with the combined EO constituents more effective than the isolated counterparts [26]. Recent studies also have been pointing out the therapeutic potential of the individual constituents of EOs, such as the work of Dias and colleagues (2017), which showed a possible association between the oxygenated monoterpenes of EOs extracted from *Lavandula luisieri* and *Cymbopogon citratus* and the antifungal activity against dermatophytes [27]. This was because an inhibitory effect was observed on the conidial germination, demonstrating the strong antifungal activity of these EOs components [27]. The mentioned studies indicate that minor constituents possess both synergistic and antagonistic activities on the major constituents, playing an important role in the overall properties of EOs on a variety of cell types.

*1.2. EOs as Therapeutic Agents.* Only 5 to 15% higher order plants have been addressed for their bioactive compounds [28]. As EOs are a coevolutionary product of plants, functioning to protect them from herbivore attack, they often elicit undesirable and potentially harmful effects on animal cells and bodily functions [5]. However, these undesirable effects of EOs can be exploited and used to treat diseases and symptoms. Examples include emetics and laxatives, muscle relaxants, cardiac stimulants, and cardiac depressants resulting in hypotension and induction of bradycardia [8].

Atherosclerosis is the arterial build-up of fats and other compounds and is a large contributor to thrombosis and arterial occlusion [29]. The main driver of this disease is the oxidation of low-density lipoproteins (LDLs), and it was shown that phenolic-rich EOs such as thymol and eugenol exhibit the highest LDL antioxidative effect, with their capabilities increased through also reducing LDLs' affinity for the LDL receptor [7]. Other benefits for treating cardiovascular disease, thus reducing the risk of atherosclerosis, include the reduction of cholesterol and triglyceride levels in plasma, in which black cumin oil achieved this reduction in rats over a period of 12 weeks, with low toxicity and no adverse effects in kidneys or liver [7]. Additionally, recent studies have demonstrated the capability of EOs to act on inflammatory and other cellular processes associated with cardiovascular diseases, by preventing the secretion of proinflammatory factors through the reduction of lipopolysaccharide (LPS) [30, 31]. EOs may be used in both analgesics and anti-inflammatories, such as black cumin and eucalyptus oils [32, 33]. It is clear, with respect to recent research, that Eos' ability to bind various cellular receptors has therapeutic value and potential for both treatment of infectious diseases, and for inborn and intrinsic diseases. Importantly, these mechanisms of action of EOs leading to cellular and metabolic responses make them attract new sources of anticancer therapeutic strategies.

The aim of this review is to evaluate the research behind the application of EOs as anticancer agents, both *in vitro* and *in vivo*. Cancer cell target specificity without noncancerous tissue toxicity will be explored, as well as the use of EOs in combination in conventional chemotherapeutic strategies.

## 2. Anticancer Proprieties of EOs

According to the International Agency for Research on Cancer (IARC), in 2012 there were 14.1 million new cancer cases worldwide and 8.2 million cancer deaths [34]. Cancer is now the leading cause of death and is expected to increase by 70% in the next two decades, with lung, liver, stomach, colorectal, breast, prostate, and oesophageal cancer accounting for most of the deaths [34, 35]. These statistics support the need for new and novel chemotherapeutic drugs in the coming years.

Cancer is broadly divided into three stages: (1) initiation, in which cellular DNA damage and mutation occur on carcinogen exposure and due to failure of DNA repair mechanisms; (2) promotion, in which hyperproliferation, tissue remodelling, and inflammation occur due to expansion of initiated cell/s; and (3) progression, in which preneoplastic cells form tumors through clonal expansion, further facilitated by an increase in genomic instability and altered gene expression [36]. The different stages of carcinogenesis require different chemotherapeutic approaches, due to the evolutionary nature of cancer, which lead to alterations in sensitivity to therapy. Specifically, tumour progression is associated with genomic instability, through accumulation of mutations for factors involved in cell proliferation, apoptosis, and DNA repair, amongst others [36, 37]. Chemotherapy drugs act on the promotion stage, in ways including cellular proliferation inhibition, increased rate of cell death, and induction of tumor cell differentiation [38].

Although research on the application of EOs as anticancer therapeutic agents is relatively new, approximately half of conventional chemotherapy agents have plant origin, with roughly 25% directly derived from plants, and 25% being chemically modified versions of phytoproducts [28]. One such molecule is paclitaxel. Paclitaxel (of which the most common brand name is Taxol) was originally derived from the bark of the tree *Taxus brevifolia* [39]. Its mechanism of action is based on the induction of a mitotic arrest via the targeting of the cytoskeleton component tubulin, resulting in mitotic checkpoint activation, and subsequent apoptosis [39]. It is used as a therapeutic agent either as a single agent or in combination therapy strategies for various cancer types, including ovarian, breast, and pancreatic cancer [39]. Laboratory synthesis of this drug was needed due to depletion of the natural source, primarily through a synthesis route involving EO constituent patchoulol (Figure 1) to produce patchoulol oxide [40]. More recently, Altshuler and collaborators found that the enantiomer (+)-citronellal, a major component of *Corymbia citriodora* and *Cymbopogon nardus* EOs, is also an effective microtubule-disrupting compound, similarly to better-known microtubule-disrupting agents colchicine and vinblastine [41].

EOs have been shown to possess anticancer properties through various mechanisms, including cancer preventative mechanisms, as well as acting on the established tumor cell

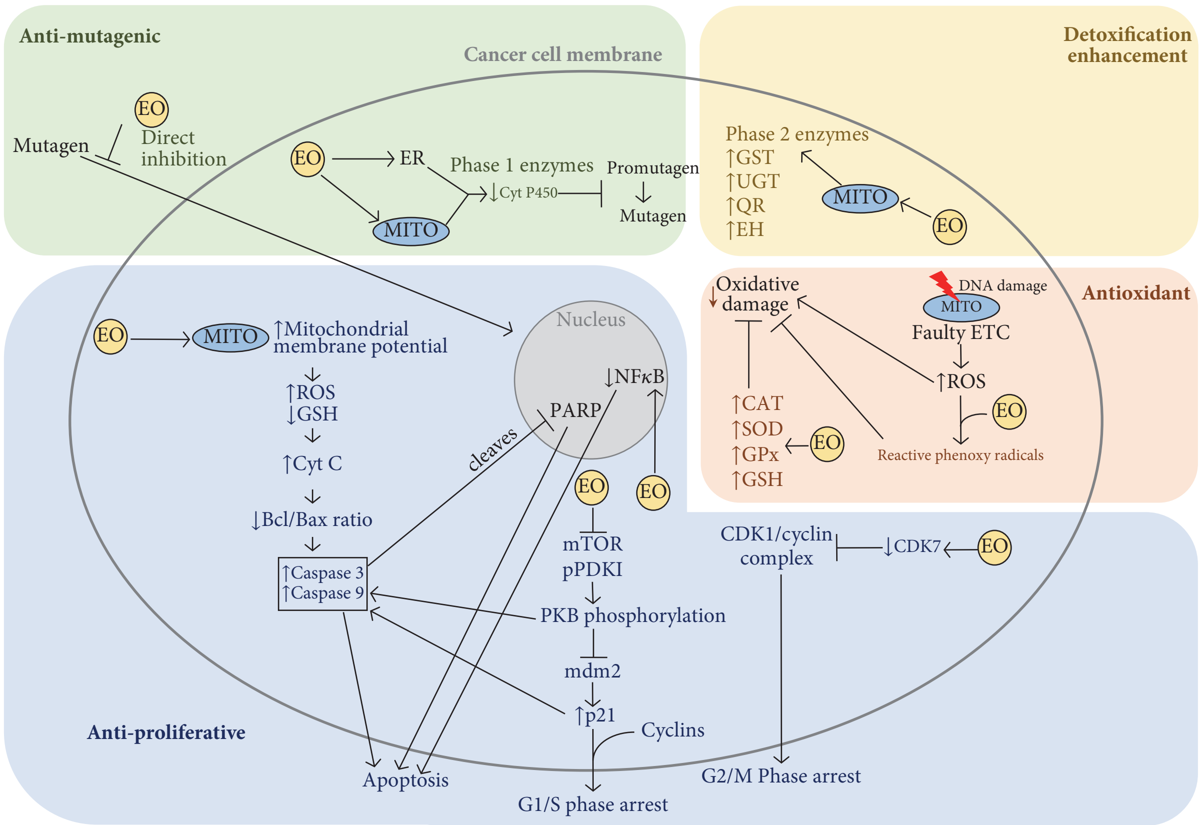


FIGURE 2: *Essential oils cancer preventative and anticancer mechanisms of action*. EOs possess antimutagenic, antiproliferative, antioxidant, and detoxifying capabilities acting on various pathways in the cancer cell as well as cancer preventative capabilities. EOs may directly inhibit mutagen entry into the cell. EOs can decrease phase I enzymes such as CytC, preventing mutagen formation, and increase phase II enzymes such as GST, UGT, QR, and EH for enhanced detoxification. EOs bind ROS forming reactive phenoxy radicals which bind further ROS and increase antioxidative enzymes CAT, SOD, GPx, and GSH thus preventing oxidative damage as a cancer preventative mechanism. EOs disrupt mitochondrial membrane potential causing an increase in ROS and decrease in GSH, release of CytC, resulting in a cascade of disruption in Bcl/Bax ratio, increase in caspase 3 and caspase 9 activity, and PARP cleavage, resulting in apoptosis. EOs suppress mTOR and pPDK1 causing PKB dephosphorylation, which dually acts to initiate caspase activity and deactivate mdm2, causing an increase in p21 to further initiate caspase activity resulting in apoptosis. Increased p21 also induces G1/S phase cell cycle arrest. EOs cause a decrease in CDK7, blocking CDK1/cyclin complex causing G2/M phase cell cycle arrest. Bax: B-cell lymphoma 2-associated X protein; Bcl-2: B-cell lymphoma 2; CAT: catalase; CDK: cyclin-dependant kinase; CytC: cytochrome C; CytP450: cytochrome P450; EH: epoxide hydrolase; EO: essential oil; ER: endoplasmic reticulum; ETC: electron transport chain; GPx: glutathione peroxidase; GSH: glutathione; GST: glutathione *S*-transferase; mdm2: murine double minute 2; mTOR: mechanistic target of rapamycin; MITO: mitochondria; NFκB: nuclear factor-κB; PARP: poly ADP ribose polymerase; pPDK1: protein pyruvate dehydrogenase kinase 1; PKB: protein kinase B; QT: quinone reductase; ROS: reactive oxygen species; SOD: superoxide dismutase; UGT: uridine 5′-diphospho-glucuronosyltransferase.

itself and interaction with the microenvironment (Figure 2) [7, 42].

*2.1. Antimutagenic Proprieties and Detoxification Enhancement.* EO cancer preventative mechanisms include direct inhibition of the mutagen entering the cell, although underlying mechanisms remain unexplained [7, 43]. Other cancer preventives and antimutagenic properties include a decrease of enzymes involved in drug metabolism. These include phase I enzymes such as cytochrome P450 [44, 45]. Phase II enzymes are responsible for detoxification and are mainly comprised of transferases [46]. Glutathione *S*-transferase (GST), uridine 5′-diphospho-glucuronosyltransferase (UGT), quinone reductase (QR), and epoxide hydrolase (EH) were observed to be increased on sulfur-containing EO activity such as that from garlic and onions [47–52]. The EO component citral, a monoterpene obtained from plants such as lemongrass, has been shown to induce phase II enzymes in a dose-dependent manner [53]. The mechanism of action of citral is due to its geranial isoform component [53]. Recent studies have shown citral to inhibit cell proliferation and tumor growth by increasing the intracellular levels of oxygen radicals and, consequently, inducing oxidative stress, leading to reduction of cancer cell proliferation and ultimately resulting in cell death [54, 55].

*2.2. Antiproliferative Mechanisms of Action of EOs.* Key hallmarks of cancer include resisting cell death, sustained

proliferative signaling, and evading growth suppressors [28]. Therefore, therapeutic strategies focused on inducing apoptosis and cellular arrest are of clear significance. EOs have been shown to induce both the intrinsic (or mitochondria-dependent) and extrinsic (or death receptor-dependent) apoptosis pathways.

Girola and coworkers (2015) tested the antitumor properties of a camphene isolated from the EO of *Piper cernuum* in melanoma cells. The study demonstrated that this compound was able to induce apoptosis through the caspase-3 pathway activation, as well as activating the endoplasmic reticulum (ER) stress signaling [56]. Another study focused on the evaluation of the mechanism of action of carvacrol, a phenolic monoterpenoid abundant in the EOs of oregano and thyme [57]. In the metastatic breast cancer cell line MDA-MB-231, carvacrol induced apoptosis via mitochondrial membrane permeabilization, resulting in cytochrome C release, induction of caspases indicated through poly ADP ribose polymerase (PARP) cleavage, and DNA fragmentation [57]. Frankincense extracts obtained from *Boswellia sacra* induced PARP cleavage with apoptosis in MDA-MB-231 cells, with higher cancer cell specificity [14]. Citral was also shown to induce caspase activation and subsequent apoptosis induction in several cancer cell types, including colorectal cancer and glioblastoma [58–60]. Other studies have shown that citral treatment can lead to reduction of expression of prostemness and prosurvival factors such as aldehyde dehydrogenase 1A3 (ALDH1A3) and microtubule affinity regulating kinase 4 (MARK4) in cancer, respectively [61, 62].

PKB (Protein kinase B) is a key molecule with roles regarding cellular metabolism, transcription, cell cycle progression, and survival [63]. The vapor of *Litsea cubeba* seed oil induced cell cycle arrest and apoptosis of nonsmall cell lung carcinoma cells, a cancer type with a high mortality rate [64]. In this study, apoptosis occurred due to a significant decline in the expression of mTOR (mechanistic target of rapamycin) protein, and a decline in the phosphorylating ability of PPDK1 (protein pyruvate dehydrogenase kinase 1), leading to dephosphorylation of PKB and initiating the caspase-dependent apoptosis pathway [64]. Furthermore, PKB dephosphorylation inactivated mdm2 (murine double minute 2), leading to an increase in p21 expression, and subsequent caspase initiation after G1/S phase arrest [64]. This dual mechanism offers antiproliferative as well as antioxidant proprieties, and the vapor can be inhaled directly to the site of cancer in the lung, offering a clear advantage in administration [64].

Wu and colleagues showed that administering organosulphur components of garlic significantly decreased cell viability ($P$ =< 0.05) compared with control in a dose and time-dependent manner, with diallyl trisulphur being the most effective [65]. This was observed in J5 liver tumor cell line through a G2/M cycle arrest, leading to cell death *via* a decrease in expression of cyclin-dependent kinase (CDK) 7 and subsequent CDK1/cyclin complex inhibition [65].

Expression of NF$\kappa$B (nuclear factor-$\kappa$B) is abnormally increased in cancer cells and is particularly associated with cancer initiation and progression [66–68]. $\alpha$-terpineol, a monoterpenoid alcohol, was able to downregulate the transcription of NF$\kappa$B in a range of tumor cells, with the strongest inhibitory effect on small cell lung carcinoma cell line NCI-H69 [69]. Finally, $\alpha$-terpineol was further shown to have synergistic properties with another monoterpene, linalyl acetate, in colon cancer cells, inhibiting NF$\kappa$B expression and resulting in apoptosis [70].

*2.3. Antioxidant Proprieties of EOs.* Mitochondrial DNA damage can result from oxidative stress, and defects on the electron transport chain (ETC) result in the further release of reactive oxygen species (ROS) and further DNA, lipid, and protein damage [71]. Antioxidant properties of EOs can, therefore, contribute to cancer preventative mechanisms [36, 72]. Specific EO components such as eugenol, the main constituent extracted from clove oil, can react with ROS to form reactive phenoxy radicals, which can then combine with further ROS and prevent further damage [73]. Other cancer protective mechanisms induced by EOs include the induction of the expression of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione, as shown by Manjamalai and Berlin Grace [74]. Treatment with EO extracts of *Wedelia chinensis* (96% of the components being carvacrol and trans-caryophyllene) lead to an increase in intracellular antioxidant activity, subsequently leading to a significant reduction in tumor mass volume as well and regeneration of surrounding healthy tissue [74].

However, research by Le Gal and colleagues (2015) showed that increased intracellular antioxidant activities can actually increase tumor cell survival, both using *in vitro* and *in vivo* models [75]. Specifically, oxidized glutathione, an indicator of oxidative stress levels, was increased on antioxidant administration, thus offering protection for the melanoma metastasis cancer cells [75]. This is a similar mechanism as the one observed with conventional chemotherapy drug methotrexate, which is a prooxidant and increases cellular glutathione levels [76]. Therefore, EO extracts with these types of antioxidant properties are likely to be more beneficial as chemopreventive agents for nontumor tissue.

Finally, Legault and colleagues (2000) showed that balsam fir oil extracts led to decreased glutathione levels, mediated by the EO component gamma-caryophyllene, which promotes ROS increase and glutathione decrease due to $\alpha$-humulene in a dose-dependent manner [77].

## 3. Cancer Cell Specificity of Essential Oils

Conventional chemotherapy drugs are more cytotoxic to cancer cells due to their higher rate of cell division; however, due to this mechanism of action, there are issues with tumor cell specificity and associated cytotoxicity to healthy cells [78]. The subsequent side effects in the patient can hinder recovery and even prove to be life-threatening. Currently, combined therapeutic approaches of surgery followed by chemotherapy, radiotherapy, and immunotherapy offer increased chances of treating cancer and remission [78]. However, this does not address the need for cancer cell-specific therapy, or an increased therapeutic window between normal and cancer cells. Novel targeted strategies are a significant improvement but still have issues with cell specificity, and more importantly,

a very high attrition when moving these agents from preclinical studies to clinical applications [78]. The use of monoclonal antibodies is highly selective, though it has limited cytotoxic activity [79]. Combined administration of monoclonal antibodies and conventional chemotherapy drugs is one potential route for solving this problem, delivering the highly cytotoxic agent specifically to cancer cells [79].

The use of EOs extracts as single agents has been shown in various *in vitro* studies to specifically target cancer cells, with absent or markedly less cytotoxicity exhibited towards healthy cells with a range of mechanisms of action (Table 1).

*Boswellia sacra* extracts have shown very promising results *in vitro* and *in vivo*. *Boswellia sacra* extracts were shown to be cytotoxic to three breast cancer cell lines (T47D, MCF7, and MDA-MB-231) at varying concentrations, which were noncytotoxic to immortalized normal human breast cells MCF10-2A [14]. This study also showed that *Boswellia sacra* extracts that were hydrodistilled for 12 hours at 100°C were more potent than the essential oil extracts prepared at 78°C, with a higher amount of boswellic acid present. Apoptosis markers activated caspase 3 activity, PARP cleavage, and DNA fragmentation rapidly in MDA-MB-231 but not MCF10-2A cells [14]. Importantly, treatment with the extracts blocked the growth of multicellular tumor spheroids from T47D, indicating the potential for efficacy in *in vivo* models [14]. Similarly, *Boswellia sacra* showed cell-specific cytotoxicity in a dose-dependent manner to bladder transitional cell carcinoma cell line J82, in contrast to no cytotoxicity observed in normal bladder cell line UROtsa [17]. Treatment of J82 cells rapidly led to cell shrinkage and detachment from the plate, whereas no changes were observed for UROtsa cells. This effect was associated with decreased expression of 47 genes after treatment with the EO extracts, whose functions include transcription factors, cell cycle regulation, and cell proliferation [17]. Finally, *Boswellia sacra* also showed cytotoxicity towards human pancreatic cells, both cultured and in a xenograft mouse model, exhibiting repression of cell cycle regulators and activation of the caspase pathway in *in vitro* cultures, and causing decreased tumor cell growth and tumor cell death *in vivo* [80]. Similarly, to the work by Suhail et al. (2011) [14], EO extract potency was increased with the increase of hydrodistillation temperature, associated with the extraction of higher levels of boswellic acids and sesquiterpenes, which is indicated to be positively correlated with cytotoxicity [80].

EO extracts from *Amomum tsaoko* exhibited cytotoxicity towards various human cancer cell lines, including liver cancer (HepG2 and Bel-7402), cervical cancer (HeLa), gastric adenocarcinoma (SGC-7901), and prostate cancer (PC-3) [15]. Importantly, these extracts were less effective towards normal hepatocytes HL-7702 and umbilical vein endothelial (HUVEC) cell lines [15]. The individual components of this EO mixture, eucalyptol and geraniol, were also tested [15]. Eucalyptol was not cytotoxic to any cancer cell line, and geraniol exhibited a minimal cytotoxic effect towards all cancer cell lines but was markedly lower than the complete EO mixture [15]. Synergism of eucalyptol and geraniol with each other and/or other EO components, therefore, must contribute to the cytotoxic activity [15].

## 4. Synergism of EO Extracts with Conventional Chemotherapeutic Agents: Potential of Combination Therapy Using EOs

Specific EO constituents have been shown to enhance the cytotoxic activity of chemotherapy drugs in various cell lines (Table 2), thus increasing the therapeutic window, that is, lowering the required drug concentrations whilst providing the same effect [22, 23].

Docetaxel is the first line therapy for hormone-refractory prostate cancer, which has a median survival of 20 months [22]. Docetaxel is associated with serious side effects and is currently used in combination with treatment exhibiting dose-dependent toxicity to the patient [22]. *d*-limonene showed cytotoxic activity alone towards prostate cancer cell line DU-145, and when administered alongside docetaxel, sensitized the cells towards this drug in a dose-dependent manner allowing for a markedly lower dose of docetaxel to be used, achieving the $IC_{50}$ in concentrations from 2.8 nM to 1.9 mM [22]. Limited toxicity was also shown towards normal prostate epithelial cells. Further analysis on the effects of combined treatment showed an increase in ROS production from both mitochondrial dependent and independent pathways, as well as increased cytochrome C release, p53 stabilisation, and caspase and PARP cleavage after 0-48 hours [22]. In addition to decreasing the amount of toxic docetaxel required, *d*-limonene showed low toxicity towards humans. It is possible that this combination may also be effective in docetaxel-resistant cell lines [22].

$\beta$-caryophyllene, which was not cytotoxic as a single agent, was shown to markedly increase the cytotoxic activity of paclitaxel in various cancer cell lines (Table 2). Specifically, the largest effect was observed on DLD-1 cells treated with paclitaxel combined with 10 $\mu$g/mL$^{-1}$ $\beta$-caryophyllene, increasing paclitaxel activity approximately 10 times [23]. It was shown that $\beta$-caryophyllene increased cell membrane permeability for paclitaxel uptake, likely due to $\beta$-caryophyllene accumulation in the lipid bilayer, and thus altering the permeability for substances such as paclitaxel [23].

Neutropenia is a common side effect of both cancer itself and therapies including chemotherapy and radiotherapy, the latter especially if targeted to active sites of bone marrow proliferation [81]. Cancer-related neutropenia has a high mortality rate due to susceptibility to infectious diseases, particularly from gram-negative bacterial infections, and combined with fever is considered an oncological emergency [81]. Currently, there are limited adjunctive treatments, one of which is the administration of granulocyte colony-stimulating factors (G-CSFs), in selected patients only, which promotes bone marrow production of granulocytes [81]. Alternatively, chemotherapy dose-modification may be deemed appropriate [81]. A study by Zhuang and coworkers (2009) which included 105 cancer patients with nonterminal breast, colorectal, nasopharyngeal, or lung cancer showed significant results in preventing the depletion of leukocytes (14.2%) and neutrophils (11%), versus control (29.1%) over a 6-week period [82]. Flow cytometry analysis showed a

Table 1: Essential oils bearing plants and main constituents with targeted cancer cell cytotoxicity in *in vitro* studies.

| Species | Major EO constituent(s) | Cancer cell lines | Noncancer cell lines | Major findings and EO concentrations | Mechanisms | REF |
|---|---|---|---|---|---|---|
| *Thymus fallax* | Carvacrol, p-cymene, thymol and $\gamma$-terpinene | DLD-1 (CRc) | Mouse fibroblast (L.929) | Cytotoxic to cancer cells ($IC_{50}$ 0.347 mg/mL) and noncytotoxic to normal cells ($IC_{50}$ 22 mg/mL) | Antioxidant activity | [13] |
| *Boswellia sacra* | $\alpha$-pinene, $\alpha$-thujene, $\beta$-pinene, myrcene and boswellic acid | T47D, MCF7, MDA-MB-231 (Bc) | Immortalized normal human breast (MCF10-2A) | Cytotoxic to cancer cells (EO dilution $IC_{50}$ 1 : 900 for TD47, 1 : 1000 for MCF7, 1 : 950 for MDA-MB-231) and noncytotoxic to immortalised normal cells (EO dilution $IC_{50}$ 1 : 680) | Antiproliferative | [14] |
| *Amomum tsaoko* | 1,8-cineole, $\rho$-propylbenzaldehyde, geraniol, geranial, $\alpha$-terpineol, $\alpha$-phellandrene, neral and $\beta$-pinene | HepG2 and Bel-7402 (Lc) HeLa (Cc), A549 (Lc), SGC-7901 (GAC), PC-3 (Pc) | Hepatocyte (HL-7702) and umbilical vein endothelial (HUVEC) | Cytotoxic to cancer cells, particularly HepG2 ($IC_{50}$ 31.8 $\mu$g/mL), Hela ($IC_{50}$ 66.46 $\mu$g/mL) and Bel-7402 ($IC_{50}$ 96.08 $\mu$g/mL), with less cytotoxicity towards HL-7702 ($IC_{50}$ 272.4 $\mu$g/mL) and HUVEC ($IC_{50}$ 163.91 $\mu$g/mL). No cytotoxicity towards A549 | Antiproliferative | [15] |
| *Lippia alba* (Citral chemotype) | Geranial, neral, geraniol, *trans*-$\beta$-caryophyllene, 6-methyl-5-hepten-2-one, limonene, linalool | HeLa (Cc) | African green monkey kidney (Vero) | Cytotoxic to cancer cells ($CC_{50}$ 3.5 $\mu$g/mL) and noncytotoxic to normal cells ($CC_{50}$ > 100 $\mu$g/mL) | Citral-dependent cytotoxicity | [16] |
| *Boswellia sp.* (1,200 mg/ml frankincense gum resin) | Duva-3,9,13-trien-1,5alpha-diol-1-acetate, octyl acetate, o-methyl anisole, naphthalene decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2-pentenyl), thunbergol (Mikhaeil et al., 2003) | J82 (Blc) | Human urothelium (UROtsa) | Cytotoxic to cancer cells (no viable cells after EO dilution 1 : 1,100 after 24 hours) and noncytotoxic to normal cells (no viable cells after EO dilution 1 : 400) | Antiproliferative | [17] |
| *Casearia sylvestris* | Bicyclogermacrene, $\beta$-caryophyllene, spathulenol, $\alpha$-humulene, $\alpha$-pinene | HeLa (Cc), A549 (Lc) HT-29 (CRc) | Monkey kidney (Vero) and mice macrophages | Cytotoxic to HeLa ($CD_{50}$ 63.3 $\mu g{\cdot}ml^{-1}$), A549 ($CD_{50}$ 60.7 $\mu g{\cdot}ml^{-1}$) and HT-29 ($CD_{50}$ 90.6 $\mu g{\cdot}ml^{-1}$) with less cytotoxicity to Vero ($CD_{50}$ 210.1 $\mu g{\cdot}ml^{-1}$) and macrophages ($CD_{50}$ 234.0 $\mu g{\cdot}ml^{-1}$) | Cytotoxicity | [18] |
| *Zanthoxylum rhoifolum* Lam | ß-caryophyllene, $\alpha$-humulene, $\alpha$-pinene, myrcene and linalool | HeLa (Cc), A549 (Lc) HT-29 (CRc) | Monkey kidney (Vero) and mice macrophages | Cytotoxic to HeLa ($CD_{50}$ 90.7 $\mu$g/ml), A549 ($CD_{50}$ 82.3 $\mu$g/ml), and HT-29 ($CD_{50}$ 113.6 $\mu$g/ml) and noncytotoxic to normal cells ($CD_{50}$ > 600 $\mu$g/ml) | Cytotoxicity | [19] |
| *Commiphora gileadensis* | Sabinene, ß-caryophyllene, germacrene D, $\alpha$-pinene | BS-241 (Mouse T-cell lymphoma) MoFir (Epstein Barr virus transformed human B lymphocytes) | Normal human skin fibroblasts (FB) | EO dilution of 1 : 5000 killed 87% of BS-24-1 cells and 40% of MoFir cells | Antiproliferative | [20] |
| *Aniba rosaeodora* | Rosewood essential oil (REO), linalool | A431 (Ec), HaCaT (pre-cancerous) | Epidermal keratinocytes (HEK001, NHEK) | Cytotoxicity to cancer cells A431 and HaCaT (<20% viability) and minor cytotoxicity to normal cells HEK001 and NHEK (>70% viability) | Cytotoxicity | [21] |

*Note*. Cytotoxicity is expressed as the concentration of the essential oils inhibiting cell growth by 50%; CRc: colorectal cancer; Bc: breast cancer; Lc: lung cancer; Cc: Cervical cancer; GAC: gastric adenocarcinoma; Pc: prostate cancer; BLc: bladder carcinoma; Ec: epidermoid carcinoma; $IC_{50}$: inhibitor concentration 50; $CC_{50}$: cytotoxic concentration.

Table 2: *In vitro* studies of essential oils in combination with conventional chemotherapy agents.

| Cell lines | Chemotherapy drug used alone and concentration | EO constituent used alone and concentration | Combined EO and chemotherapy drug | | Reference |
|---|---|---|---|---|---|
| Prostate cancer cell (DU-145) | Docetaxel $IC_{50}$ 2.8 nM | *d*-limonene $IC_{50}$ 2.8 mM | $IC_{50}$ docetaxel 1.9 mM and d-limonene 0.2 mM | | [22] |
| Human breast cancer (MCF-7) | Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 28% cell growth inhibition | $\beta$-caryophyllene resulted in no inhibition of cell growth | $\beta$-caryophyllene 2.5 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 50% cell growth inhibition | $\beta$-caryophyllene 10 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 68% cell growth inhibition | [23] |
| Human colorectal adenocarcinoma (DLD-1) | Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 17.3% cell growth inhibition | $\beta$-caryophyllene resulted in no inhibition of cell growth | $\beta$-caryophyllene 2.5 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 91% cell growth inhibition | $\beta$-caryophyllene 10 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 189% cell growth inhibition | [23] |
| Mouse fibroblast (L-929) | Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 18.4% cell growth inhibition | $\beta$-caryophyllene resulted in no inhibition of cell growth | $\beta$-caryophyllene 2.5 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 36% cell growth inhibition | $\beta$-caryophyllene 10 $\mu g/mL^{-1}$ and Paclitaxel 0.025 $\mu g/mL^{-1}$ resulted in 123% cell growth inhibition | [23] |

larger depletion of CD4 and natural killer cells in the placebo receiving group versus the Chinese medicinal herb complex (CCMH) receiving group [82]. The largest component of the CCMH was the EO component citronellol (273.6 mg per capsule), a known strong antioxidative compound, also exhibiting anticancer and anti-inflammatory properties, as well as promoting wound healing [82]. It is not clear from this study how exactly citronellol and each other component contributed to results. So, to date the mechanism of action remains to be elucidated.

Geraniol has been shown previously to sensitize cancer cells to the conventional chemotherapeutic agent 5-fluorouracil (5-FU), also causing an increased uptake of the drug [83, 84]. Geraniol has also been shown to be chemoprotective towards normal colon cells in rats when administered with the potent carcinogen dimethylhydrazine [85]. This effect occurs through mediating the reduction of DNA damage when compared with controls where no EO extract was used [85].

## 5. Conclusions and Future Directions

EO have been shown to possess a wide range of anticancer properties and mechanisms. Considering the myriad of components present and the mechanism and synergistic capabilities of EO extracts, it is of paramount importance to perform further studies regarding evaluation on how EO minor components contribute to the overall effect of the EO extract mixture. Further *in vitro* and *in vivo* research into achieving the most effective cytotoxic EO mixture composition would allow for more targeted therapy, and with increased specificity to cancer cells over non-cancer tissue. Furthermore, the currently used concentrations of conventional chemotherapy drugs could potentially be reduced combined with specific EO, which could also decrease chemotherapy-associated toxicity. Moreover, synthetic modification of these molecules may allow improving their overall efficacy further. However, there is still a significant lack of preclinical studies for EOs as anticancer agents; thus many EOs require further safety and toxicity studies before they can take part in clinical trials.

Cancer cell specificity is a sought-after propriety that is lacking in conventional chemotherapeutic strategies [79, 86]. As well as addressing cellular specificity, another strategy to increase cell specificity includes novel drug delivery strategies [86]. Specifically, a new field addressing this involves the use of microspheres made of proteins or synthetic polymers containing the anticancer agent or EO, for delivery to the specific organ or another site of cancer [86]. These can be administered intravenously or intra-arterially depending on the target site [86]. The use of microspheres has promising potential due to multiple types of drugs being successfully contained and delivered in a single vehicle, offering the potential for combination therapies, but also, the use of nanoemulsions is an improvement to transport and to deliver the EOs with anticancer properties, improving their therapeutic effect [87]. Cancer cell specificity can also be enhanced by the use of ligands added to the surface, targeting overexpressed cell surface proteins on the cancer cell [88]. Crucially, EOs can be degraded through physical, chemical, or enzymatic processes, so microsphere encapsulation may prevent this for optimised delivery [88, 89]. This way, EOs and other drugs may be released in a controlled manner, potentially reducing excess dosage and increasing the overall safety of these constituents, and offering a promising strategy for targeted drug and EO delivery to cancer cells [89].

In conclusion, although this is a relatively new and emerging area of cancer research, the ability of EOs and their components of having such diverse anticancer effect through acting on various pathways and cellular mechanisms is compelling. Thus, it is warranted that more studies be performed to expand the present knowledge of these mechanisms with the aim of promoting cell-specific and individualized cancer therapy.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

K. Blowman and M. Magalhães contributed equally to this work.

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*Review Article*

# Flavonoids Effects on Hepatocellular Carcinoma in Murine Models: A Systematic Review

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The hepatocellular carcinoma (HCC) is the second most common cause of cancer deaths worldwide. It occurs primarily as manifestation of other pathological processes, such as viral hepatitis, cirrhosis, and toxin exposure that affect directly the cellular process. Studies were selected from PubMed and Scopus databases according to the PRISMA statement. The research filters were constructed using three parameters: flavonoids, hepatocellular carcinoma, and animal model. The bias analysis of the 34 selected works was done using the ARRIVE guidelines. The most widely used flavonoid in the studies was epigallocatechin gallate extracted from green tea. In general, the treatment with different flavonoids presented inhibition of tumor growth and antiangiogenic, antimetastatic, antioxidant, and anti-inflammatory activities. The bias analysis evidenced the absence of methodological processes in all studies, such as the age or weight of the animals, the method of flavonoids' extraction, or the experimental designs, analytical methods, and outcome measures. It has been known that flavonoids have a protective effect against HCC. However, the absence or incomplete characterization of the animal models, treatment protocols, and phytochemical and toxicity analyses impaired the internal validity of the individual studies, making it difficult to determine the effectiveness of plant-derived products in the treatment of HCC.

## 1. Introduction

The hepatocellular carcinoma (HCC) is a widely distributed type of cancer with high prevalence, being the second most common cause of cancer deaths worldwide. It is responsible for high mortality rates, with approximately 745,000 annual deaths, according to world the cancer report in 2014 [1]. HCC occurs primarily as a manifestation of other pathological processes, such as viral hepatitis, cirrhosis, and toxin exposure, which affect directly the liver, increasing inflammatory processes and leading to necrosis and accumulation of the connective tissue [2].

The viral hepatitis B (VHB) and viral hepatitis C (VHC) are considered risk factors in the HCC [1], causing alteration by modification in the cell environment and producing genetic pathways instability. One of the pathways affected in the infection process is the P53 protein. This pathway is one of the main regulators of the cell cycle, which is suppressed by the HBx gene expression in the viral hepatitis infection [2]. P53 protein is affected by the action of other factors, such as aflatoxin exposure, which is a toxin produced by the fungus *Aspergillus* spp. The cytochrome P450 converts this toxin into exo-8,9 epoxide, which is able to affect this gene, damaging the DNA [3].

The Wnt/catenin signaling pathway is involved in liver development, amino acid metabolism, and oxidative stress, also acting in the HCC progression by the mutation of $\beta$-catenin and inactivation of the negative regulation of the APC/Axin1/GS3KB complex over Wnt/catenin. This process leads to the transcription of different genes involved in cell

proliferation [4]. Meng et al. [5] demonstrated that MAP kinases and PI3K/AKt/mTor pathways are affected in HCC by the action of CAMK2$^{\gamma}$, causing hyperproliferation of hepatocytes. Other studies demonstrated that IL-6 in HCC is overproduced in malignant transformation of the hepatic tissue [6, 7].

Conventional drugs have shown a toxic effect in the liver, thymus, and spleen of patients with HCC and have not also been effective due to the resistance of cancer cells [8]. Currently, there is growing interest in the study of metabolites from plants that have presented beneficial effects to human health. These components have effects on disease prevention for its antioxidant activity, anti-inflammatory, and anticancer potential [9]. Phenolic compounds of plants, mainly flavonoids, are known by its beneficial effects in treatments for various diseases. Many studies have suggested chemopreventive effects of flavonoids on different cancer types, including HCC. They can act regulating several pathways associated with the progression of HCC [8]. Flavonoids are heterogeneous polyphenols found in a wide variety of plants, mainly in fruits and vegetables. The flavonoid structure has two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C [10]. The variations in substitution patterns to ring C result in more flavonoids classes, like flavonols, flavones, flavanones, flavanols (or catechins), flavan-3,4-diol, isoflavones, and anthocyanidins [11]. These metabolites have been effectively providing protection and enhancing the repair process through the control of different molecular pathways, which regulates the expression and suppression of genes, protein activities, and the cell cycle [2, 12]. The flavonoids have shown to protect the cell against cancer progression by the activation of proapoptotic and antiproliferative pathways, or inactivation of the antiapoptotic pathways, such as the inactivation of ERK/2 and inhibition of proteins like MMP2 [8, 13] and COX-2 gene [14], which are widely expressed in tumor cells.

Some clinical and preclinical studies have tried to demonstrate the positive effect of plant compounds and their derivatives in the treatment of HCC. However, this hypothesis is not always confirmed, mainly due to the large methodological variations involving the obtaining of the compounds, the therapeutic schemes, and the mechanisms of action. Therefore, it is critical to compile data from various studies in order to clarify the aforementioned discrepancies. In this context, the systematic review, although little used in animal studies, is a powerful tool that incorporates the variability among the studies and allows the obtaining of an overall estimate of the use of plant extracts in the treatment of HCC in murine models. Besides this systematically reviewing, the preclinical evidence in an objective manner (unlike the widely used narrative reviews) has never been carried out before and might provide us with reliable and solid new evidence on whether or not plant extracts and its derivatives could be beneficial in the treatment of HCC. Based on this, our systematic review was developed to determine if there is a rational basis in the selection of all plant species and subclasses of investigated flavonoids. In addition, we performed a critical analysis of preclinical studies in order to improve the quality of the reports and thus prevent the reproduction of methodological failures that could compromise the development of clinical studies.

## 2. Material and Methods

The systematic review adhered to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) [49] guidelines, including search strategy, selection criteria, data extraction, and data analysis.

*2.1. Literature Research.* The papers analyzed in this review were selected from two electronic databases, PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and Scopus (https://www.scopus.com/freelookup/form/author.uri), completed in October 05, 2015, at 12:31 p.m. The keywords used were based on filters constructed by three criteria: flavonoids, hepatocellular carcinoma, and animal model. The strategy used in the construction of the filters on PubMed platform was the hierarchical distribution of the MESH terms. In the Scopus database, a standardized filter for animal studies was applied, and the same PubMed search strategy was adapted and used on it [50] (Table S1). Only experimental studies published in English were included. Reviews, comments, and notes, as well as unpublished studies, were not considered. The studies were selected based on the inclusion criteria mentioned below:

(i) Studies showing the effect of flavonoids in the HCC in murine model for animal experimentation were included.

(ii) Studies only *in vitro*, performed in humans and using synthetic flavonoids, were excluded.

(iii) Studies reporting the use of flavonoids in the treatment of other tumors in organs other than the liver were also withdrawn.

Exception was done with works that used commercial green tea extracts or synthetic flavonoids derived from green tea, which was included for the commercial value of the plant. We searched the references of the select studies for others studies that met the inclusion criteria.

*2.2. Extraction and Data Management.* Abstract selection: three independent reviewers (ERG, RDN, and RVG) selected eligible studies based on title and abstract analysis. In case of disagreements a fourth reviewer (FCSAM) decided whether the study met the inclusion and exclusion criteria. In order to discard subjectivity in the data collection and selection process, the information was independently extracted by the two reviewers (ERG, EAG) and analyzed separately. Data from each study were extracted and tabulated using standard information, such as features of the publication (author, year, country, and title); plant (plant family and species, used part, flavonoid type, and extraction and purification method); *in vitro* assay; experimental model (animal model, strain, sex, age, body weight, administration, and frequency of treatment); induction of hepatocellular carcinoma/concentration and volume; tumor measurement; *in vivo* analysis (Table 1).

TABLE 1: Description of the main characteristics of the studies using flavonoids in the treatment of hepatocellular carcinoma in murine models.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wan et al., 2014 [15] | China | Preparation of morusin from *Ramulus mori* and its effects on mice with transplanted H22 hepatocarcinoma | Moraceae/*Ramulus mori* | Root bark | Isoprenylated flavone (morusin) | Ethanol extraction/Sephadex gel LH-20 and RF-HPLC | ? | Mice/SPF ICR | ♂ | 3–5 weeks/18–20 g | Intraperitoneal/ daily for 10 days | Transplanted H22 cells $1 \times 10^7$ cells/ml/0.1 ml | Weight of tumor/2 weeks | H&E, qRT-PCR (P53, survivin, cyclin D1, caspase-3, NF-K$\beta$) |
| Shu et al., 2014 [16] | China | Kurarinol induces hepatocellular carcinoma cell apoptosis through suppressing cellular signal transducer and activator of transcription 3 signaling | Leguminosae/ *Sophora flavescens* | Root | Kushenol H, kurarinol, norkurarinol, kushenol N, kurarinone | Ethanol extraction, HPLC and Sephadex LH-20 column | HepG2, Huh-7, Bel-7402 cells humans, HL-7702, H22 cells mouse | Mice/Kunming | ♂ | ?/18–22 g | Intraperitoneal/ daily for 10 days | Transplant of H22 cells $1 \times 10^6$ cells/ml/? | Weight of tumor/experiment end | Serum (white blood, red blood cells, platelets, ALT, AST, BUN, uric acid, and CK), TUNEL, immunohistochemistry (STAT3) |
| Wang et al., 2014 [17] | China | Liquiritigenin induces tumor cell death through mitogen-activated protein kinase- (MPAKs-) mediated pathway in hepatocellular carcinoma cells | ?/*Glycyrrhiza radix* | ? | Liquiritigenin (LQ) | Purchased | PLC/PRF/5, HepG2 human cells | Mice/BALB/cA nude | ♂ | 5 weeks/? | Intraperitoneal/ daily for 18 days | Transplanted PLC/PRF/5 $5 \times 10^7$ cells/ml/0.1 ml | Volume and diameters of tumor (mm3), body weight/every day | ? |
| Zhang et al., 2014 [18] | China | Dihydromyricetin promotes hepatocellular carcinoma regression via a p53 activation-dependent mechanism | ?/*Hovenia dulcis* | ? | Dihydromyricetin (DHM) | Purchased | HepG2, SMMC-7721, HL7702, L02 cells, primary cell: 4401, 4403, 1204 | Mice/BALB/cA nude | ♂ | 8–10 weeks/? | ?/daily for 21 days | Transplanted HepG2 cells $1 \times 10^7$ cells/ml/0.2 ml | ? | Immunohistochemistry (P53) |
| Darweish et al., 2014 [19] | Egypt | Chemopreventive and hepatoprotective effects of epigallocatechin gallate against hepatocellular carcinoma: role of heparan sulfate proteoglycans pathway | ?/*Camellia sinensis* | ? | Epigallocatechin gallate (EGCG) | ? | HepG2 cell | Rats/Sprague Dawley | ♂ | ?/180–200 g | ?/twice for week for 16 days | Thioacetamide (200 mg/Kg b.w.) | ? | H&E, immunohistochemistry (HSPGs), qRT-PCR (FGF-2), in serum (AST, albumin, and bilirubin), enzymatic determination (MDA, SOD, MPO, ELISA MMP-9, HSPGs, AFP, and syndecan-1) |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Zheng et al., 2014 [20] | China/USA | Anticancer effects of Baicalein on hepatocellular carcinoma cells | ?/*Scutellaria* sp. | Radix | Baicalein | Purchased | MIHAs, H22, BEL-7404, HepG2 cells | Mice/ICR | ♂ | ?/18–22 g | Intraperitoneal/ daily for 12 days | Transplanted H22 $2 \times 10^5$ cells/ml/0.2 ml | Weight of tumors/experiment end | Immunohistochemistry (AKT, p-AKT Ser473, $\beta$-catenin, cyclin D1) |
| Fan et al., 2014 [21] | China | Luteoloside suppresses proliferation and metastasis of hepatocellular carcinoma cells by Inhibition of NLRP3 inflammasome | ?/*Gentiana macrophylla* | ? | Luteoloside | Purchased | Hep3B, SNU-449, Huh-7, SMMC-7721, MHCCLM3, MHCC97-H cell | Mice/BALB/cA nude | ♂ | 6 weeks/? | Oral/daily for 4 weeks (subcutaneous) and 8 weeks (metastasis) groups | Transplanted SMMC-7721 $2 \times 10^6$ cells 7/0.2 ml | Volume of tumor (cm3)/every 3 to 4 days | H&E |
| Feng et al., 2014 [22] | China | Effect of grape procyanidins on tumor angiogenesis in liver cancer xenograft models | ? | Seed | Proanthocyanidins (GPC) | ? | ? | Mice/ SPF Kunming | ? | 4–6 weeks/20 g | Intraperitoneal/ daily for 10 days | Transplanted H22 cells $1 \times 10^6$ cell/ml/0.2 ml | ? | Immunohistochemistry (VEGF and CD34), RT-PCR (VEGF and $\beta$-actin) |
| Zhang et al., 2013 [23] | China | Silybin-mediated inhibition of notch | ?/*Silybum marianum* | ? | Silybin (SIL) | ? | HepG2 cells | Mice/athymic nude | ♂ | 4–6 weeks/? | Intraperitoneal/5 times for week for 20 days | Transplanted HepG2 $1 \times 10^6$ cells/ml/? | Size of tumor/every 3 days | Western blot (Notch1, Hes1, RBP-J$\kappa$, survivin, cyclin D1, Bcl2, BAX, $\beta$-actin) |
| Yang et al., 2013 [24] | China | Antitumor effects of two extracts from *Oxytropis falcata* on hepatocellular carcinoma in vitro and in vivo | ?/*Oxytropis falcata* | Whole plant | Flavonoids (FOF) $2',4'$-dihydroxychalcone | Ethanol extraction/? | SMMC-7721 cells | Mice/ICR | ♂ | 5-6 weeks/18–22 g | Intragastric/? | Transplanted H22 cells $5 \times 10^6$ cell/ml/0.2 ml | Tumor growth inhibitory ratio/experiment end | ? |
| Yu et al., 2013 [25] | China | A study on the antitumor effect of total flavonoids from *Pteris multifida* poir in H22 tumor-bearing mice | ?/*Pteris multifida* | Whole plant | Total flavonoids | Ethanol extraction/? | ? | Mice/Kunming | 1/2 ♂ and 1/2 ♀ | ?/18–22 g | Intraperitoneal/ daily for 10 days | Transplanted H22 cell $5 \times 10^7$ cells/ml/0.3 ml | Tumor inhibition rate and organ index (spleen and thy-mus)/experiment end | ELISA (TNF-$\alpha$ and IL-2), enzymatic determination (T-AOC, MDA) |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Xiang et al., 2013 [26] | China | Chemical composition of total flavonoids from *Salvia chinensia* Benth and their proapoptotic effect on hepatocellular carcinoma cells: potential roles of suppressing cellular NF-kB signaling | ?/*Salvia chinensia* | Whole plant | Total flavonoids | Alcoholic extraction 95%/HPLC and chromatography in silica gel column | HepG2, Huh-7 HCC cells | Mice/Kunming | ♂ | ?/18–22 g | Intraperitoneal/ daily for 10 days | Transplanted H22 cells $1 \times 10^6$ cells/ml/? | Weight of tumor, organ index/experiment end | Automatic counting (red blood cells, hemoglobin, white blood cells, platelets), serum (AST, ALT, BUN, CK, uric acid), ELISA (caspase-3, caspase-8, caspase-9) |
| Hashimoto et al., 2014 [27] | Japan | Methylated-($3''$)-epigallocatechin gallate analog suppresses tumor growth in Huh7 hepatoma cells via inhibition of angiogenesis | ?/*Camellia sinensis* | Green leaves | Polyphenolic, catechin, methylated-(3)-epigallocatechin gallate (MethylEGCG) | Purchased/HPLC purification | HUVECs, Huh7 cells | Mice/BALB/cA nude | ♂ | 6 weeks/20 g | Intraperitoneal/ daily for 21 days | Transplanted Huh7 cells $5 \times 10^6$ cell/ml/? | Size, volume of tumor/every week | Immunohistochemistry (CD31) |
| Saleem et al., 2013 [28] | India and Saudi Arabia | Anticancer potential of rhamnocitrin 40-b-D-galactopyranoside against N-diethylnitrosamine-induced hepatocellular carcinoma in rats | ?/*Astragalus hamosus* | Leaves | Flavonol glycoside (rhamnocitrin 40-b-D-galactopyranoside RGP) | MeOH/H2O extraction/column Sephadex LH-20 | ? | Rats/Wistar | ? | ?/150–220 g | Intraperitoneal/ daily for 20 weeks | Single intraperitoneal dose of N-nitrosodiethylamine (NDEA) (200 mg/kg b.w.) | ? | Serum (ALT, AST, ALP, total bilirubin, protein content, LPO), enzymatic determination (SOD, CAT, GPx, GST), H&E |
| Monga et al., 2013 [29] | India | Growth inhibition and apoptosis induction by (+)-Cyanidan-3-ol in hepatocellular carcinoma | Fabaceae/*Acacia catechu* | Heart wood | Cyanidan-3-ol (CD-3) | Ethanol extraction/HPLC | HepG2 cells | Mice/BALB/cA | ♂ | 6-7 weeks/26-27 g | Oral/daily for 20 weeks | Single intraperitoneal dose of NDEA (200 mg/kg b.w.) and carbon tetrachloride (CCl4, 3 ml/kg b.w.) thrice a week for six weeks | Relative liver weight/experiment end | Serum (AST, ALT, ALP, γ-GT, TSA, LASA), enzymatic determination (MDA, SOD, CAT, GPx, GR, GST, T-SH, GSH, PrPr-SHs), H&E, immunohistochemistry (p53, p65, c-jun) |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Zhao et al., 2013 [30] | China | Enhanced 5-fluorouracil cytotoxicity in high COX-2 expressing hepatocellular carcinoma cells by Wogonin via the PI3K/AKT pathway | ?/*Scutellaria radix* | ? | Wogonin 5,7-dihydroxy-8-methoxyflavone (WOG) | ? | HepG2, SMMC-7721 cells | Mice/BALB/cA nude | ♀ | 35–40 days/18–22 g | Intravenous/once every two days for 10 times | Transplanted SMMC-7721 cells $1 \times 10^6$ cells/mouse/? | Tumor inhibitory ratio, weight, size, volume of tumor/experiment end | ? |
| Wei et al., 2012 [31] | China | Antitumor and antiangiogenic effects of *Macrothelypteris viridifrons* and its constituents by HPLC-DAD/MS analysis | ?/*Macrothelypteris viridifrons* | Air dried roots | 2-(1,4-Dihydroxy-cyclohexyl)-5,7-dihydroxy-chromone-4′-O-glucoside; protoapigenin-4′-O-glucoside; 2-(1,2-dihydroxy-4-oxo-cyclohex-5-enyl)-5,7-dihydroxy-chromone; 5,7,2′,5′-tetrahydroxy-flavanone-2′-O-glucoside; protoapigenin; 2-(1,4-dihydroxy-cyclohexyl)-5,7-dihydroxy-chromone; 5,7,2′,5′-tetrahydroxy-flavanone-2′-O-6′′-O-acetylglucoside; 5,6-dihydro-6-methoxyprotoapigenone; quercetin-3-O-rutinose; protoapigenone; apigenin-4-O-glucoside; kaempferol-3-O-rutinose; kaempferol-3,7-di-O-rhamnoside; kaempferol-3-O-rhamnoside; apigenin-4-O-rhamnoside | ?/HPLC-DAD/MS Amethyst C18-P column | HUVECs, H-22 cells | Mice/Kunming | ♂ | ?/18–22 g | Intraperitoneal/ daily for 10 days | Transplanted H22 cells $2 \times 10^6$ cells/ml/0.2 ml | Weight of tumor, tumor inhibitory ratio/experiment end | Serum (red blood cell, white blood cell, platelet and hemoglobin), BUN, CRE, AST and ALT, TSA, LASA, enzymatic determination (MDA, SOD, CAT, GPx, GR, GST, T-SH, GSH, PrPr-SHs), H&E, immunohistochemistry (CD34, VEGF) |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wu et al., 2011 [32] | Taiwan | Suppression of hepatitis B virus × protein-mediated tumorigenic effects by ursolic acid | Rubiaceae/*Morinda citrifolia* | Leaves | Ursolic acid and silymarin | ? | Huh7, HepG2, Hep3B cells | Mice/nude | ? | 3 weeks/? | Intraperitoneal/?/8 weeks | Transplanted 6 2.2.15 cells $1 \times 10^6$ cell/ml/0.1 ml | Diameters of tumor/experiment end | Serum (ALT, AST, BUN, CRE) |
| Wang et al., 2011 [33] | Taiwan | *Solanum nigrum* L. polyphenolic extract inhibits hepatocarcinoma cell growth by inducing G2/M phase arrest and apoptosis | ?/*Solanum nigrum* | Whole plant | Total flavonoids | Water extract (SNWE)/? | HepG2 cells | Mice/Athymic nude | ? | ? | Intraperitoneal/?/35 days | Transplanted HepG2 cells $5 \times 10^6$ cell/ml/0.4 ml | Volume of tumor/every week, final volume, wet weight of tumor/experiment end | ? |
| Cai et al., 2011 [34] | China | Apigenin inhibits hepatoma cell growth through alteration of gene expression patterns | ? | ? | Apigenin | ? | Huh7 cells | Mice/BALB/c nude | ♀ | 5 weeks/16–18 g | Intraperitoneal/?/30 days | Transplanted Huh7 cells $2 \times 10^6$ cell/ml/? | Number of tumors, diameters, wet weight of tumor/experiment end | H&E |
| Huang et al., 2010 [35] | China | Carbonyl reductase 1 as a novel target of (−)-epigallocatechin gallate against hepatocellular carcinoma | ?/*Camellia sinensis* | ? | Epigallocatechin gallate (EGCG) | ? | Hep G2, Hep 3B, SMMC-7721 cells | Mice/? | ? | ? | Injected/?/15 days | Transplanted SMMC-7721 cells and Hep3B/?/? | Tumor growth, body weight/? | Serum (ALT, AST, LDH, CK-MBB, LDH, MDA, cTnT) |
| Liang et al., 2010 [36] | China and USA | Green tea catechins augment the antitumor activity of doxorubicin in an *in vivo* mouse model for chemoresistant liver cancer | ?/*Camellia sinensis* | ? | Catechin and epigallocatechin-3-gallate | ? | BEL-7404, BEL-7404/DOX cells | Mice/ BALB/cA nude | ♂ and ?? | 4-5 weeks/13–17 g | Intraperitoneal/?/33 days | Transplanted BEL-7404/DOX HCC cells $5 \times 10^7$ cell/ml/0.2 ml | Dimensions of tumor, volume of tumor/every 2 days, tumor growth, body weight/experiment end | Fluorospectrophotometry (DOX), immunohistochemistry, RT-PCR (MDR1) |
| Zhou et al., 2010 [37] | China | Inhibition of hepatoma 22 tumor by liquiritigenin | ?/*Glycyrrhiza glabra* | ? | Liquiritigenin (LQ) | Purchased | ? | Mice/ICR | ♂ | ?/20–22 g | Intragastric/?/15 days | Transplanted H22 cells $10 \times 10^6$ cell/ml/0.2 ml | Size, volume of tumor, body weight/every 3 days, Inhibition ratio of tumor growth, weight of tumor, organ index/experiment end | H&E, electron microscopy (tumor ultrastructure), enzymatic determination (MDA) |
| Yang et al., 2009 [38] | China | Antiproliferative efficacy of icariin on HepG2 hepatoma and its possible mechanism of action | ?/*Herba epimedii* | ? | Icariin (5-hydroxy 4-methoxy 8-isopentenyl 3-O-$\alpha$-rhamnosyl 7-O-$\beta$-glucosyl flavone) | Purchased | HepG2 cells | Mice/NMRI nude | ♂ | ? | Oral/28 weeks | Transplanted HepG2 cells $1 \times 10^7$ cell/ml/? | Volume of tumor, tumor growth inhibition/twice per week | Immunohistochemistry (CD4, CD8, CD19) |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Zhao et al., 2010 [39] | China | Synergistic effect of 5-fluorouracil and the flavonoid Oroxylin A on HepG2 human hepatocellular carcinoma and on H22 transplanted mice | ?/*Scutellariae Radix* | ? | Oroxylin A | ? | HepG2 cells | Mice/Kunming | ♂ | ?/18–22 g | Oral/?/7 days | Transplanted H22 cells $5 \times 10^6$ cells/ml/0.9 ml | Volume of tumor/experiment end | ? |
| Cui et al., 2009 [40] | USA | Effects and mechanisms of Silibinin on human hepatocellular carcinoma xenografts in nude mice | ?/*Silybum marianum* | ? | Silibinin | ? | HuH7 cells | Mice/nude | ? | ? | Oral/?/5 weeks | Transplanted HuH7 cells $5 \times 10^6$ cell/ml/0.25 ml | Volumes of tumor/every week, weight of tumor/experiment end | Immunoprecipitation (AFP, PTEN, binding interaction between p21, p27 with CDK4, binding of DP1 with E2F1), Western blotting (Ki-67, p21, p27, E2F1, CDK4, p-Rb, caspase-3, caspase-9, PTEN, AC-H3, AC-H4, p-AKT, p-survivin and p-ERK, Plk1, Chk1, SOD1), ELISA (NF-kB) |
| Umarani et al., 2008 [41] | India | Protective effect of *Kalpaamruthaa* in combating the oxidative stress posed by aflatoxin B1-induced hepatocellular carcinoma with special reference to flavonoid structure-activity relationship | Anacardiaceae/ *Semecarpus anacardium* | Fruit | Flavonoids | Dried and powdered fruit/? | ? | Rats/? | ♂ | 8–10 weeks/120–150 g | Oral/?/28 days | Intraperitoneal dose of AFB1 (2 mg/kg b.w.) | ? | Serum (protein, LPO, lipid peroxides, G6PD, vitamin E, vitamin C, uric acid), enzymatic determination (SOD, CAT, GPx, GR) |
| Miura et al., 2007 [42] | Japan | Effect of apple polyphenol extract on hepatoma proliferation and invasion in culture and on tumor growth, metastasis, abnormal lipoprotein profiles in hepatoma-bearing rats | ?/*Malusdomestica* | Unripe apples | Apple polyphenol extract (APE) | Purchased | AH109A cells | Rats/Donryu | ♂ | 5 weeks/? | Oral/?/21 days | Transplanted AH109A cells $1 \times 10^7$ cells/ml/0.5 ml | Size of tumor/every day | Serum (T-Ch, HDL-Ch), LPO, fecal steroid, AI |

Table 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Selvendiran et al., 2006 [43] | Japan | Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids | ? | Seeds | Luteolin | ?/HPLC | HepG2, HLF, HAK-1B, IMR-32 cells | Mice/BALB/cA nude | ♂ | 5 weeks/? | Oral/?/6 weeks | Transplanted HAK-1B cells $1 \times 10^7$ cell/ml/? | Size, volume (mm3) of tumor/weekly | Immunoblotting (Tyr705-P-STAT3) |
| Nishikawa et al., 2006 [44] | Japan | A green tea polyphenol, epigallocatechin-3-gallate, induces apoptosis of human hepatocellular carcinoma, possibly through inhibition of Bcl-2 family proteins | ?/*Camellia sinensis* | ? | Epigallocatechin-3-gallate | ? | HLE, HepG2, HuH-7, PLC/PRF/5 cells | Mice/BALB/cA nude | ♂ | 28 weeks/? | Oral/?/25 days | Transplanted HLE cells $1 \times 10^6$ cell/ml/? | Volume of tumor/daily | TUNEL, immunohistochemistry (Bcl-2a, Bcl-xl) |
| Premalatha and Sachdanandam, 1999 [45] | India | *Semecarpus anacardium* L. nut extract administration induces the in vivo antioxidant defense system in aflatoxin B1 mediated hepatocellular carcinoma | ?/*Semecarpus anacardium* L | Nuts | Total flavonoids | Purchased | ? | Rat/Wistar | ♂ | ?/100 g | Oral/?/14 days | Single intraperitoneal dose of AFB1 (2 mg/kg b.w.) | Weight of liver and kidney/experiment end | Total protein, enzymatic determination (GSH, uric acid, vitamin E, vitamin C, CYP, T-SH, NPSH) |
| Nishida et al., 1994 [46] | Japan | Inhibitory effects of (−)-epigallocatechin gallate on spontaneous hepatoma in C3H/HeNCrj mice and human hepatoma-derived PLC/PRF/5 cells | ?/*Camellia sinensis* | ? | Epigallocatechin gallate (EGCG) | ? | PLC/PRF5 cells | Mice/C3H/HenCrj | ♂ | 8 weeks/? | Oral/?/65 weeks | Spontaneous hepatocarcinogenesis | # of tumor, diameter of liver/experiment end | H&E, albumin, bilirubin, GPT, γ-GT, total cholesterol |
| Zhang et al., 2002 [47] | Japan | Effects of dietary powdered green tea and theanine on tumor growth and endogenous hyperlipidemia in hepatoma-bearing rats | ?/*Camellia sinensis* | ? | ? | ? | ? | Rat/Donryu | ♂ | 4 weeks/? | Oral/?/14 days | Transplanted AH109A cells $5 \times 10^5$ cell/ml/0.5 ml | Size, volume, diameter of tumor, radius tumor/every day, weight of tumor/experiment end | Precipitation method (HDL, LDL, VLDL), enzymatic determination (T-Ch and HDL-ChM, TBARS), fecal extraction (neutral sterol, bile acid) |

TABLE 1: Continued.

| Ref. | Country | Article | Plant family/species | Used part of plant | Flavonoids type | Extraction and purification method | *In vitro* assay | Animal model/strain | Sex | Age/weight | Administration/ frequency | Induction of carcinoma concentration/ volume | Tumor measurement/ frequency | *In vivo* analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Klaunig, 1992 [48] | USA | Chemopreventive effects of green tea components on hepatic carcinogenesis | ?/*Camellia sinensis* | ? | Epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate | Methanol extraction/steel column with silicic acid | Primary cells mouse hepatocytes | Rat/$B_6C_3F_1$ | ♂ | ? | Oral/?/28 weeks | Single intraperitoneal dose NDEA (90 mg/kg b.w.) | ? | H&E |

?: not specified; ♀: female; ♂: male; b.w.: body weight; ELISA: enzyme-linked immunosorbent assay; H&E: hematoxylin and eosin histological staining; qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; AC-H3: acetilated histone H3; AC-H4: acetilated histone H4; AKT: protein kinase B; P-$AKT_{Ser473}$: phosphorylated AKT serine 473; Bcl-2: B-cell lymphoma 2; BAX: Bcl-2 associated X protein; CD4: *cluster of cuadruple differentiation; CD8:* cluster of differentiation 8; CD19: cluster of differentiation 19; CD31: cluster of differentiation 31; CD34: hematopoietic progenitor cell antigen CD34; CDK4: cyclin-dependent kinase 4; P21: cyclin-dependent kinase inhibitor 1; P27: cyclin-dependent kinase inhibitor 1B (CDKN1B); P-ERK: phosphorylated extracellular signal-regulated kinases; Plk1: polo-like kinase 1; Chk1: checkpoint kinase 1; cTnT: cardiac troponin T; CYP: cytochrome P450; DOX: doxorubicin; DP1: transcriptional factor DP1; E2F1: transcriptional factor E2F1; Hes1: transcription factor HES1; FGF-2: fibroblast growth factor; VEGF: vascular endothelial growth factor; IL-2: interleukin 2; Ki-67: Ki-67 antigen; MDR1: multidrug resistance protein 1; MMP9: matrix metallopeptidase 9; NF-$\kappa$B: nuclear factor kappa-light-chain-enhancer of activated B cells; Notch 1: Notch homolog 1; P53: P53 protein; P65: nuclear factor NF-kappa-B p65 subunit; P-Rb: retinoblastoma protein; PTEN: phosphatase and tensin homolog; RBP-jk: recombining binding protein suppressor of hairless; STAT3: signal transducer and activator of transcription 3; TNF-$\alpha$: tumor necrosis factor-alpha; Tyr 705-P-STAT3: phosphorylated signal transducer and activator of transcription 3; CAT: catalase; LPO: lipid peroxidation; MDA: malondialdehyde; SOD: superoxide anion; SOD1: superoxide dismutase 1; T-AOC: total antioxidant capacity; TBARS: thiobarbituric acid reactive substances; AFP: alpha-fetoprotein; AI: atherogenic index; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CK-MB: creatinine kinase-MB; CK: creatinine kinase; G6PD: glucose-6-phosphate dehydrogenase; GPx: glutathione peroxidase; GPT: glutamic-pyruvic transaminase; $\gamma$-GT: gamma-glutamyl transferase; GR: glutathione reductase; GSH: reduced glutathione; GST: glutathione transferase; HDL-Ch: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein cholesterol; HSPGs: heparan sulfate proteoglycans; LASA: lipid associated sialic acid; LDH: lactate dehydrogenase; MPO: hepatic myeloperoxidase; PrPr-SHs: protein thiols; TBARS: thiobarbituric acid reactive substances; T-Ch: triglyceride; TSA: total sialic acid; T-SH: total thiol; NPSH: nonprotein thiol.

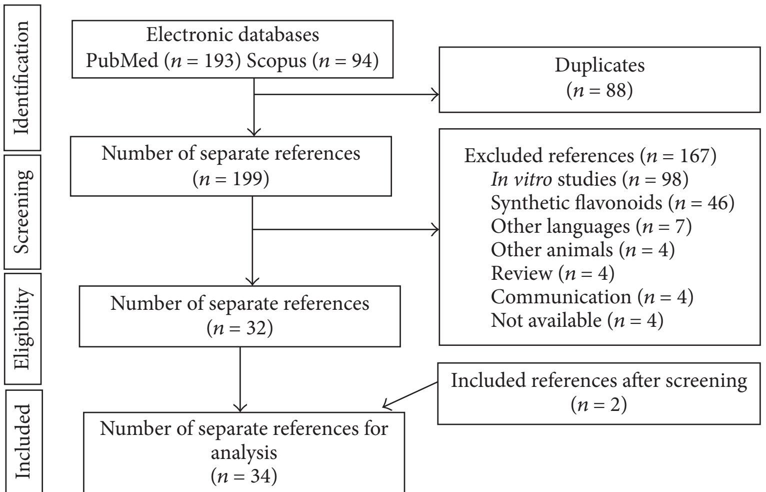


Figure 1: Flow diagram of the systematic review literature search results. Based on "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement." http://www.prisma-statement.org. From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097. For more information, visit http://www.prisma-statement.org/.

When there was difficulty in extracting the data or obtaining the studies, the authors were contacted by e-mail to provide the necessary information. Subsequently, the data were compared and the conflicting information were identified and corrected by discussion and consensus among the reviewers.

*2.3. ARRIVE Analysis.* The quality of the articles was analyzed according to the criteria described on Animal Research: Reporting of *In Vivo* Experiments (ARRIVE). These criteria are based on brief descriptions of essential characteristics of all studies using animal models, such as theoretical and methodological basis, research objective and improvement of analytical methods, statistical design, sample calculations, and outcome measures [51]. Considering that this systematic review aims to evaluate important aspects of the referenced publications, a table summarizing all the investigated aspects was built, as well as their relevance, describing positive and negative aspects of the recovered studies.

## 3. Results

*3.1. PRISMA Guideline.* The PRISMA diagram illustrates the selection process of the studies (Figure 1). The initial search resulted in 287 studies (193 on PubMed and 94 on Scopus), out of which 88 were duplicates. After reading the title and abstract, 167 studies were excluded, since they addressed subjects not related to the chosen topic. Among the excluded studies, we can highlight 98 about *in vitro* studies, 46 studies related to the use of synthetic flavonoids, and 23 excluded by other criteria listed in Figure 1. After analysis of the eligibility criteria, 32 studies were included in this review. The references' list of the 32 articles selected was analyzed and 2 of them fitted the inclusion criteria, totalizing 34 studies.

*3.2. Data Extraction.* The selected studies were conducted in 6 different countries, mainly China ($n = 19$, 55.88%), followed by Japan ($n = 6$; 17.64%), India ($n = 4$; 11.76%), Taiwan ($n = 2$; 5.88%), USA ($n = 2$; 5.88%), and Egypt ($n = 1$; 2.94%). The number of plant species was 26 and 34 flavonoids were extracted and studied. The most widely used flavonoid was epigallocatechin gallate (EGCG) (17.64%) extracted from green tea, followed by total flavonoid (14.7%), catechin (8.82%), liquiritigenin (LQ) extracted from *G. radix* (5.88%), and Silibinin (5.88%) extracted from *S. marianum* (Figure 2). Five studies used the whole plant (14.7%), other studies extracted flavonoids from the leaf (8.82%), root, and seed (5.88%), and different studies used the radix, fruit, nut, and heartwood (2.94% each). Seventeen studies did not specify the used part of the plant to obtain the flavonoid (49.98%) (Table 1).

Considering the animal strain, most studies used mice of the BALB/c strain ($n = 10$; 29.41%), Kunming ($n = 6$; 17.64%), followed by ICR ($n = 4$; 11.76%). Other studies used Wistar rats ($n = 2$; 5.88%), Donryu rats ($n = 2$; 5.88%), and Sprague Dawley and B6C3F1 rats ($n = 1$; 2.94% each), and 1 study did not specify the rat strain (2.94%). Most studies described the sex of the animals ($n = 28$; 82.35%), where 24 of the works used males (70.58%), 2 used females (5.88%), and other 2 works used males and females (5.88%). Six studies did not specify the sex of the animals (17.64%). The age and the weight of the animals were specified in 9 studies (26.47%), 10 studies only described the age of the animals (29.41%), 10 studies only described the weight of the animals (29.41%), and 5 works did not specify the weight of the animals (14.70%) (Table 1).

The process of carcinoma induction was determined in all studies. The induction of HCC by transplanted cancer cell lines was made in 27 studies (79.41%), 3 studies induced by the administration of N-nitrosodiethylamine (NDEA) (8.82%), 2 studies by the administration of aflatoxin B1 (AFB1) (5.88%), 1 study by the administration of thioacetamide (2.94%), and

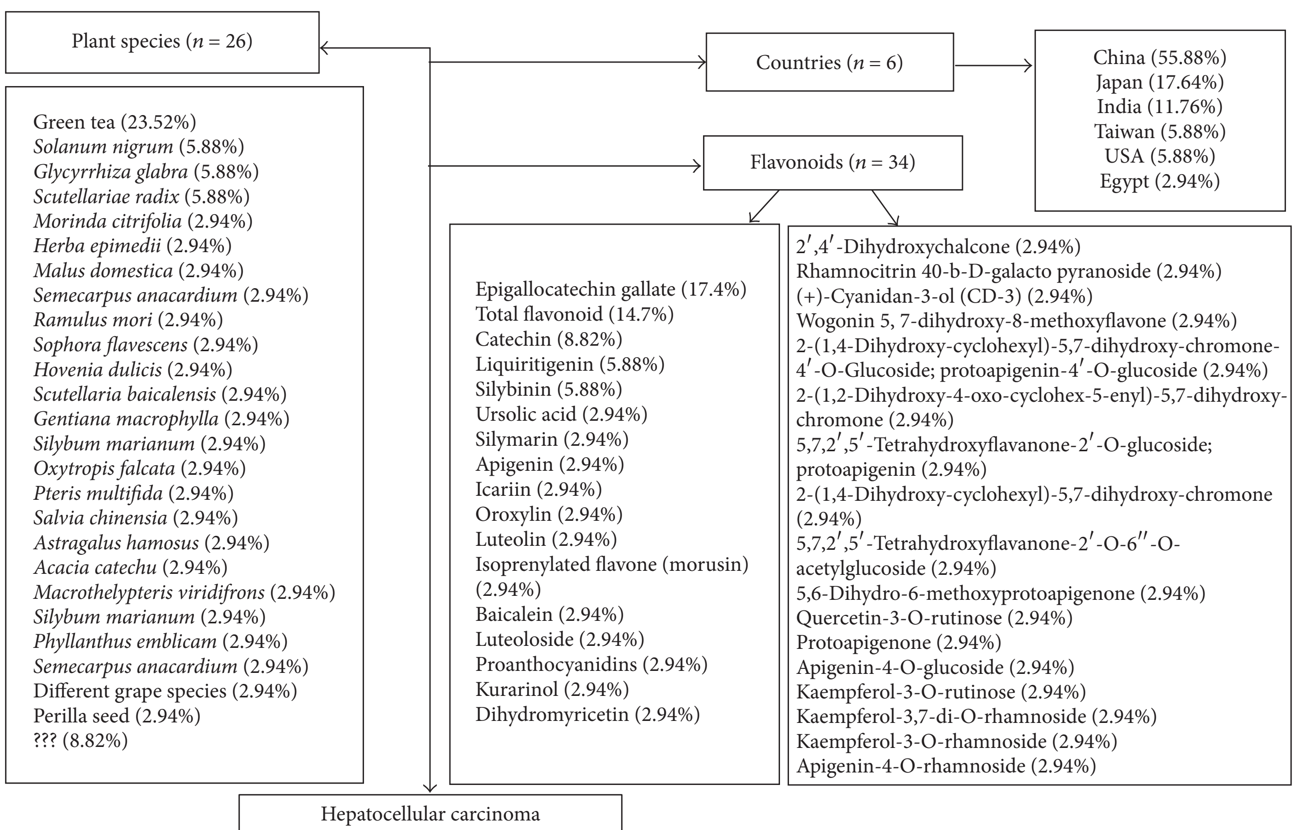


Figure 2: Schematic representation of plant species, used flavonoids, and countries with researches about benefits of flavonoids in hepatocellular carcinoma.

others by spontaneous hepatocarcinogenesis (2.94%). The main transplanted cell lines by the induction were H22, with 10 studies (29.41%), HepG2 with 4 studies (11.76%), Huh7 and SMMC7721 with 3 studies each (8.82%), and AH109A with 2 studies (5.88%) (Table 1).

The treatment with flavonoids in the animal models studied (rats and mice) was shown to be efficient in decreasing the size and volume of tumors by the inhibition of tumor growth, suppression of antiapoptotic proteins expression, and increase in proapoptotic proteins expression. Proteins that favor cell proliferation and the process of metastasis were inhibited, and proteins that disrupt the process of cell proliferation were expressed in greater quantity in the treatment groups, when compared to the control groups of the different works. A decrease in angiogenesis was also reported, and an antioxidant effect was evidenced mainly with the decrease of markers of oxidative stress. Other parameters, such as the presence of necrosis in the tumor tissue, inflammatory infiltrate, and blood markers were analyzed (Figure 3). The results of our work suggest an increase in the interest for products of plant origin in recent years, mainly due to the use of flavonoids in the treatment of HCC in 2013 and 2014, when compared to 1990–2009 (Figure 4).

*3.3. ARRIVE Analysis.* From the articles analyzed in this systematic review, 64.71% reported the permission from the ethics committee for performing the research. Regarding animal experiment, 61.76% specified the total number of animals used in each experiment, 82.35% specified the number of experimental and control groups, and 44.11% reported the number of animals in each group included in the analysis. No study reported if any animals or data were not included in the analysis or how the number of animals was decided, while 17.64% of the works described that the results can be interpolated to other species, such as humans. 55.88% of the studies specified how the animals were allocated to experimental groups, and 14.70% indicated the characteristics and health status of the animals before the treatment. The origin of the animals was specified in 85.29% of the papers, where 64.71% specified the sex of the animals, 52.94% reported the weight, and all studies specified the animal species. The developmental stage was reported in 50% of the works, 58.82% indicated any steps taken to minimize the effects of subjective bias when allocating animals to treatment, 41.17% specified the housing of the animals, and 50% reported the husbandry conditions. Regarding the statistical methods, 70.58% of the studies provided details of the statistical methods used for each analysis and specified the unit of analysis for each dataset. The modifications made to reduce adverse events in the experimental protocols were specified only in 2.94% of the studies (Table 2).

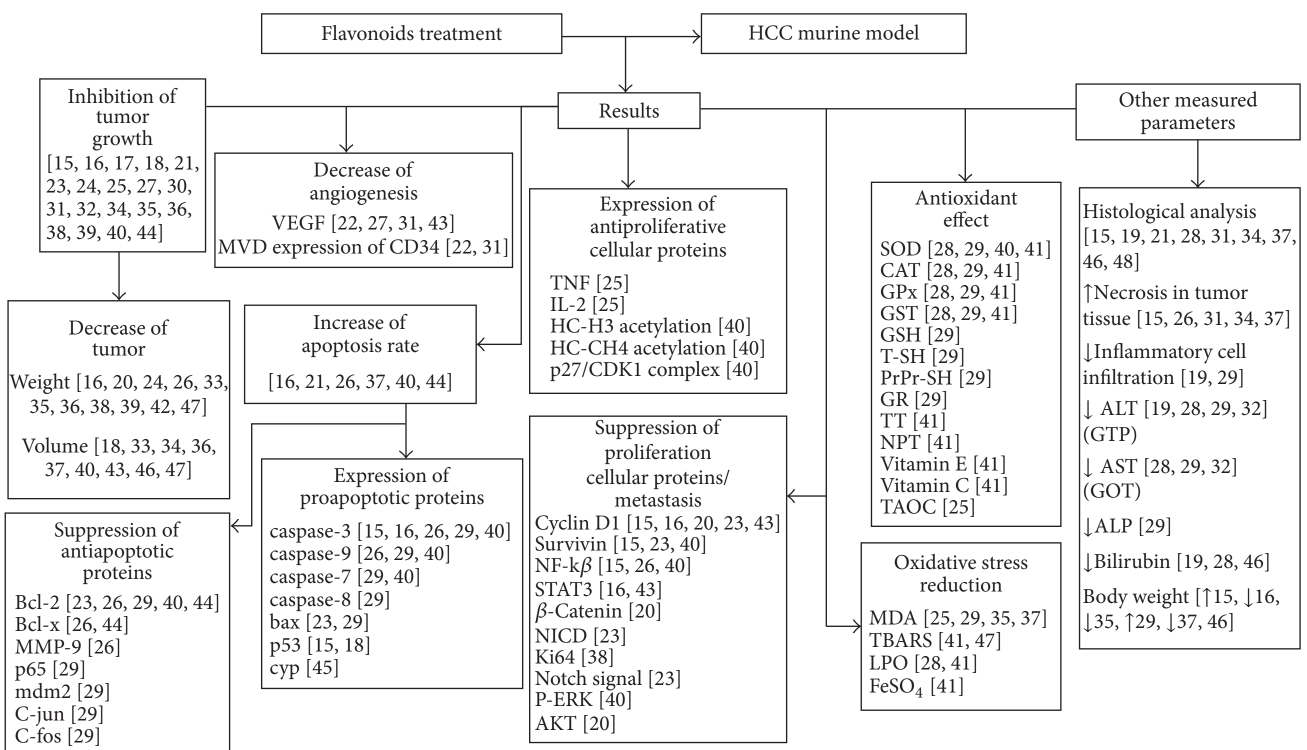


Figure 3: Main results demonstrating the action of flavonoids in the treatment of hepatocellular carcinoma in murine models.

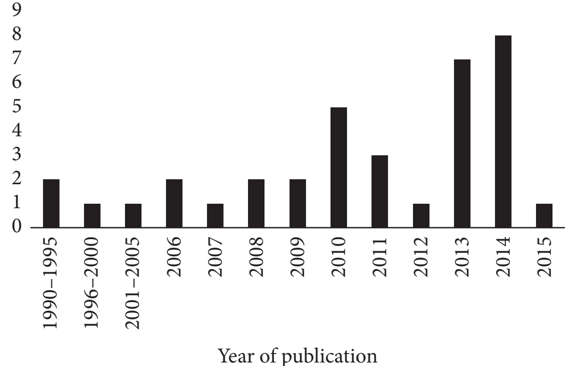


Figure 4: Number of papers published by worldwide scientists using flavonoids in the treatment of hepatocellular carcinoma in the last 25 years (between 1990 and 2015).

## 4. Discussion

In this study we conducted a systematic review to describe the main findings in the literature regarding flavonoids effects in the treatment of HCC in murine models. Despite the great heterogeneity of the studies it is possible to conclude that, in general, the use of flavonoids is effective in the treatment of HCC. The option for plant research is due to the fact that they are the oldest and most important source of bioactive compounds for the treatment of several diseases and still contribute directly to the development of new drugs [52, 53]. The results of our work suggest an increase in the interest for products of plant origin in recent years, mainly natural products in treatment of HCC.

The use of natural products for the treatment of different tissue changes is an old practice, and its use is closest to popular utilization. It is noteworthy that the biological activity of a natural product is often due to the synergism between its constituents, which potentiates the therapeutic properties [54]. Therefore, natural products therapy represents a promising alternative for the treatment of cancer, especially HCC, which is classified as an extremely aggressive type of cancer with high morbidity and mortality worldwide [1]. Our findings indicated that although there are punctual research initiatives in developed countries, the search for new treatment options for HCC using plant-derived natural resources is a concern of developing countries. Well-defined geographic patterns were expected, in which research initiatives on a particular disease concentrate in regions that have tradition in research using natural products, such as China, Japan, and India. This type of observation was possible because the systematic review allows evaluating, from multiple studies, the variability of individual works, allowing the establishment a global estimate on the use of flavonoids in the treatment of HCC [55]. However, although systematic review papers present a high level of scientific evidence, and the selection process is based on widely accepted practices, the results presented here should be interpreted with caution. This is related to the selection process of studies that may be biased due to different factors, such as initial exclusion based on just reading the titles and abstracts, or the inclusion of more than one study of the same group of researchers. Even so, the

Table 2: ARRIVE of the studies using flavonoids for the treatment of hepatocellular carcinoma in murine models.

| | Reference | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [15] | [16] | [17] | [18] | [19] | [20] | [21] | [22] | [23] | [24] | [25] | [26] | [27] | [28] | [29] | [30] | [31] | [32] | [33] | [34] | [35] | [36] | [37] | [38] | [39] | [40] | [41] | [42] | [43] | [44] | [45] | [46] | [47] | [48] | |
| **Title** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Accurate and concise a description of the content of the article | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| **Abstract** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Summary of the background, objectives, methods, principal findings, and conclusions | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| **Introduction** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Background | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| Objectives | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| **Materials and methods** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Ethical statement* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Indicate the nature of the ethical review permissions, relevant licenses | X | X | X | X | X | X | X | X | X | X | | | X | | X | X | X | X | | X | | X | X | | X | | X | X | X | | | | | | 64.71% |
| *Study design* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Number of experimental and control groups | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | X | X | X | X | X | X | X | | | | | | 82.35% |
| Any steps taken to minimize the effects of subjective bias when allocating animals to treatment | X | X | | X | | X | X | X | X | | X | X | X | X | X | X | X | | X | | | X | X | X | | | X | X | | | | | | | 58.82% |
| The experimental unit | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | | | | | | | | | X | | | | | | | | 52.94% |
| *Experimental procedures* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Doses | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| Method of administration | X | X | X | | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | X | X | | | X | | | X | X | | | | | | | 64.71% |
| Time of day | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | X | | | | | | | | | | | | | | | 55.88% |

TABLE 2: Continued.

| | Reference | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [15] | [16] | [17] | [18] | [19] | [20] | [21] | [22] | [23] | [24] | [25] | [26] | [27] | [28] | [29] | [30] | [31] | [32] | [33] | [34] | [35] | [36] | [37] | [38] | [39] | [40] | [41] | [42] | [43] | [44] | [45] | [46] | [47] | [48] | |
| *Experimental animals* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Origin of animal | X | | | X | | | | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 85.29% |
| Species | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| Sex | X | X | X | X | X | X | X | | X | X | X | X | X | | X | X | X | | | X | X | X | X | X | | | X | X | | | | | | | 64.71% |
| Developmental stage | X | | X | X | | | X | X | X | X | | | X | | X | X | | X | X | X | | X | | | X | | X | X | | | | | | | 50% |
| Weight | X | X | | | X | X | | X | | X | X | X | X | X | X | X | X | | | X | | X | X | | X | | X | | | | | | | | 52.94% |
| *Housing and husbandry* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Housing | X | X | X | | X | | | X | | X | | | | X | X | | X | | X | | | | X | | X | | X | X | | | | | | | 41.17% |
| Husbandry conditions | X | | X | X | X | X | | X | | X | | X | | X | X | | X | | X | | | X | X | | X | | X | X | | | | | | | 50% |
| *Sample size* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Specify the total number of animals used in each experiment | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | | | | X | | | | X | | | | | | | 61.76% |
| Explain how the number of animals was decided | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0% |
| *Allocating animals to experimental groups* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| How animals were allocated to experimental group (AZAR) | X | X | X | X | | X | X | X | X | | X | X | | | X | X | X | X | X | X | | | X | X | | | | X | | | | | | | 55.88% |
| *Experimental outcomes* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Clearly define the primary and secondary experimental outcomes assessed | X | | X | | X | | X | | | | | X | | X | X | | X | X | X | X | X | | X | X | | | X | X | | | | | | | 47.05% |
| *Statistical methods* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Provide details of the statistical methods used for each analysis | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | | X | X | | | X | X | | | | | | | 70.58% |
| Specify the unit of analysis for each dataset | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | | X | X | | | X | X | | | | | | | 70.58% |
| Describe any methods used to assess whether the data met the assumptions of the statistical approach | | X | | | | | | | X | | | X | | | | X | X | X | X | X | | | X | X | | | X | X | | | | | | | 35.29% |

Table 2: Continued.

| | Reference | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [15] | [16] | [17] | [18] | [19] | [20] | [21] | [22] | [23] | [24] | [25] | [26] | [27] | [28] | [29] | [30] | [31] | [32] | [33] | [34] | [35] | [36] | [37] | [38] | [39] | [40] | [41] | [42] | [43] | [44] | [45] | [46] | [47] | [48] | |
| **Results** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Baseline data* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| For each experimental group, report relevant characteristics and health status of animals before treatment or testing | X | | X | | | | | X | | | | | | | X | | | | | | | | X | | | | | | | | | | | | 14.70% |
| *Numbers analyzed* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Report the number of animals in each group included in each analysis | X | X | | | X | | X | | X | X | | | X | X | X | | X | X | X | | | | X | X | | | X | | | | | | | | 44.11% |
| If any animals or data were not included in the analysis, explain why | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0% |
| *Outcomes and estimation* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Report the results for each analysis carried out | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| *Adverse events* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Give details of all important adverse events in each experimental group | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | 2.94% |
| Describe any modifications to the experimental protocols made to reduce adverse events | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | 2.94% |

Table 2: Continued.

| | Reference | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [15] | [16] | [17] | [18] | [19] | [20] | [21] | [22] | [23] | [24] | [25] | [26] | [27] | [28] | [29] | [30] | [31] | [32] | [33] | [34] | [35] | [36] | [37] | [38] | [39] | [40] | [41] | [42] | [43] | [44] | [45] | [46] | [47] | [48] | |
| **Discussion** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Interpretation/ scientific implications* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Interpret the results, taking into account the study objectives and hypotheses, current theory, and other relevant studies in the literature | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | 100% |
| Comment on the study limitations including any potential sources of bias | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | 2.94% |
| *Generalizability/ translation* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Comment on whether and how the findings of this study are likely to translate to other species or systems, including any relevance to human biology | | | | | | X | X | | | X | | | X | | | | | | | | | | | | X | | X | | | | | | | | 17.64% |
| *Funding* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| List all funding sources | X | X | X | X | | X | X | X | X | | | X | X | X | X | | X | X | X | X | X | X | X | X | X | | | X | X | | X | X | | | 73.52% |
| % | 80% | 68.57% | 68.57% | 62.85% | 62.85% | 65.71% | 68.57% | 71.42% | 68.57% | 71.42% | 57.14% | 68.57% | 68.57% | 65.71% | 80.00% | 65.71% | 80.00% | 62.85% | 62.85% | 65.71% | 40.00% | 45.71% | 68.57% | 65.71% | 48.57% | 28.57% | 71.42% | 68.57% | 34.28% | 25.71% | 28.57% | 28.57% | 25.71% | 25.71% | |

results presented will serve as a basis for a critical analysis of the main findings that demonstrate the effect of plant-derived flavonoids on HCC treatment, since it is the first systematic review work to study the effect of these compounds on HCC.

In this review, the research has taken into account works carried out with murine models, which are widely used as a model for the development of human diseases. Several researches have reported the ease in handling these animals, as well as their similarity to humans regarding the metabolic and molecular processes that occur in different diseases, including cancer [56, 57]. In the reviewed studies, most of the investigations used mice from different strains, mainly BALB/c nude as a model for the induction of HCC. Besides this, genetic engineering has advanced in the genotypic modification of mice and rats. Strains of nude mice provide a greater development of the carcinogenic process because they favor the appearance of spontaneous tumors and provide a more stable biochemical environment [58, 59]. Athymic mice also have advantage when the transplantation of tumors is present, as this prevents immunological rejection [60]. Moreover, in addition to the decrease in maintenance time, the experimental processes with mice, compared to rats, also decrease the time of tumors induction [61]. Despite the percentage of used rats, this murine is also a good model for the induction of HCC, since different strains present appropriate characteristics for cancer induction, such as increased neovascularization, predisposition to metastasis, and rapid development of the tumor [62]. However, it presents limitations, such as the low availability of genetically knock out rats of specific genes [63].

In these investigations, there was a strong tendency to use male murine, and the percentage of researches that used females or the two genders was low. This is probably because males have been shown to be more predisposed to HCC, and this is attributed to the increase of IL-6 concentrations released by Kupffer cells in response to hepatic stress [64]. That does not occur in the same way in females due to the action of estrogens that inhibit the action of IL-6, disrupting the activity of NF-$\kappa$B and other transcript factors [65].

The induction process of HCC in most investigations is performed by the transplantation of cell lines and has been shown to be effective in murine model for HCC, especially in mice [63, 66, 67]. The use of xenograft models is widely used due to the rapidity with which the tumor is established and the carcinogenic process is developed [60]. In the case of reproducibility of metastases, the use of orthotopic models has given better results. In the study made by Fan et al. [21], the observation of the metastatic process in the lung is performed by means of an injection of HCC cells into the caudal vein of the animal, the induction of metastasis in the lung being effective. Miura et al. [42], on the other hand, performed the induction of hepatocarcinoma by subcutaneous injection and, subsequently, observed the metastatic process in lungs and inguinal and axillary lymphatic nodes. Several authors suggest that the use of orthotopic models for the visualization of metastatic processes has more effective and reproducible results in the case of HCC with transplants of HCC cells directly in the murine liver [62, 63, 68]. Other induction methods were performed using toxic components as NDEA, aflatoxin B1 (AFB1), and thioacetamide, which are reported to have an action in the instability of different pathways involving the cellular cycle control [1]. Investigations on these toxic agents are important to study the damage caused by them. AFB's damage, for example, is directly related to the chromosomal damage and DNA degeneration [69]. Works of this type give a more specific approach to the problems that occur in countries such as China and Africa, where the prevalence of people affected by pollution with AFB is still high [70].

All models used by the researchers showed efficiency on the carcinoma induction, with physiological changes in the animals and tumor growth. However, a murine model is far from the human condition, since these animals present a high mortality rate and frequently do not develop the chronic disease. Although canine and primate models are indicated as the most closed related to human disease [71], the limited availability of dogs and monkeys, the high cost of maintenance in large animal facilities, and ethical issues related to animal welfare hamper the widespread of these models [72].

In our study, the parameters evaluated in the HCC development in murine models showed mainly the inhibition of tumor growth and physiological changes in the animals treated with flavonoids when compared to control groups. Flavonoids such as Wogonin stopped tumor growth with an inhibition of 65% when associated with a drug commercially used in the treatment of HCC [30]. He et al. [73] reported the antiproliferative activity of Wogonin by the control of the oncoprotein CDK8 involved in the proliferation process in colorectal cancer and by acting on the Wnt/b-catenin pathway. Flavonoids have also been shown to have an effect on reducing the size and weight of different types of tumors [74, 75]. Reducing tumor size was reported in five studies, in which the flavonoid CD-3 showed beneficial effects both in *in vivo* and *in vitro* analyzes [29], due to the activation of caspases leading to an increase in the apoptotic pathway. The tumor weight was measured in 6 studies, where in most treatments a decrease in a dose dependent manner was observed. The flavonoid Baicalein [20] showed a reduction of 78% of the tumor weight. Other studies reported that Baicalein induced apoptosis in human gastric carcinoma, breast cancer, and hepatocellular carcinoma line cell when combined with the alkaloid 10-hydroxycamptothecin, usually used alone in cancer treatment [76]. This result can suggest that the apoptotic process is related to the suppression of tumor growth and reduction of tumor weight.

In addition to the morphological factors, the activation or inactivation of molecular pathways that halt the progression of the disease is of great importance in this type of research. The expression levels of proteins and growth factors in tumor tissue are an important factor that showed the progression of the carcinogenesis. In general, the use of flavonoids promoted decrease of VEGF and micro vesicular density of vessels and showed a decrease in angiogenic markers CD31, supporting the findings of other studies that have demonstrated reduced vascularization in metastasis processes in lung and kidneys tumors [67]. Associated with vascular increase, tumor cells acquire the property of evading apoptotic signals and show

increased activity to promitotic signals [77]. These characteristics lead to uncontrolled cell division and decreased apoptosis, which results in rapid tumor growth [78]. An effective therapeutic agent for controlling tumor growth should be able to activate the proapoptotic signaling pathways and/or inhibit the antiapoptotic pathways. In addition, it must be able to control cell division by modulating the cell cycle; for this, it must be effective in controlling important cellular pathways. One of the central components in the regulation of apoptosis and the cell cycle is NF-k$\beta$, an important factor that induces the transcription of several antiapoptotic proteins and promotes cell replication [78, 79]. Among the main apoptotic pathways, we can highlight the caspase pathway, which is responsible for a series of processes that cleave the DNA and the cytoskeleton [80], altering the permeability of the mitochondrial membrane [79]. In this systematic review, the studies have shown that, in general, flavonoids treatment activates proapoptotic pathways and decreases cell replication, which results in decreased tumor growth. These findings show the therapeutic potential that flavonoids represent for the treatment of cancer.

Another important signaling pathway in cell survival is MAP kinases proteins, which have a regulatory role in proliferation, differentiation, and cell migration and has an overexpression in carcinogenic and metastatic processes [81]. The flavonoids used in the experiments selected had a control over this pathway, inhibiting the proliferation of cancerous cells. One of the major proteins in the activation cascade MAPKs are ERKs that can be activated by reactive oxygen species (ROS) and induce activation of cell survival signals, promoting cell proliferation [82]. The enzyme threonine kinase (AKT), another cell survival pathway, has been extensively studied because of its action on inhibition and blocking of protein-related activation of apoptosis [83]. AKT also blocks pathways that induce cell death under stress and is responsible for mediating activation complex IPK3/AKT, which regulates the IKK activation on the cascade for the degradation of IKB that will again lead to the activation of NF-k$\beta$ [26, 30]. Baicalein, Wogonin, and Silibinin were shown to have an effect over the expression and activation of AKT, promoting the activation of apoptotic pathways [20, 30, 40]. Proteins like BAX and BAK, with proapoptotic activity, were upregulated in the tissue and showed suppressed expression of antiapoptotic proteins in culture tumor cells when treated with silibinin flavonoid (SIL) [23]. These targets are important in cancer therapy and the findings of this work showed that the use of flavonoids inhibits cell survival important pathways, such as MAP kinases, and IPK3/AKT, while it also favors the proapoptotic action of BAX/BAK proteins by acting positively on regulation of the mechanisms involved in cell survival and proliferation. In addition to the pathways mentioned above, other markers of cellular proliferation and formation of metastases were analyzed in the papers included in this review. Among them, we highlight cyclin D1, Notch signal, and $\beta$-catenin. These pathways appear to act individually or be in association with localized tumor development and metastasis formation [15, 20, 23].

Other important targets related to the anticancer therapy are the control of the cell cycle by the recognition of arrest points, where protein families such as cyclin regulated other proteins like CDKs and controlled the cellular cycle progress [84]. One of the most studied is the cyclin D1, which promotes the transition from G1 to S and is regulated by the GSK activation protein [85]. In our review, the use of flavonoids showed a positive effect in the treatment of HCC, since it inhibits the progression of the cell cycle. Baicalein [20], *Silybum marianum* [23], $2',4'$-dihydroxychalcone, and Luteolin [43] flavonoids showed inhibition on the activity of cyclin D1, suggesting cell cycle arrest and representing a promising therapy in the regulation of cell cycle. Some studies also measured the activation of proapoptotic factors on HCC, including the family of Ps. P38 proteins are activated for ASK, TNF receptor, and JNK that together promote apoptosis [86]. P53 proteins are important in the activation of metabolic pathway genes like CYP, a fundamental gene in the metabolism of lipids and sex steroids, and are known by their important activity in the cell cycle arrest, promoting genetic stability and control over DNA damage [87]. Mutations in the P53 gene are found in early cases of HCC in patients affected by AFB [88]. The results found in our review showed that P53 was upregulated in the cell culture of HCC treated with Dihydromyricetin [18], Cyanidan-3-ol [29], and Oroxylin [39] flavonoids, suggesting an induction of apoptosis in cancer cell lines, which would lead to the elimination of the tumor.

Flavonoids have been shown to exhibit high antioxidant activity, being effective in the protection of tissues from damage caused by ROS [89, 90]. In the reviewed articles, flavonoids like Silibinin were shown to be effective in reducing tumor in xenografts mice, acting on p-ERK activation, and reducing their activity [40]. Other flavonoids showed strong antioxidant activity, reducing stress oxidative markers like MDA [19, 25, 29, 31, 35] and stimulating the increase of antioxidant enzymes such as SOD [19, 28, 29, 31, 41].

Tumor metastasis encompasses a series of interrelated phenomena consisting of invasion and metastatic colonization, where malignant cells spread from primary tumor to organs [91, 92]. In this context, angiogenesis is a prerequisite for advancing the tumorigenic process, increasing the vascularization, allowing the tumor nurturing and the migration of cancer cells, favoring metastasis [93]. Involved in this process are enzymes and proteins that allow the development production of blood vessels, such as growth factors and metalloproteinases (MMPs) [33]. One of the MMPs involved in this process is the MMP9, which together with Symdecam 1 and FGF-2 regulates leukocyte cell migration during inflammation and tissue regeneration in wound healing, but in the oncogenic process, they are released by invasive cells, which degrade the extracellular matrix and favor cell migration and consequently the formation of metastasis [5]. The results of this work showed that all these markers are increased in the presence of metastases and have shown reductions in the treatment with flavonoids [19]. It shows that flavonoids protect the extracellular matrix from degradation, hindering the migration of tumor cells, delaying the invasion of capillaries, and, consequently, decreasing the formation of metastases and invasion of other tissues.

*4.1. Limitations.* Although our systematic review represents a proposal to group and critically analyze the evidence on the applicability of plant derivatives in the treatment of HCC, results' interpretation should consider some limitations. Our sampling frame was based on a specific number of databases. Thus, some articles may be not recovered due to the boundaries applied in the search strategy, as well as limitations in algorithms adopted in the search interfaces of each database. These aspects affect directly the sensitivity and specificity of the search strategy, which may have contributed to identify key articles. We attempted to mitigate these limitations by screening the reference lists of all articles, which are not limited to databases or any keywords-based search model. The relevant number of papers additionally recovered indicates the utility of this approach in a heterogeneous area, such as the evaluation of plant extracts in the treatment of HCC.

## 5. Conclusion

In general, flavonoids are effective in the treatment of HCC due to their capacity to reduce the tumor growth and inhibit the metastatic and angiogenic processes. Its repressive action of tumor responses occurs in different metabolic pathways. As a consequence, modulation of the carcinogenic signaling pathways reduces disordered replication, evasion of apoptosis, tissue invasion, and formation of metastasis, angiogenesis, and inflammation. However, the relevance of studies using flavonoids in the treatment of HCC is hampered by the lack of methodological rigor. Absence or incomplete characterization of the animal models, experimental groups, treatment protocols, phytochemical screening, and toxicity analysis of the plant products impairs the internal validity of the individual animal studies. Together with these limitations, contradictory results based on heterogeneous studies of the same plant species compromise the external validity of the evidence, making it difficult to translate animal data into clinical practice, as well as the relevance of the plant species as potential biotechnological targets in the development of new drugs to treat HCC. We believe that, in order to reduce the margin of error, the results should correlate *in vivo* and *in vitro* studies, since *in vitro* experiments are important for the identification of molecular markers to understand the action of the flavonoids in cancer treatment *in vivo*. Taking into account the fact that poor reporting quality does not always reflect the quality of the research actually carried out, we hope that our critical analysis may help to streamline preclinical researches in order to reduce methodological bias, improving data reliability and generalizability.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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## Supplementary Materials

Table S1: descriptors used for advanced search in PubMed and Scopus. *(Supplementary Materials)*

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*Research Article*

# Propolin C Inhibited Migration and Invasion via Suppression of EGFR-Mediated Epithelial-to-Mesenchymal Transition in Human Lung Cancer Cells

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Controlling lung cancer cell migration and invasion via epithelial-to-mesenchymal transition (EMT) through the regulation of epidermal growth factor receptor (EGFR) signaling pathway has been demonstrated. Searching biological active phytochemicals to repress EGFR-regulated EMT might prevent lung cancer progression. Propolis has been used as folk medicine in many countries and possesses anti-inflammatory, antioxidant, and anticancer activities. In this study, the antimigration and anti-invasion activities of propolin C, a c-prenylflavanone from Taiwanese propolis, were investigated on EGFR-regulated EMT signaling pathway. Cell migration and invasion activities were dose-dependently suppressed by noncytotoxic concentration of propolin C. Downregulations of vimentin and snail as well as upregulation of E-cadherin expressions were through the inhibition of EGFR-mediated phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinase (ERK) signaling pathway in propolin C-treated cells. In addition, EGF-induced migration and invasion were suppressed by propolin C-treated A549 lung cancer cells. No significant differences in E-cadherin expression were observed in EGF-stimulated cells. Interestingly, EGF-induced expressions of vimentin, snail, and slug were suppressed through the inhibition of PI3K/Akt and ERK signaling pathway in propolin C-treated cells. Inhibition of cell migration and invasion by propolin C was through the inhibition of EGF/EGFR-mediated signaling pathway, followed by EMT suppression in lung cancer.

## 1. Introduction

Propolis is a resinous material collected from buds and exudates of plants to build and defend the hive by honeybee. Propolis has been used as folk medicine for a long time in many countries. Numerous studies have indicated the biological activities of propolis, such as antimicrobial, antiviral, anti-inflammatory, antioxidant, and antitumor activities [1–4]. The biological components of propolis are highly diverse due to different plant species of propolis source. According to plant origins and chemical compositions, propolis can be divided into six categories in the world [5]. The main bioactive compounds of propolis from Europe and China are flavonoids and phenolic acids [6, 7]. Otherwise, Brazilian propolis mainly contains terpenoids and prenylated derivates of *p*-coumaric acids [8, 9]. The c-prenylflavanones are specific active constitutes of propolis from east Pacific regions, such as Taiwan and Okinawa [10, 11]. Eight prenylflavanones of Taiwanese propolis, propolin A to H, have been identified and originate from the surface resinous materials of *Macaranga tanarius* L. fruits [12, 13]. The anticancer activities of propolins have been characterized in lung cancer, melanoma, and glioma cell models [14–18]; however, the anti-invasion and antimigration activities of these components are still unclear.

Lung cancer is the leading cause of cancer death worldwide. Histologically, non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer [19]. Aberrant activation of epidermal growth factor receptor (EGFR) signaling pathway has been identified to advance lung cancer

tumorigenesis and results in the increase of patients mortality [20, 21]. EGFR is a receptor tyrosine kinase of ErbB family including EGFR (ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4). EGFR activation is induced by ligand and epidermal growth factor (EGF) binding and leads to dimerization, autophosphorylation, and activation of downstream signaling pathways. PI3K/Akt and MEK/ERK are the dominant two downstream signaling pathways of EGFR and are involved in EGFR-mediated cell proliferation, differentiation, and metastasis [22]. Dysregulation of EGF/EGFR signaling pathway is a well-known critical factor of lung cancer tumorigenesis and for the poor prognosis of this cancer [21]. Therefore, targeting EGF/EGFR signaling pathway to attenuate cancer cell tumorigenesis is the strategy for preventing and/or improving lung cancer patient prognosis.

Epithelial-to-mesenchymal transition (EMT) is a critical mechanism to regulate embryonic development, wound healing, and cancer cell metastasis [23]. EMT is a process in which cells lose their epithelial properties and convert to mesenchymal characteristics. During EMT development, the cell-cell cohesive ability is lost, and the migration capacity emerges [24]. In this process, molecularly, the epithelial-type molecules, such as E-cadherin and cytokeratin intermediate filament proteins, are downregulated; and, in contrast, the mesenchymal-type markers, such as N-cadherin, vimentin, and EMT-associated transcription factors, are upregulated [25]. Cumulative evidences demonstrate that EMT is a critical mechanism in tumor metastasis and impact on prognosis patient [26]. Induction of EMT by various growth factors, such as hepatocyte growth factor, transforming growth factor, and EGF, has been clarified in many cancer cell models [27, 28]. EGF-induced EMT has been demonstrated via ERK-mediated downregulation of E-cadherin and upregulation of vimentin and snail [29]. Furthermore, suppression of PI3K/Akt signaling pathway by quercetin has been examined in EGF-induced EMT in prostate cancer. EGF-induced expression of mesenchymal-like molecules, such as N-cadherin, vimentin, snail, and slug, is repressed by quercetin. Meanwhile, decreasing the expression of E-cadherin by EGF is reversed in quercetin-treated prostate cells [30].

Although the biological active components [12] and the anticancer properties of Taiwanese propolis have been examined [14–18], many biological functions and molecular mechanisms of Taiwanese propolis are still mystery, especially in EGF/EGFR-regulated tumorigenesis. In this study, the antimigration and anti-invasion properties of propolin C, a biological active compound of Taiwanese propolis, were examined in EGF/EGFR-regulated EMT in lung cancer. The results implicated that propolin C could be developed as a potential preventive agent for lung cancer metastasis.

## 2. Materials and Methods

*2.1. Purification of Propolin C.* Taiwanese propolis was purchased from Hualian, Taiwan. A voucher specimen (CGU-PE-1) was deposited in the herbarium of Chang Gung University, Taoyuan, Taiwan. Taiwanese propolis (985 g) was extracted with ethanol (5 L × 6) at room temperature. The filtered ethanol extracts were collected and concentrated to gain brown syrup (902.2 g). The brown syrup of ethanol extract was subjected to column chromatography (column: 14 cm i.d. × 75 cm) over silica gel (SiliaFlash G60, SiliCycle) and eluted with $CH_2Cl_2$ and EtOAc step gradients to acquire seven fractions. The second fraction ($CH_2CL_2$ : EtOAc = 9 : 1, 6 L) was concentrated and purified by recrystallization to obtain propolin C (22.206 g). The structure of propolin C was identified by comparison of the spectral data with literature values (Figure 1(a)) [14].

*2.2. Chemicals, Reagents, and Antibodies.* Anti-p-EGFR, anti-EGFR, anti-p-ERK, anti-ERK, anti-p-Akt, anti-slug, and anti-snail antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Akt and anti-vimentin antibodies were acquired from GeneTex, Inc. (Irvine, CA, USA). Anti-E-cadherin antibody was purchased from BD Biosciences, Inc. (San Jose, CA, USA). Anti-$\beta$-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

*2.3. Cell Culture and Cell Viability Assays.* The A549 and HCC827 lung cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Both of the cell lines were maintained in 5% fetal bovine serum-containing RPMI-1640 (HyClone Laboratories, Logan, UT, USA) and cultured at 37°C in 5% $CO_2$ atmosphere. Cells ($1 \times 10^4$/well) were seeded in 96-well plates for 24 h and then incubated with propolin C (0, 2.5, 5.0, 7.5, 10, and 20 $\mu$M) for 24 h. After treatment, cell viability was examined by MTT assay.

*2.4. Cell Cycle Analyses.* Cell cycle analysis was performed as described previously [31]. Briefly, cells were seeded and synchronized. After synchronization, propolin C-containing 5% fetal bovine serum medium was incubated for 24 h. Cells were harvested and stained with propidium iodide (50 $\mu$g/mL, Sigma-Aldrich, St. Louis, MO, USA), and FACScan laser flow cytometer analysis system (Beckman Coulter, Fullerton, CA) was used to detect cell cycle distribution.

*2.5. In Vitro Wound Closure.* A549 and HCC827 cells ($1 \times 10^5$ cells/well) were plated in 6-well plates for 24 h. Cells wounded by scratched with a pipette tip, incubated with or without propolin C (0, 2.5, 5.0, 7.5 and 10 $\mu$M)-containing 0.5% FBS RPMI medium for 24 h. Cells were photographed using a phase-contrast microscope (×200), as the descriptions in Kao et al. [32].

*2.6. In Vitro Invasion and Migration Assays. In vitro* invasion and migration assays were measured by modified protocols from Kao et al. [32]. Briefly, HCC827 and A549 cells were treated with serial concentrations of propolin C (0, 2.5, 5.0, 7.5, and 10 $\mu$M) for 24 h and cells were collected to be plated on Boyden chamber (BD Biosciences, Bedford, MA, USA) at cell density of $1 \times 10^5$ cells/well in serum-free medium for 24 h incubation. For *in vitro* invasion assay, 8 $\mu$m

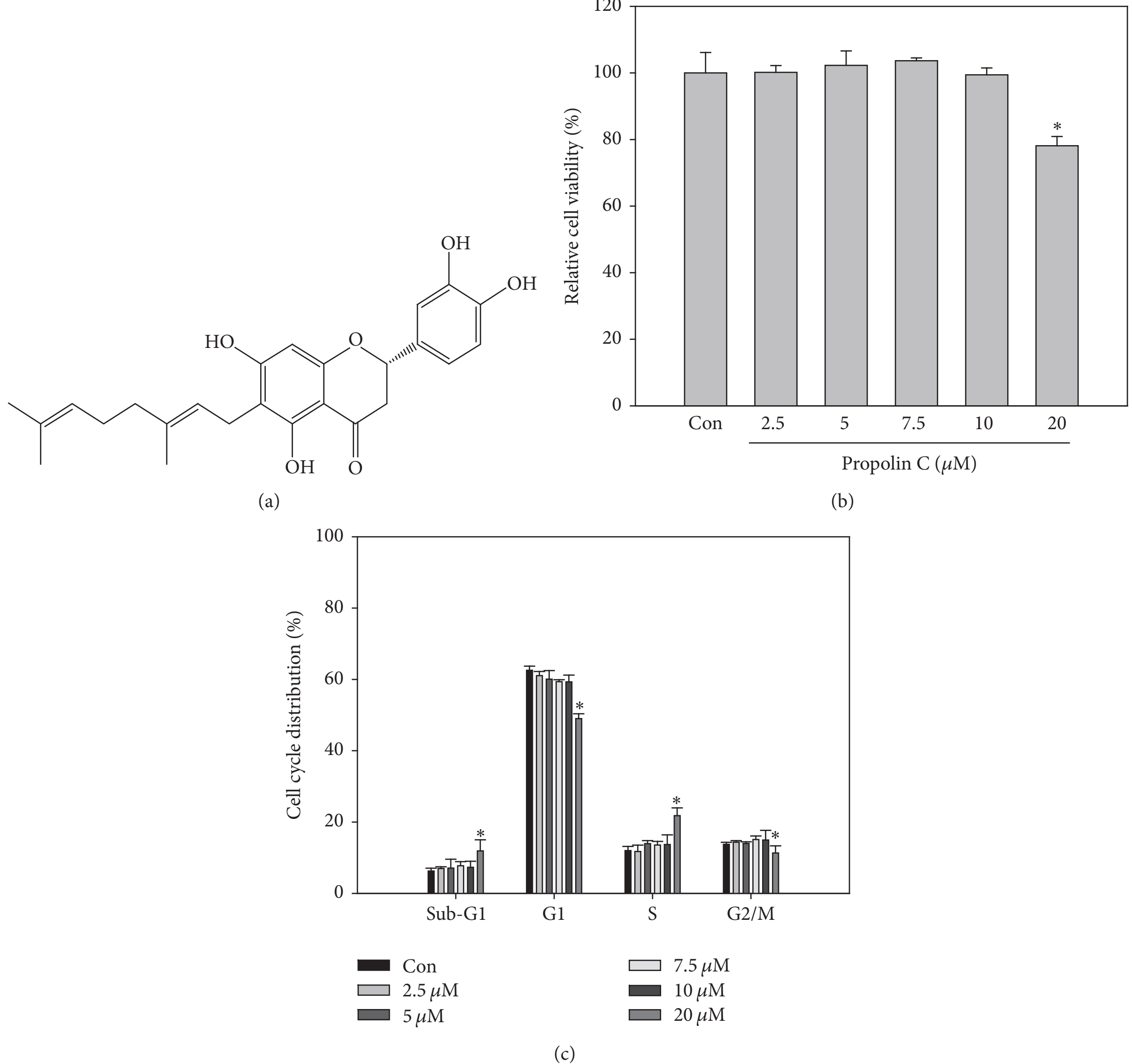



FIGURE 1: *Inhibitory effects of propolin C on cell viability of human lung carcinoma cancer cell line*. (a) Chemical structure of propolin C. (b) HCC827 cells were cultured in 96-well plates and treated with propolin C (2.5, 5, 7.5, 10, and 20 $\mu$M) for 24 h. After incubation, cell viability and (c) cell cycle distribution were detected by MTT assay and flow cytometry with PI labeling, respectively. Data were the mean ± SD of triplicate samples. $^{*}p < 0.05$ compared with control cells.

pore polycarbonate filters were coated with 10 $\mu$l Matrigel (25 mg/mL; BD Biosciences, Bedford, MA, USA) and the lower chamber was contained in 5% FBS-containing RPMI-1640 medium. The invaded cells were fixed with methanol and stained with 0.1% crystal violet. Cell numbers were counted under a light microscope. *In vitro* migration assay, 8 $\mu$m pore polycarbonate filters were not coated with Matrigel and experimental processes were the same as *in vitro* invasion assay. Triplicate samples were conducted, and data were expressed as average cell number.

*2.7. Western Blot Analyses.* Western blot analyses were performed as described previously [31]. Briefly, cell lysates were prepared and then quantitated, electrophoresed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA). After transfer, the membranes were blocked and incubated with the indicated antibodies. The signals were detected by chemiluminescence (ECL Kit, Amersham Pharmacia Biotech, IL, USA). The intensities of protein expression were then quantitated by a BioSpectrum Imaging System ChemiDoc-It2 810 (UVP, LLC, CA, USA). The expression of $\beta$-actin was used as the internal control.

*2.8. Statistical Analyses.* The results were expressed as the mean ± SD calculated from at least three independent determinations. One-way analysis of variance (ANOVA) tests

were used to compare individual experiment with the control value. ANOVA with Duncan's post hoc test in SAS statistical software was used for statistical analysis for the comparisons with different treatment groups. A $p < 0.05$ was considered as a significant difference.

## 3. Results

*3.1. The Antiproliferative Effects of Propolin C in HCC827 Lung Cancer Cells.* To evaluate the antiproliferative activity of propolin C on EGFR-mutated lung cancer cells, HCC827 cells were incubated with serial dosages of propolin C for 24 h and cell viability was then detected. As shown in Figure 1, there was no significant difference in cell viability after treatment with 10 $\mu$M of propolin C. However, cell viability decreased about 20% after treatment with 20 $\mu$M of propolin C (Figure 1(b)). Furthermore, treatment with 10 $\mu$M of propolin C did not affect cell cycle distribution. The sub-G1 and S phase cell accumulations were only observed in cells treated with 20 $\mu$M of propolin C (Figure 1(c)). According to these results, concentration of propolin C with no cytotoxicity effect for further examination was chosen.

*3.2. Effects of Propolin C on Cell Migration and Invasion in HCC827 Lung Cancer Cells.* To examine the antimigration and anti-invasion activities of propolin C, HCC827 cells were incubated with serial dosages of propolin C (0, 2.5, 5, 7.5, and 10 $\mu$M) for 24 h and *in vitro* migration and invasion assay was assessed subsequently. The results revealed that propolin C inhibited cell migration in a dose-dependent matter by wound healing and *in vitro* migration analyses (Figures 2(a) and 2(b)). Meanwhile, suppression of cell invasion was also observed in propolin C-treated cells in a dose-dependent manner (Figure 2(c)).

*3.3. The Role of PI3K/Akt and ERK Signaling Pathway in Propolin C-Regulated EMT.* To understand antimigration and anti-invasion effect of propolin C on EMT regulation, HCC827 cells were incubated with serial dosages of propolin C (0, 2.5, 5, 7.5, and 10 $\mu$M) for 24 h and EMT molecule expressions were detected by Western blot. The expression of epithelial-like cell marker, E-cadherin, was upregulated with the treatment of 7.5 $\mu$M propolin C, and the expression of mesenchymal-like cell marker, vimentin, was inhibited in a dose-dependent matter. Meanwhile, transcription factor, snail, but not slug, was dramatically decreased with propolin C treatment (Figure 3(a)).

EMT has been well known to be regulated by PI3K/Akt and ERK signaling pathways. To investigate suppression of EMT by propolin C, the expressions of phosphorylated Akt and ERK were evaluated. As shown in Figure 3(b), the expressions of phospho-ERK and phospho-Akt were dose-dependently inhibited by propolin C. Furthermore, protein expression of E-cadherin in the treatment with PI3K/Akt inhibitor (LY294002) or ERK inhibitor (PD98059) alone was upregulated, resulting in the inhibition of vimentin and snail (Figure 3(c)). Interestingly, treatment with LY294002 or PD98059 enhanced propolin C-induced E-cadherin expression. In addition, the inhibitions of vimentin and snail expression were also enhanced in combination treatment, compared with propolin C treatment alone (Figure 3(c)).

*3.4. The Effects of Propolin C-Regulated EMT on EGFR Signaling Downregulation.* EGFR-mediated PI3K/Akt and ERK signaling pathways are important in EMT regulation. Suppression of PI3K/Akt and ERK signaling pathway by propolin C inhibited EMT in propolin C-treated HCC827 cells (Figure 3). To measure the target of propolin C treatment, phosphorylation of EGFR was estimated in propolin C-treated HCC827 cells. The results revealed that the expression of phospho-EGFR was decreased in a dose-dependent manner (Figure 4(a)). To further assess the role of EGFR in propolin C-regulated EMT, clinical EGFR inhibitor, ZD1839, was used. The expression of E-cadherin was increased, whereas the expressions of vimentin and snail were decreased in ZD1839-treated cells (Figure 4(b)). Meanwhile, the expression of EMT molecular prolife in propolin C-treated cells was similar to ZD-treated cells.

*3.5. The Suppression of EGF-Induced Cell Migration and Invasion by Propolin C in A549 Lung Cancer Cells.* To further investigate the role of EGFR signaling pathway in propolin C-suppressed lung cancer cell migration and invasion, low endogenous EGFR-expressive A549 cells was chosen as study model. A549 lung cancer cells were pretreated with serial dosages of propolin C (2.5, 5, 7.5, and 10 $\mu$M) for 30 min and then stimulated with 50 ng/mL of EGF for 24 h. After EGFR treatment, wound healing assay and *in vitro* cell migration and invasion assay were performed as described in Materials and Methods. As shown in Figure 5, the wound healing ability was induced after EGF stimulation (Figure 5(a)). EGF-induced wound healing ability was repressed in a dose-dependent manner in the cells pretreated with propolin C (Figure 5(a)). Meanwhile, EGF-induced *in vitro* migration and invasion activities were dose-dependently inhibited in A549 lung cancer cells pretreated with propolin C.

*3.6. The Inhibition Mechanism of EGF-Induced EMT by Propolin C.* To demonstrate the inhibition mechanism of propolin C in EGF-induced lung cancer cell migration and invasion, EMT molecules expressions were evaluated. A549 lung cancer cells were pretreated with serial dosages of propolin C for 30 min and then incubated with EGF for 24 h. After EGF treatment, EMT molecule expressions were examined by Western blot analyses. Neither downregulation of E-cadherin expression was observed with EGF treatment nor upregulation of E-cadherin expression was measured in the pretreatment with propolin C compared with the control (Figure 6(a)). Interestingly, mesenchymal-like markers were only detected in the expression of EGF-regulated EMT. The expressions of vimentin, snail, and slug were upregulated in EGF treatment. Furthermore, EGF-induced expressions of vimentin, snail, and slug were inhibited in the pretreatment with propolin C.

The results revealed that propolin C-regulated EMT molecule expressions might be through downregulation of

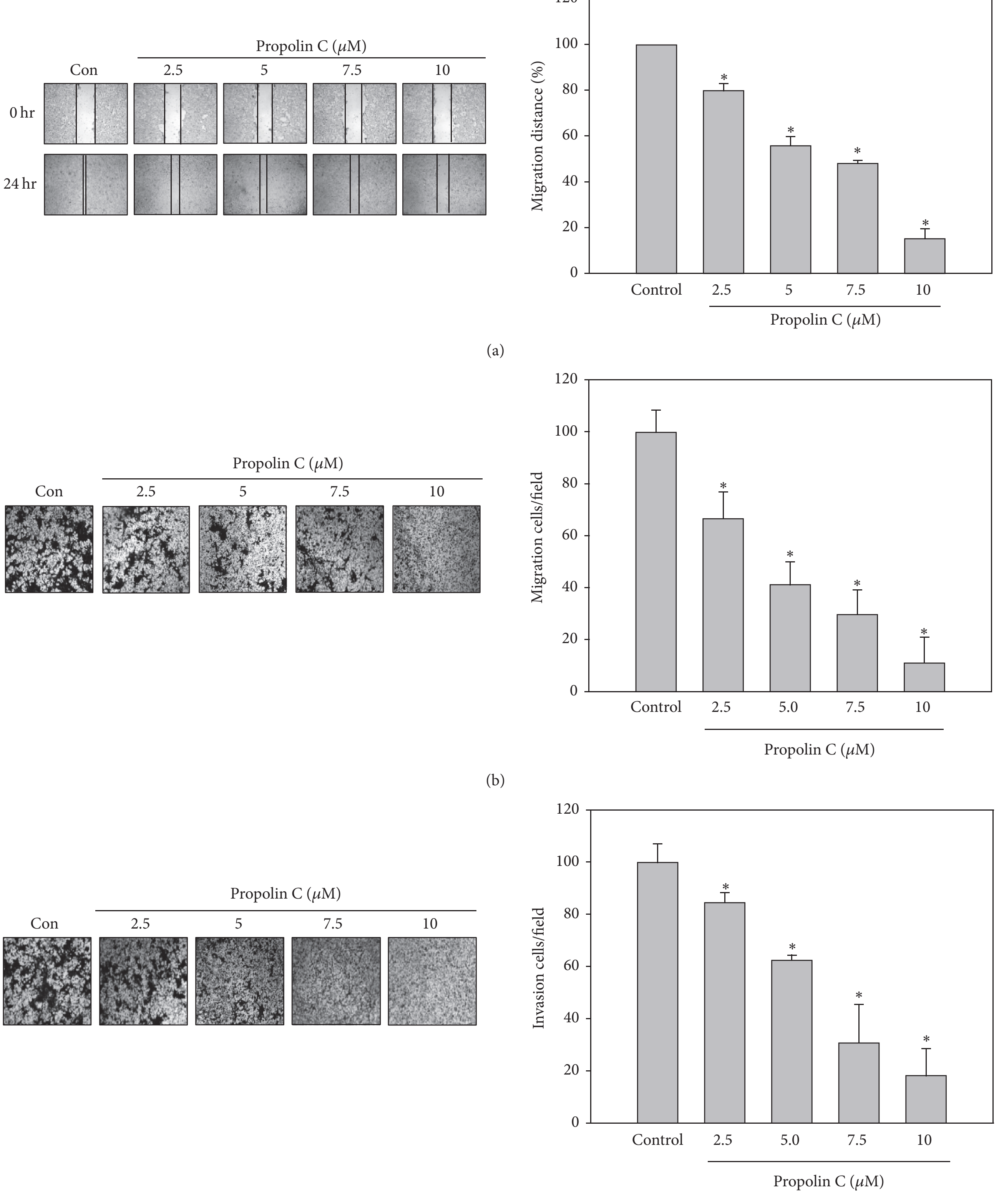



Figure 2: *Inhibitory effects of in vitro migration and invasion by propolin C in HCC827 lung cancer cells*. HCC827 cells were incubated with serial dosages of propolin C (2.5, 5, 7.5, and 10 μM) for 24 h and (a) *in vitro* wound healing, (b) Transwell migration, and (c) invasion analyses were performed as described in Materials and Methods. Data were represented as the mean ± SD of triplicate samples. Significant difference was observed from the control group ($^{*}p < 0.05$).

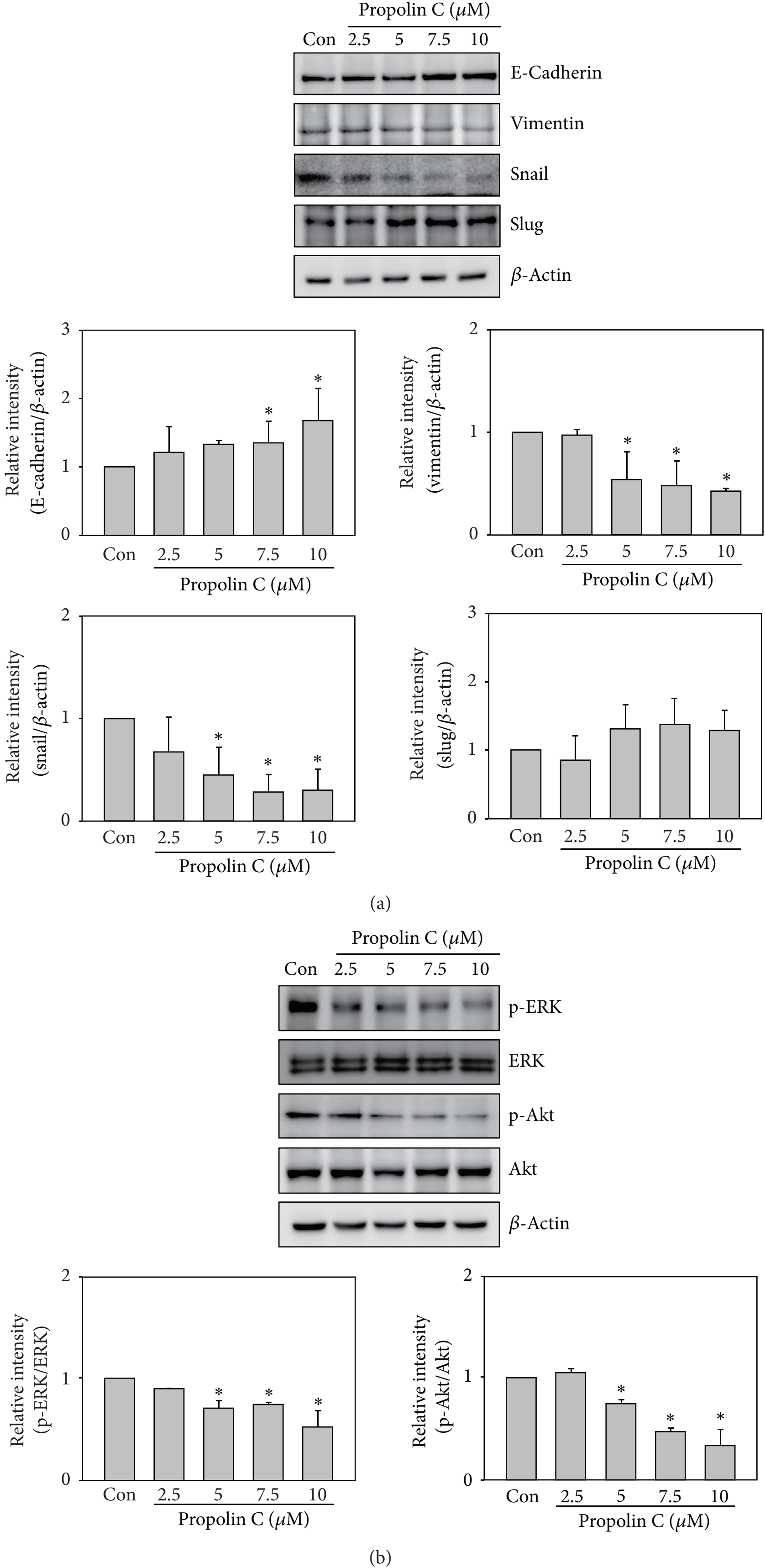



Figure 3: Continued.

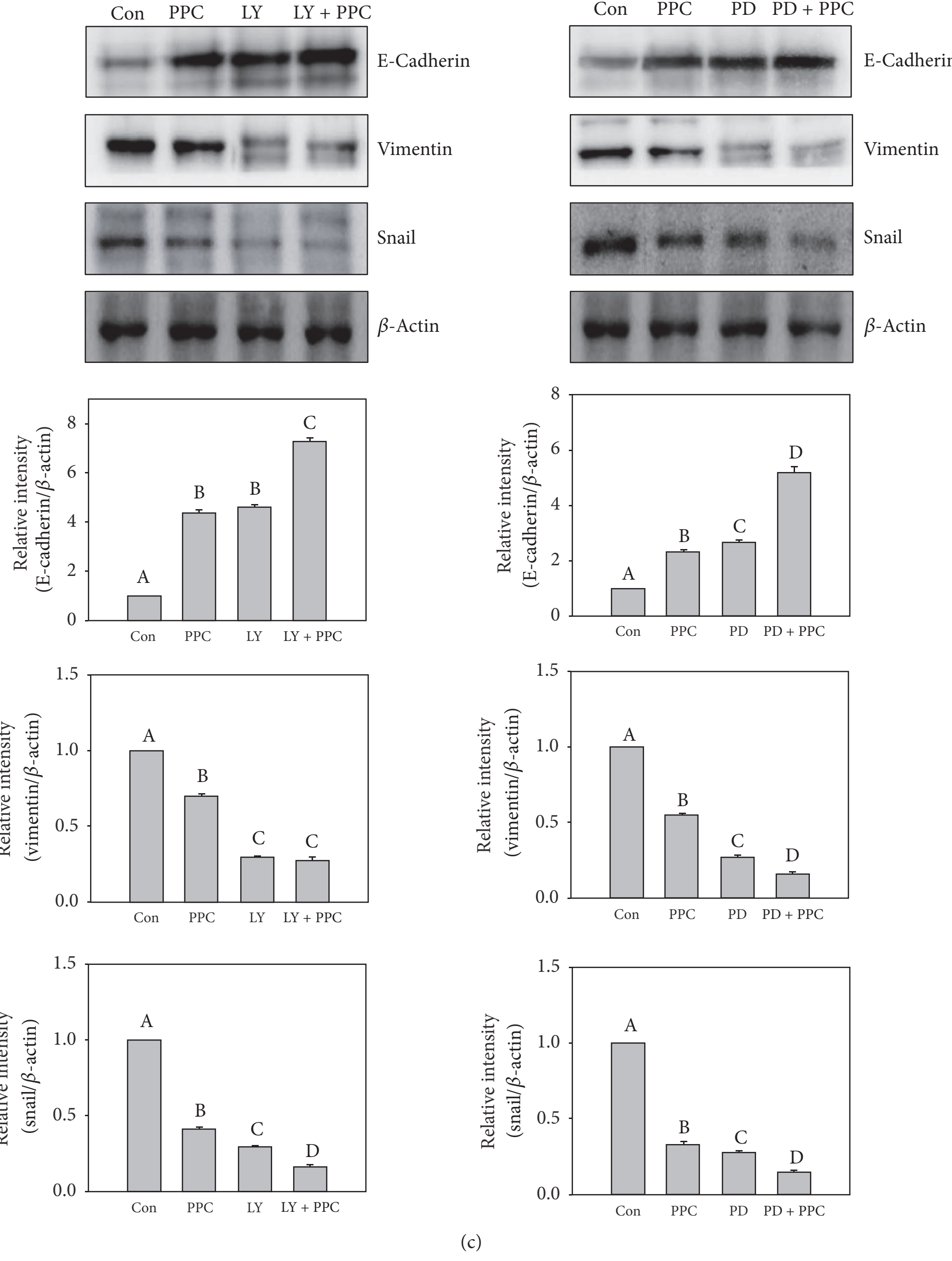



Figure 3: *Effects of propolin C on PI3K/Akt and ERK-mediated EMT marker expressions in HCC827 lung cancer cells*. HCC827 cells were treated with propolin C (2.5, 5, 7.5, and 10 $\mu$M) for 24 h. After treatment, the expressions of (a) E-cadherin, vimentin, slug, snail, and $\beta$-actin and (b) phospho-ERK, ERK, phospho-Akt, and Akt were analyzed by Western blot as described in Materials and Methods. Significant difference was observed from the control group ($^* p < 0.05$). (c) HCC827 cells were pretreated with LY294002 (LY, 10 $\mu$M) or PD98059 (PD, 10 $\mu$M) for 30 min and then incubated with or without propolin C (PPC, 10 $\mu$M) for 24 h. After incubation, cells were harvested and Western blot analyses were used to detect E-cadherin, vimentin, snail, and $\beta$-actin expressions. Data were shown as mean ± SD ($n = 3$). Different uppercase letters (A–D) indicate statistical differences among group ($p < 0.05$), and the same letter showed no difference ($p > 0.05$).

PI3K/Akt and ERK signaling pathways in EGFR-mutated HCC827 lung cancer cells (Figure 3). To verify the effects of propolin C in EGF/EGFR signaling pathway, the downstream EGF/EGFR signaling effectors, PI3K/Akt and ERK, were addressed. Increase of phospho-ERK expression was detected after EGF treatment for 15 min and the expression dramatically increased after 30 min of EFR treatment. However, EGF-induced ERK phosphorylation was inhibited by 10 $\mu$M of

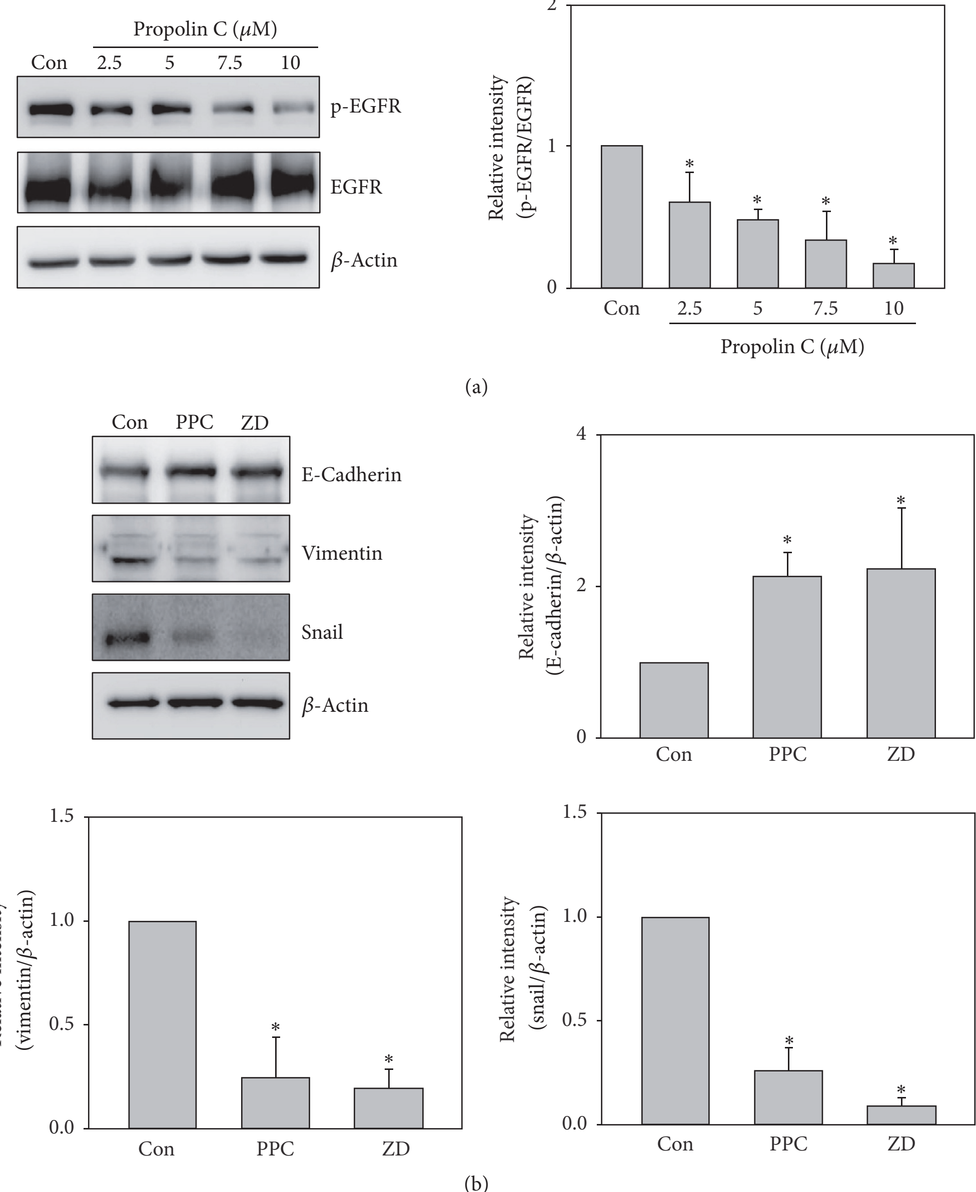



Figure 4: *Effects of propolin C on EGFR-mediated EMT marker expressions in HCC827 lung cancer cells*. HCC827 cells were incubated with (a) propolin C (2.5, 5, 7.5, and 10 $\mu$M) or (b) 10 $\mu$M of propolin C (PPC) and 1 nM of ZD1839 (ZD) for 24 h. After incubation, cell lysates were harvested and Western blot analyses were performed to detect the expressions of E-cadherin, vimentin, snail, and $\beta$-actin. Data represented at least three independent experiments. Significant difference was observed from the control group ($^*p < 0.05$).

propolin C (Figure 6(b)). In PI3K/Akt signaling pathway, increasing expression of phospho-Akt was shown after 5 min of EGF treatment and it persisted to 30 min. EGF-induced Akt phosphorylation was repressed by the pretreatment with propolin C (Figure 6(b)). Subsequently, the biological roles of PI3K/Akt and ERK signaling pathways in propolin C-repressed EGF-induced EMT were examined. A549 lung cancer cells were pretreated with propolin C, LY294002, and PD98059 alone, respectively, or were cotreated with LY294002 and PD98059 with propolin C for 30 min and then stimulated with EGF for 24 h. The expressions of EMT molecules were then addressed by Western blot. As shown in Figure 6(c), significant difference of EGF-regulated E-cadherin expression was not perceived in the pretreatments with the inhibitors alone or cotreatment with propolin C and inhibitors. However, downregulation of EGF-induced vimentin, snail, and slug expressions was discovered in LY294002- or PD98059-treated cells. Furthermore, repressing EGF-induced mesenchymal-like molecule expression via propolin C was enhanced in cotreatment with LY294002 or PD98059 (Figure 6(c)).

## 4. Discussion

Searching and characterizing phytochemicals and exploring biological activities from foods or plant sources have become

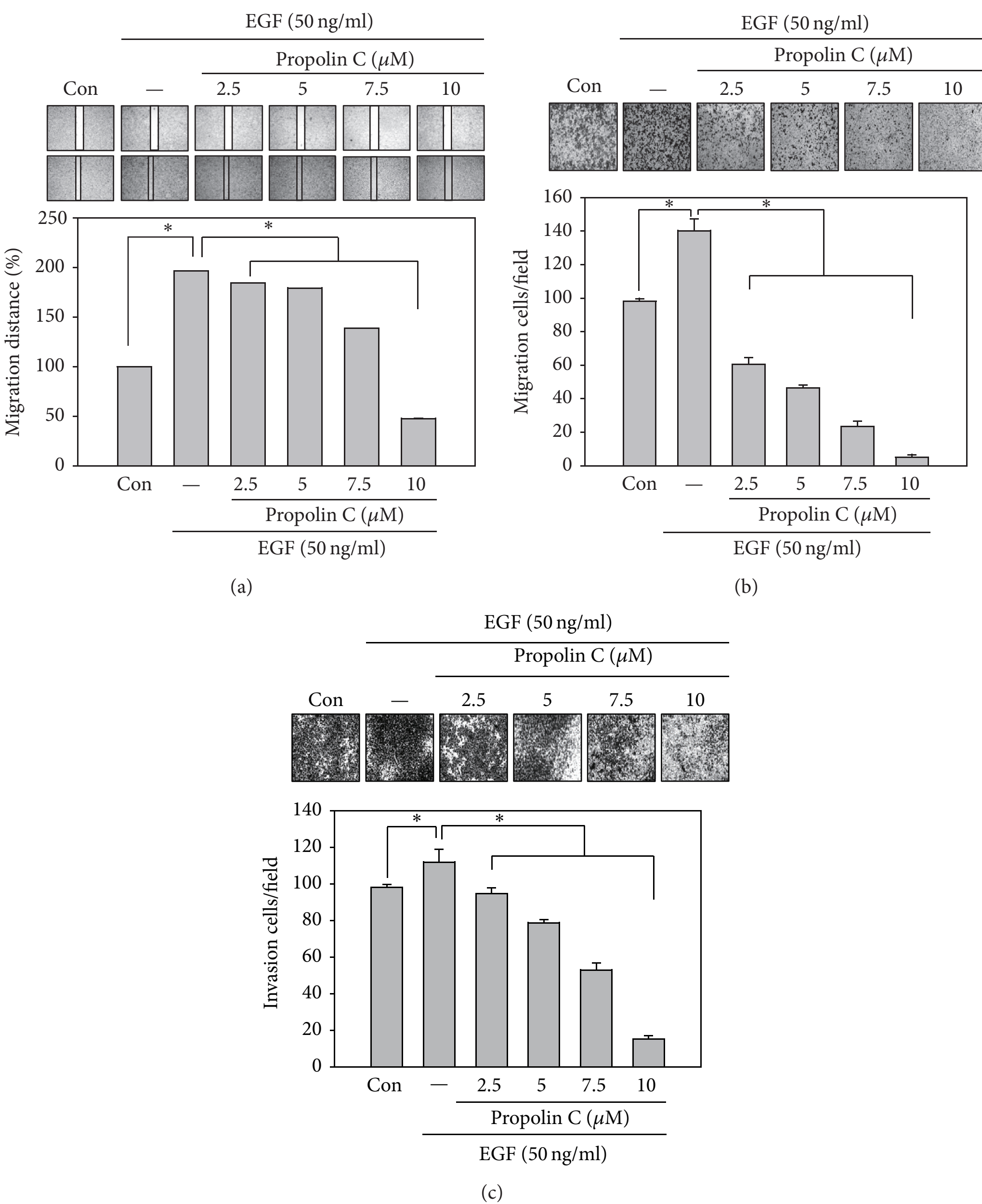
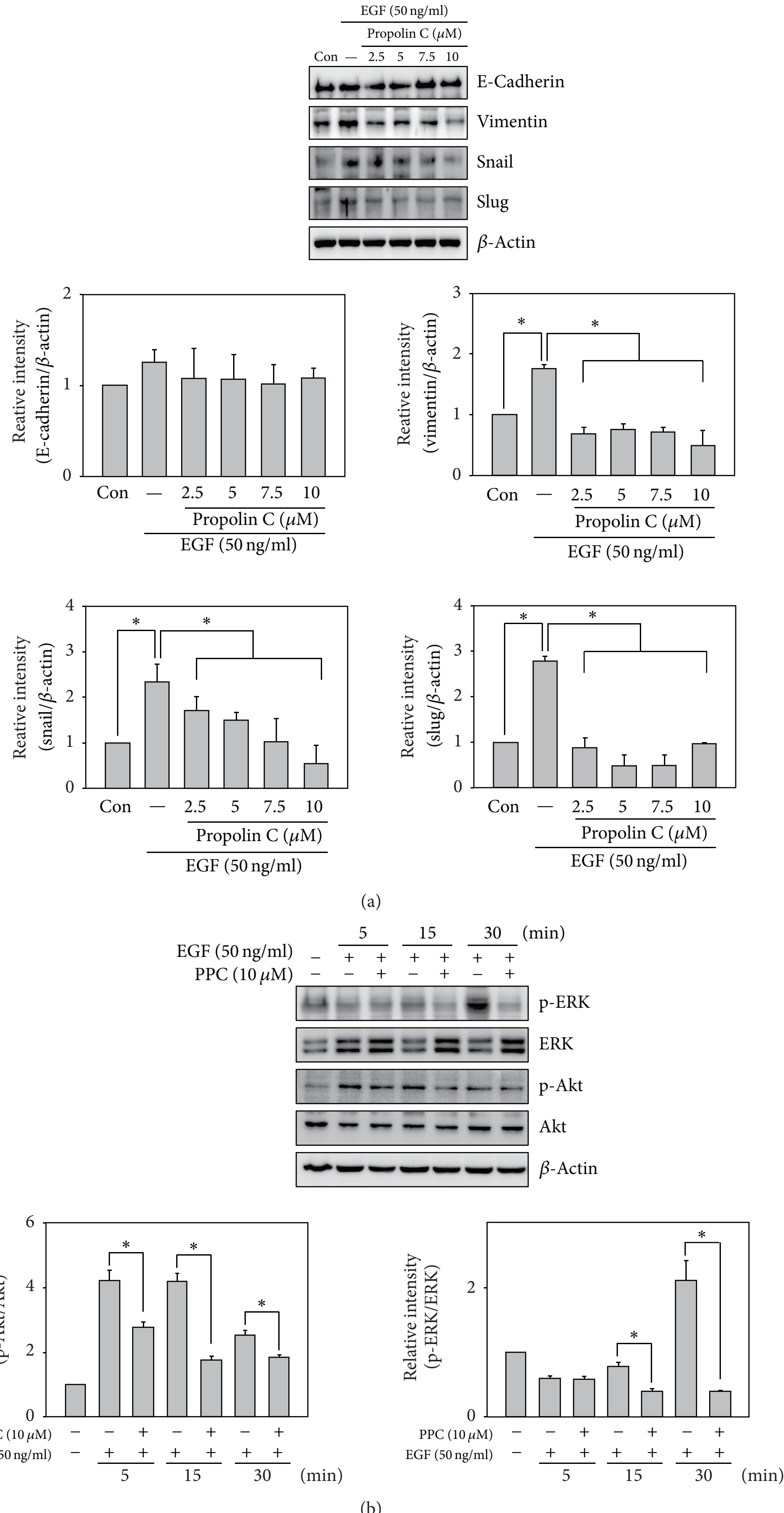



Figure 5: *Effects of propolin C on EGF-induced migration and invasion in A549 lung cancer cells*. A549 cells ($1 \times 10^4$/well) were pretreated with propolin C (2.5, 5, 7.5, and 10 $\mu$M) for 30 min and then stimulated with 50 ng/mL of EGF for 24 h. After treatment, (a) wound healing, (b) Transwell migration, and (c) invasion analyses were performed as described in Materials and Methods. Data were represented as the mean ± SD of triplicate samples. Significant difference was observed from the control group ($^*p < 0.05$).

the prominent strategy for cancer chemoprevention [33]. Propolins belong to the family of c-prenylflavanones and 8 related compounds have been identified from Taiwanese propolis [13]. Although propolins have been demonstrated to possess anticancer activities in numerous cancer cell models, the antimigration and anti-invasion activities of propolins are still unclear. In present studies, the anti-migration and anti-invasion activities of propolin C in EGF/EGFR-mediated EMT in lung cancer cells were examined. The results revealed that inhibition of migration and invasion by propolin C was through downregulation of EGFR/PI3K/Akt and ERK-mediated EMT signaling pathways in EGFR-mutated HCC827 lung cancer cells. Moreover, reversing EGF-induced migration and invasion and EMT changes were observed in propolin C-treated EGFR wild-type A549 lung cancer cells. Activation of EGFR/PI3K/Akt and ERK signaling pathway by EGF was repressed via propolin C.

Malignant neoplasm metastasis is highly correlated with majority of deaths in cancer patients. Changing cells from immobile epithelial phenotype to more invasive mesenchymal phenotype via EMT is critical process for tumor metastasis [26]. During EMT progression, the

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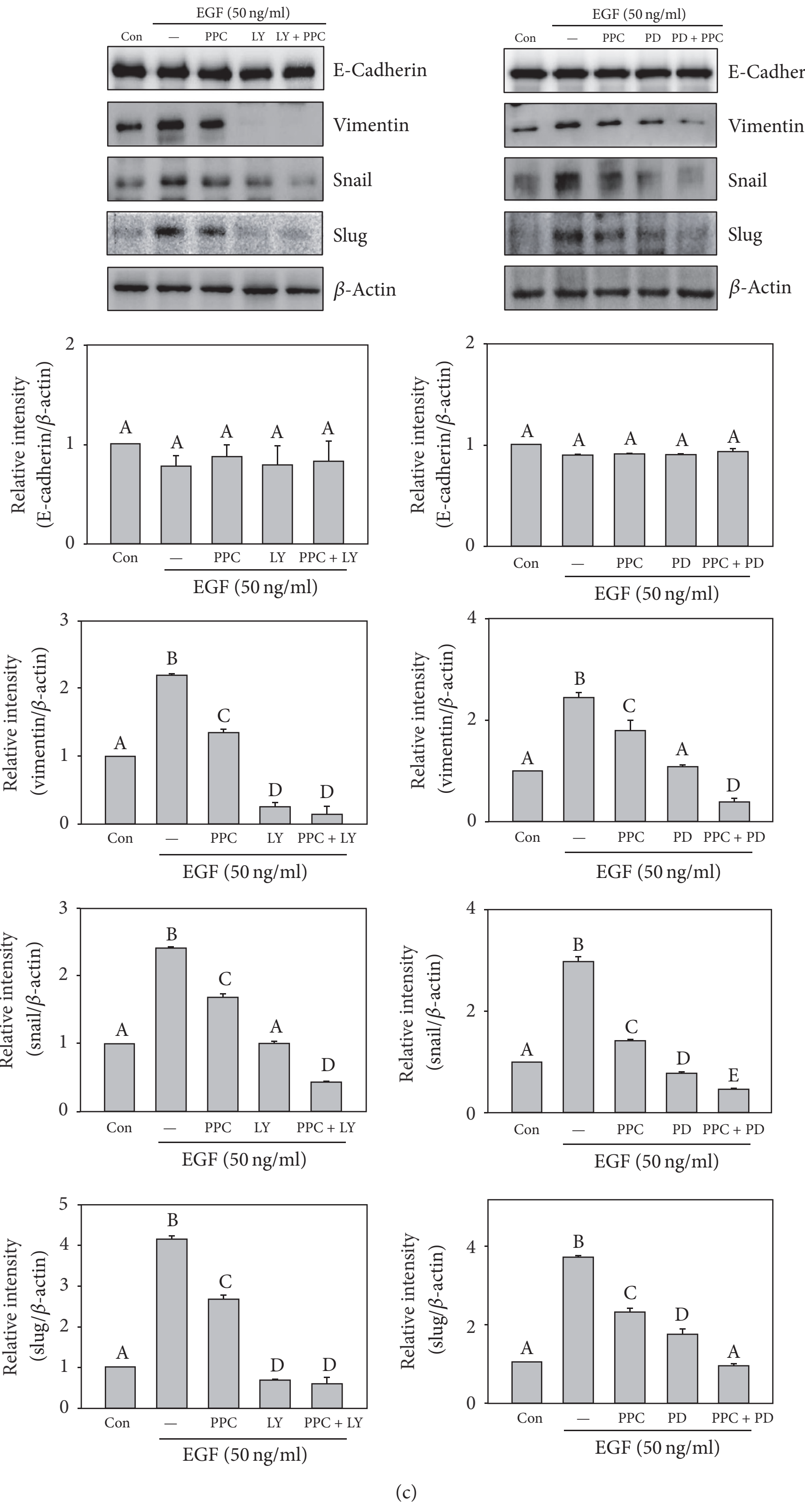


(c)

Figure 6: *Degeneration of EGF-induced EMT via PI3K/Akt and ERK inhibition in propolin C-treated A549 lung cancer cells*. A549 cells were synchronized and (a) pretreated with propolin C (2.5, 5, 7.5, and 10 $\mu$M) or (b) pretreated with 10 $\mu$M of propolin C (PPC) for 30 min and then stimulated with 50 ng/mL EGF for 5, 15, and 30 min. After EGF incubation, cell lysates were harvested and Western blot analyses were then performed to detect the expressions of E-cadherin, vimentin, slug, snail, phospho-ERK, ERK, phospho-Akt, Akt, and $\beta$-actin. Significant difference was observed from the compared groups ($^*p < 0.05$). (c) HCC827 cells were pretreated with propolin C (PPC, 10 $\mu$M), LY294002 (LY, 10 $\mu$M), and PD98059 (PD, 10 $\mu$M) alone or cotreated with LY294002 or PD98059 with propolin C for 30 min and then incubated with EGF (50 ng/mL) for 24 h. After incubation, cells were harvested and Western blot analyses were used to detect E-cadherin, vimentin, snail, slug, and $\beta$-actin expressions. Data were shown as mean ± SD ($n = 3$). Different uppercase letters (A–D) indicate statistical differences among group ($p < 0.05$), and the same letter showed no difference ($p > 0.05$).

expression of E-cadherin, the major epithelial-like molecule, is decreased concomitantly with the increase of mesenchymal-like molecules expressions [25]. Numerous studies indicate the high correlation between EMT and prognosis of cancer patients. Downregulation of E-cadherin expression in colon cancer patients is associated with high Tumor-Node-Metastasis (TNM) stage and distant metastasis [34]. Gene overexpression of mesenchymal-like markers and repressive expression of E-cadherin are indicated to reduce recurrence of free survival in breast cancer and NSCLC patients [35, 36]. Therefore, inhibiting EMT progression may benefit cancer patients at the risk of developing metastasis. In the present studies, the inhibitory effects on migration and invasion by propolin C, an active biological component from Taiwanese propolis, were investigated in EMT-regulated lung cancer cells. The results revealed that propolin C did not affect cell proliferation and cell cycle distribution in EGFR-mutated HCC827 cells under treatment with 10 $\mu$M of propolin C (Figure 1). Treatment of propolin C with noncytotoxic effect suppressed HCC827 cell migration and invasion in a dose-dependent manner by wound healing and *in vitro* migration and invasion assay (Figure 2). Furthermore, Western blots showed that the expressions of vimentin and snail were dose-dependently decreased by propolin C. Inductive expression of E-cadherin was observed after treatment with 7.5 $\mu$M of propolin C. However, significant difference in slug expression in propolin C-treated cells was not detected (Figure 3(a)). To confirm the molecular mechanism of propolin C in EMT regulation, PI3K/Akt and ERK signaling pathways were inspected. As shown in Figure 3(b), the expressions of phospho-ERK and phospho-Akt were repressed in a dose-dependent manner (Figure 3(b)). Increase of E-cadherin expression and repression of vimentin and snail expression were observed by pharmacological inhibitor of PI3K/Akt (LY294002) and ERK (PD98059). Interestingly, the enhancement of expression of E-cadherin was perceived in combinational treatment with PI3K/Akt or ERK inhibitor plus propolin C, whereas the expressions of vimentin and snail were dramatically decreased (Figure 3(c)). These results revealed that PI3K/Akt- and ERK-mediated EMT was inhibited by propolin C followed by migration and invasion suppression in HCC827 cells.

EGFR has been indicated to regulate cell proliferation, survival, and metastasis [22]. Genetic mutation of EGFR has been found in numerous cancers and indicated high correlation with poor prognosis, especially in lung cancers [21, 22]. Clinical study indicates that patients with EGFR mutation possess higher invasive activity than those harboring wild-type EGFR [37]. In addition, higher concentration of EGF in serum has been observed in lung cancer patients compared to healthy group [38]. Accordingly, blockade of EGF/EGFR-mediated migration and invasion might improve prognosis. Numerous studies indicate that EMT-mediated migration and invasion are regulated by EGF/EGFR signaling pathway [29, 30, 39]. In the present studies, the results unveiled that the expression of phospho-EGFR was dose-dependently inhibited by propolin C in EGFR-mutated HCC827 lung cancer cells (Figure 4(a)). To further validate the character of EGFR signaling pathway in propolin C-regulated EMT, EGFR tyrosine kinase inhibitor, ZD 1839, was selected. As shown in Figure 4(b), the expression of E-cadherin was increased and the expressions of vimentin and snail were decreased in ZD-treated HCC827 cells. Interestingly, the profiles of ZD-regulated EMT molecule expression were similar to propolin C-regulated experiment. The results implicated that inhibition of EMT-regulated cell migration and invasion via propolin C might be over EGFR signaling repression.

To further prove migration and invasion of EGFR-mediated EMT in propolin C-suppressed lung cancer cells, EGF-induced EGFR wild-type A549 lung cancer cell model was examined. The results showed that the migration and invasion abilities were increased in EGF-stimulated cells by wound healing and *in vitro* migration and invasion analyses. EGF-induced migration and invasion were dose-dependently suppressed in A549 cells pretreated with propolin C (Figure 5). Furthermore, the expressions of mesenchymal-like molecules, vimentin, snail, and slug, were upregulated after EGF stimulation, while expressions of EGF-induced mesenchymal-like molecules were inhibited via propolin C. Nevertheless, there was no significant difference in the expression of epithelial-like molecule, E-cadherin, in EGF-stimulated A549 cells with or without propolin C pretreatment (Figure 6(a)). In addition, EGF-induced expressions of vimentin, snail, and slug were suppressed by PI3K/Akt and ERK inhibitors alone or plus propolin C. Remarkably, EGF-inhibited E-cadherin expression was not upregulated after PI3K/Akt and ERK inhibitors alone or plus propolin C (Figure 6(c)). Recent studies have shown that the expression of E-cadherin is not increased after 24 h of EGF treatment in A549 cells [40]. However, downregulation of E-cadherin expression is noticed after EGF stimulation for 72 h in MCF-7 cells [29]. A long-term effect of downregulation of E-cadherin expression via EGF stimulation is suggested in our system. 24-Hour exposure might not be enough to detect the changes of E-cadherin expression in our system. Furthermore, our results also revealed that the expression of slug did not change after propolin C treatment in HCC827 cell (Figure 3(a)) but decreased in propolin C-treated A549 cells after EGF stimulation (Figure 6(c)). Not only is the expression of slug regulated by EGFR signaling pathway but also it is induced by hepatocyte growth factor (HGF)/c-Met-mediated signaling pathway in murine colorectal and lung cancer cells [41, 42]. Analyses of the endogenous expression of c-Met showed that higher expression of c-Met signaling pathway was observed in HCC827 than in A549 lung cancer cells [42]. We speculated that high expression of c-Met signaling pathway might bypass propolin C-inhibited slug expression in HCC827 lung cancer cells.

Propolis has been indicated to possess beneficial effects on health and disease prevention. More than 300 biological components of propolis have been characterized from different countries or locations. The vast variations of these active components depend on the geographic regions, seasons, and plant sources [5–8, 12]. Propolins, which belong to c-prenylflavanones of Taiwanese propolis, have been identified and the biological activities have been characterized. The anticancer activities of propolins have been demonstrated in various cancer cell lines. Induction of

Figure 7: Proposed signal transduction pathways of the migration and invasion by propolin C in lung cancer cells.

mitochondria-dependent apoptosis by propolin A, B, and C has been observed in human melanoma cells [14, 15]. The highly radical scavenging activity of propolin G has been demonstrated to protect oxidative stress-induced cortical neuron damage [17]. Accumulation of G1 phase cells through p53-dependent and -independent $p21^{Waf1/Cip1}$ expression via propolin H is also indicated in lung cancer cells [16]. Although the antitumorigenic activities of propolins have been examined, other biological activities of propolins are still unclear. In these studies, antimigration and anti-invasion activities of propolin C through EMT regulation were discovered. Furthermore, repression of EMT-regulated migration and invasion was through downregulation of EGFR-mediated PI3K/Akt and ERK signaling pathways in EGFR-mutated HCC827 lung cancer cells (Figures 3, 4, and 5). Additionally, EGF-induced PI3K/Akt and ERK activation was inhibited by propolin C in EGFR wild-type A549 lung cancer cells (Figure 6(b)). Accordingly, propolin C might be an inhibitor of EGFR. Although the results revealed that propolin C-regulated EMT might be through EGFR signaling pathway downregulation, other signaling pathways involved in EMT regulation could not be excluded. Many signaling pathways have also been indicated to regulate EMT, such as hepatocyte growth factor (HGF)/c-Met, transforming growth factor-beta 1 (TGF-$\beta$1), and fibroblast growth factor (FGF) signaling pathways [42–44]. The roles of propolin C in these signaling pathways-mediated migration and invasion should be further explored. The antitumorigenesis of propolin C in other signaling pathways is also investigated in our future study. In conclusion, inhibition of migration and invasion via propolin C was suggested by the inhibition of EGFR-mediated signaling pathway in lung cancer cells (Figure 7). Propolin C was suggested as an antitumorigenic candidate compound for lung cancer treatment and/or prevention.

## 5. Conclusions

The present results revealed that suppression of lung cancer cells migration and invasion with propolin C treatment was through EMT regulation. Propolin C-regulated EMT was through downregulation of EGFR-mediated PI3K/Akt and ERK signaling pathways in EGFR-mutated lung cancer cells. In addition, propolin C also inhibited EGF-induced migration and invasion as well as mesenchymal-like markers expressions in EGFR wild-type lung cancer cells. EGF-induced PI3K/Akt and ERK activation was inhibited in propolin C-repressed EMT in EGFR wild-type lung cancer cells. Taiwanese propolis active component, propolin C, might become an antitumorigenic candidate compound for lung cancer treatment and/or prevention in the future.

## Conflicts of Interest

The authors declare that there are no conflicts of interest.

## Authors' Contributions

Jih-Tung Pai, Yann-Lii Leu, and Meng-Shih Weng conceived and designed the experiments. Yi-Chin Lee, Si-Ying Chen, and Yann-Lii Leu performed the experiments. Yi-Chin Lee and Meng-Shih Weng analyzed the data. Jih-Tung Pai, Yann-Lii Leu, and Meng-Shih Weng prepared the manuscript. Jih-Tung Pai and Yi-Chin Lee contributed equally to this work.

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*Research Article*

# Traditional Herbal Formula NPC01 Exerts Antiangiogenic Effects through Inhibiting the PI3K/Akt/mTOR Signaling Pathway in Nasopharyngeal Carcinoma Cells

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Antiangiogenic therapy is vital in nasopharyngeal carcinoma (NPC) treatment. NPC01 has already been successfully used in treating patients with NPC in clinical practice and exerted an excellent antiangiogenetic effect. However, the potential molecular mechanism underlying the antitumor effect of NPC01 has not been well explored. The present study demonstrated that NPC01 could significantly inhibit cell proliferation and induce cell apoptosis in a dose-dependent manner in human NPC cell lines. Furthermore, NPC01 exerted antiproliferative and antiangiogenic effects in NPC xenograft mice. Moreover, the study showed that NPC01 could significantly decrease the expression of angiogenesis-associated factors including hypoxia-inducible factor-1$\alpha$ and vascular endothelial growth factor. Additionally, the decreased expression of these angiogenesis-associated factors could be due to the inhibition of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway (PI3K/Akt/mTOR). In conclusion, the results proposed that NPC01 could exert its antitumor effect by suppressing the PI3K/Akt/mTOR signaling pathway. Further studies are warranted to elucidate the molecular mechanism.

## 1. Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck malignant epithelial tumor that shows a clear regional and racial prevalence [1, 2]. NPC is endemic in several areas, particularly in Southeast Asia. A total of 60,000 new cases of NPC and 34,000 deaths attributed to NPC in both sexes were reported in China in the year 2015 [3]. The multifactorial etiology, including infection, genetic predisposition, and environment, has been widely reported, but some other factors still remain unknown [4, 5].

Angiogenesis is the formation of new blood vessels. It is a part of growth and healing. It is also important in cancer growth and spread [6]. Emerging evidence has shown that antiangiogenic therapy is an important part of chemotherapy for several types of cancer [7–9]. Antiangiogenesis can be a potential therapeutic target for NPC treatment, as one of the important biologic features of NPC is the abnormal angiogenesis. Despite the little controversy that radiotherapy is the mainstay of primary treatment, chemotherapy can serve dual purposes of potentiating radiotherapy and eradicating subclinical micrometastasis (PMID: 2064265, 17404741, 8640688, and 2334832). Several studies have demonstrated that compounds derived from plants have anti-inflammatory, antiallergic, antiviral, antiangiogenesis, and anticancer properties [6, 10]. Additionally, increasing lines of evidence suggest that traditional Chinese herbs are sources of compounds that may serve as potential therapeutic drugs in NPC [11, 12]. However, a previous study suggested that a single compound could be effective but did not reflect the formula effects. Therefore, NPC01 was evaluated to determine its *in vitro* and *in vivo* therapeutic effects on NPC cells.

NPC01 is modified from Liang-Ge-Sang, an ancient Chinese herbal formula derived from the formula book "He

Ji Ju Fang" of the Song Dynasty (AD 960–1279) in China. "He Ji Ju Fang" has been used for treating inflammation in clinical practice, and Liang-Ge-Sang has been especially used to treat fever, constipation, pharyngitis, and rhinitis in China for centuries [13]. Since some pieces of formula similar to NPC01 have a critical modulatory effect on immune system and have already been used for NPC treatment in clinics, the present study investigated the antitumor effect of NPC01 on NPC cells [14, 15]. The study found that NPC01 could inhibit NPC cell growth *in vitro* and *in vivo* and play antiangiogenic effects putatively by suppressing the PI3K/Akt/mTOR signaling pathway.

## 2. Materials and Methods

*2.1. Reagents.* NPC01 contained five species of medicinal plants purchased from Tianjiang Pharmaceutical Co. Ltd. (Jiangyin, Jiangsu, China, one of the six approved manufacturers of Chinese herbal granules in China) [16]. Every herb of NPC01 was cut into pieces and then mixed together in the ratio of Glycyrrhizae Radix Preparata 60 g, *Rheum palmatum* L. 60 g, *Ligusticum chuanxiong* Hort 75 g, *Coptis chinensis* Franch. 75 g, and *Forsythia suspensa* 120 g. The rule of compositions is based on traditional Chinese medicinal theory, and the compatibility of herbs is due to our clinical experience. The rat doses of were converted from human doses (Chinese Pharmacopeia, 2010) based on body surface areas. According to the body surface areas of human and rat, the oral dose of formula in rats was 500 mg/kg (suspended in 1% CMC-Na), which was equal to its clinical dose (80 mg/kg). The dose was a 10-day treatment for an adult with an average body weight of 60 kg. It was extracted with purified water (390 mL) using a reflux for 2 h at 100°C and then filtered using a 25 $\mu$m sieve. The extracts were lyophilized into 27.4 g dry powder, which was stored at −80°C. For experiments, 10 mg dry powder, approximately equivalent to 1‰ of daily dose for one adult person, was dissolved in 100 mL RPMI-1640 culture medium (Gibco, China) and filtered through a 0.45 $\mu$m syringe filter before use.

*2.2. Cell Culture.* Human immortalized nasopharyngeal epithelial cell line (NP69) and NPC cell lines 5–8F (high metastasis) were purchased from ATCC and cultured in RPMI 1640 (Gibco, USA) with 10% fetal bovine serum (Gibco, USA). The immortalized nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte serum-free medium (Invitrogen) supplemented with 5% FCS, 25 $\mu$g/ml bovine pituitary extract, and 0.2 ng/ml recombinant epidermal growth factor, as suggested. 5–8F was cultured in RPMI 1640 medium (GIBCO, USA) containing 15% FCS by the manufacturer. All the cell lines were grown in a humidified incubator at 37°C with 5% CO2. The cells in the logarithmic growth phase were used for the experiment.

*2.3. Cell Proliferation Assay.* A total of $3 \times 10^4$ cells/well were seeded into 96-well plates, incubated overnight, and treated with different concentrations of NPC01 (0, 50, 100, and 200 mg/kg). MTT (Sigma–Aldrich, USA) was added and incubated in the dark at 37°C for 2 h. Absorbance was measured at a wavelength of 490 nm.

*2.4. Flow Cytometric Analysis.* The cell lines were treated with different concentrations of NPC01 (0, 50, 100, and 200 mg/kg). After 24 h, the cells were trypsinized (Sigma) and centrifuged at 1000$g$. The pellets were washed twice using phosphate-buffered saline (PBS). Subsequently, the cells were resuspended and labeled using an Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit according to the manufacturer's protocol (BD Biosciences, NJ, USA).

*2.5. Quantitative Real-Time Polymerase Chain Reaction.* Total RNA was extracted from the NPC cell lines 5–8F and an immortalized nasopharyngeal epithelial cell line, NP69, as well as three NPC biopsies and the paired normal tissues by using TRIzol reagent (Invitrogen). After reverse transcription of the total RNA, the first-strand cDNA was then used as template for detection of LATS2 expression by using quantitative real-time PCR (QT-PCR) with the SYBR Green I chemistry (ABI Inc., USA). The PCR protocol included one cycle at 95°C (3 min), followed by 40 cycles of 95°C (15 s) and 55°C (1 min). The primer sequences were as follows:

Vascular endothelial growth factor (VEGF) sense, 5′-TGCCCACTGAGGAGTCCAAC-3′

VEGF antisense, 5′-TGGTTCCCGAAACGCTGAG-3′,

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense,

5′-CGGAGTCAACGGATTTGGCC-3′

GAPDH antisense, 5′-GTGCAGAGATGGCAT-GGAC-3′.

*2.6. Western Blotting.* Approximately $5 \times 10^5$ cells/well were seeded into 6-well plates and treated with NPC01 or vehicle for 24 h. The protein content was measured using the BCA Protein Assay Reagent (Pierce), and 20 $\mu$g of each sample was diluted with 1x lysis buffer. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4.5–15% gradient gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies against phosphorylated Akt (rabbit), mTOR (rabbit), and PI3K (rabbit) which were purchased from Cell Signaling Technology (Danvers, MA, USA). HIF-1a (rabbit) and VEGF (rabbit) were obtained from Abcam company (Cambridge, UK), and antibodies for total Akt (rabbit), mTOR (rabbit), and PI3K (rabbit) were purchased from Cell Signaling Technology (Danvers, MA, USA). The treated cells were collected, and total proteins were extracted using lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl sodium pyrophosphate, 1 mmol/L $\beta$-glycerophosphate, 1 mmol/L $Na_3VO_4$, 1 $\mu$g/mL leupeptin, and 1 mmol/L PMSF; Cell Signaling Technology, USA). After centrifugation at 14,000$g$ for 15 min at 4°C, the supernatant was collected and the protein concentration was detected using the Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Inc., IL, USA) according to the manufacturer's protocol. Equal amounts of protein were separated using 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride

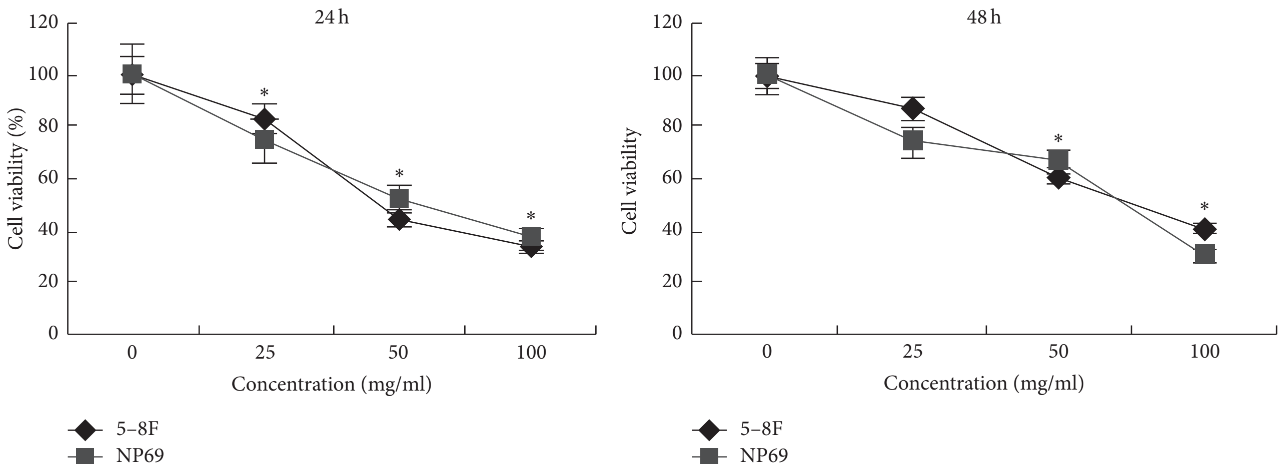


Figure 1: Effect of NPC01 on cell proliferation. 5–8F and NP69 cells were cultured in a 96-well plate and treated with different concentrations of NPC01 for 24 or 48 h. Cell proliferation was assessed using an MTT assay. Values represent the mean ± standard deviation of three independent experiments. $^{*}P < 0.05$.

membrane (Millipore, MA, USA). After blocking with 5% nonfat milk in Tris buffered saline with Tween 20 washing buffer, the membrane was incubated with the specific primary antibodies at 4°C overnight. The blots were labeled with peroxidase-conjugated secondary antibodies. The formed immune complex was visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.) according to the manufacturer's protocol and exposed to an X-ray film.

*2.7. A Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End-Labeling Assay.* Cell apoptosis in mouse tumor samples was measured using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay kit (Roche Diagnostics, IN, USA). Brown nuclei were considered apoptotic. The number of apoptotic cells/1000 cells was recorded in each field of view using a microscope (LZ12; Leica Microsystems GmbH, Wetzlar, Germany) at magnification ×200.

*2.8. Animal Procedures.* All animal procedures, including tumor transplantation, tumor volume measurement, and mouse euthanization, were approved by the Institutional Animal Care and Use Committee at Tianjin Cancer Hospital. The male nude mice, aged 6–8 weeks and weighing 18–20 g, were maintained under specific pathogen-free conditions, because 5–8F is high metastasis nasopharyngeal epithelial cell line. To evaluate the in vivo effect, we performed experiment in NPC cells and human nasopharyngeal epithelial cell line NP69 and the results showed the antitumor effect in 5–8F compared with the control NP69 cell, especially for 5–8F cells. The xenograft tumor model was established by subcutaneously injecting $1 \times 10^9$ 5–8 F cells. The mice were daily subjected to the intragastric administration of NPC01 at the indicated dose per mouse or the same volume of PBS as the control for 14 consecutive days after the injection. Tumor volume was calculated according to the formula: volume = 1/2 × length × width$^2$. These mice were killed after 2 weeks of NPC01 treatment, and the tumors were harvested for pathological observation. The tumor-inhibiting rate was calculated according to the following formula:

$$\text{Tumor inhibiting rate (\%)} = \left(1 - \frac{\text{mean of NPC01 treated tumor volume}}{\text{mean of vehicle tumor volume}}\right) \times 100\%. \quad (1)$$

*2.9. Preparation of Tissues and Histology.* The right lower lobes were collected and fixed using 4% paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 $\mu$m sections of embedded tissue were cut on a rotary microtome, placed on glass slides, deparaffinized, and stained with hematoxylin and eosin. The slides were mounted using Canada balsam (Showa Chemical Co. Ltd., Tokyo, Japan). PAS-positive cells in the epithelium and total epithelial cells were counted, and the percentage of positive cells was calculated. For quantitating airspace in the lungs, the sections with the maximum parenchymal cross sections were selected for morphometric analysis using a digitized image tool. The micrographs were obtained using Image Pro-Plus 5.1 software (Media Cybernetics, Inc. MD, USA).

*2.10. Statistical Analysis.* Data were expressed as the mean ± standard deviation, and a statistical analysis was performed using SPSS version 10.0 (SPSS, Inc., IL, USA). A $P$ value < 0.05 was considered to indicate a statistically significant difference.

## 3. Results

*3.1. NPC01 Inhibited the Proliferation of 5–8F and NP69 Cells.* 5–8F and NP69 cells were treated with different concentrations of NPC01 (0, 25, 50, or 100 $\mu$g/mL) for 24 or 48 h followed by the MTT assay to investigate the possible antiproliferative effects of NPC01. The result showed that NPC01 could significantly inhibit the proliferation of 5–8F and NP69 cells in a dose-dependent manner (Figure 1).

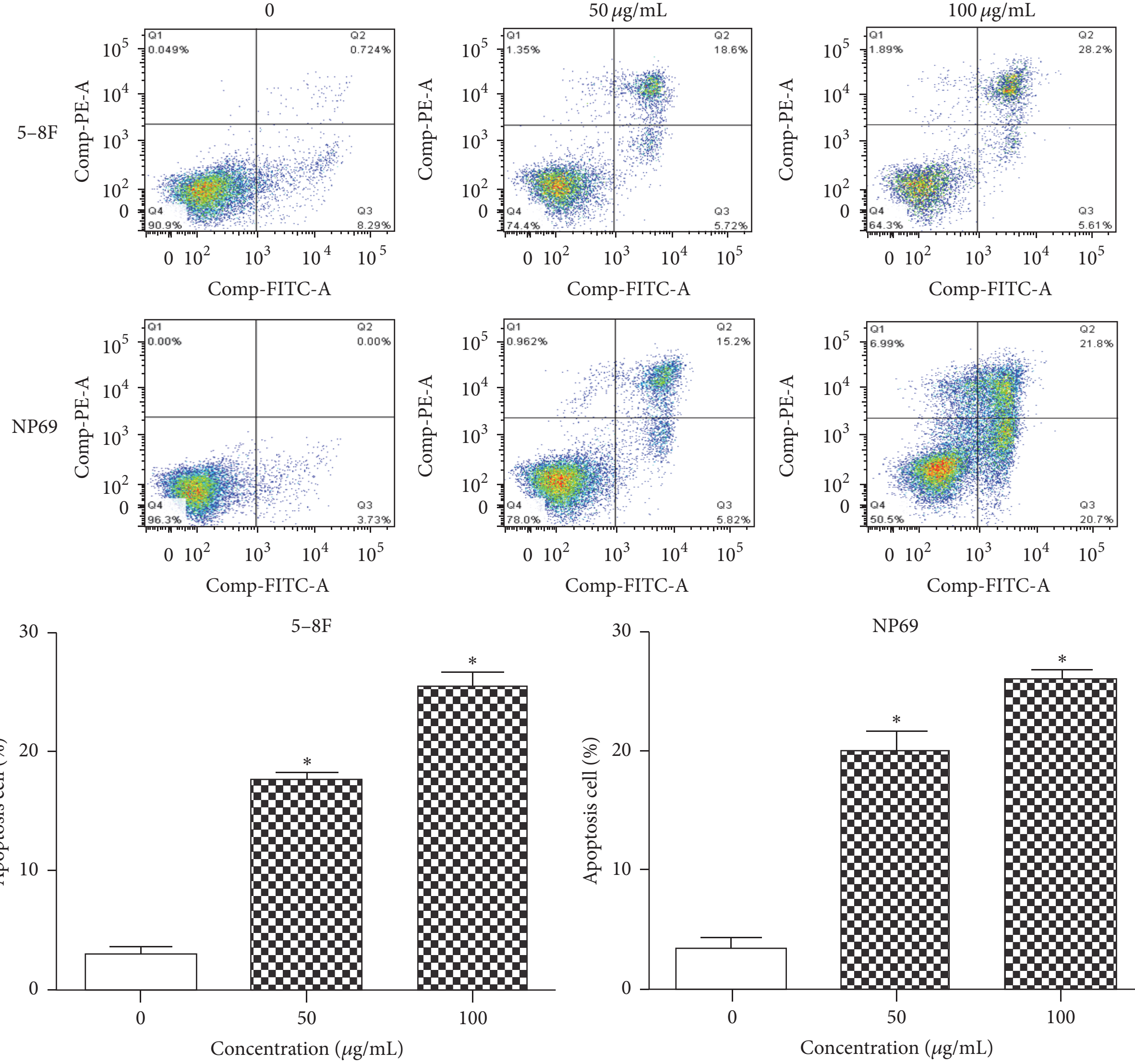


Figure 2: Effect of NPC01 on cell apoptosis. (A) 5–8F and (B) NP69 cells were treated with different concentrations of NPC01 for 24 or 48 h. The cells were collected and labeled with Annexin V–FITC/PI. The signal of Annexin V/PI was analyzed using flow cytometry. Values represent the mean ± standard deviation of three independent experiments. $^{*}P < 0.05$.

*3.2. NPC 01 Promoted NPC Cell Apoptosis.* 5–8F and NP69 cell lines were treated with 25, 50, or 100 $\mu$g/mL NPC01 for 24 or 48 h and measured using Annexin V–FITC/PI staining to investigate the possible apoptotic effect of NPC01 on NPC cells. Compared with the control group, NPC cell lines treated with NPC01 showed a significant increase in cell apoptotic population in a dose-dependent manner and 25 $\mu$g/mL which has no statistical significance when compared to the control (Data not shown) (Figure 2). These data suggested that NPC01 treatment promoted NPC cancer cell apoptosis.

*3.3. NPC01 Exerted Significant Therapeutic Activity in NPC Xenografts.* 5–8F cells were subcutaneously injected into nude mice to investigate the antitumor effect of NPC01 *in vivo*. After 12 days of s.c. 5–8F injection in which the tumor volume was 100–120 $mm^3$, the mice were randomized. Tumor volume was calculated using the equation $V\ (mm^3) = 0.5 * a * b * b$. Then NPC xenograft mice were given NPC01 (50, 100, and 200 mg/kg) or saline (negative control) for 2 weeks. At the end of the experiment, autopsies showed that NPC01-treated mice had much smaller tumor masses (Figure 3(a)). The tumor volumes (Figure 3(b)) and average tumor weights significantly decreased in a dose-dependent manner on treating with NPC01 compared with the control group. Additionally, the TUNEL assay also suggested that the number of apoptotic cells in the NPC01 treatment group increased significantly in a dose-dependent manner compared with the control group (Figure 3(c)). After 4-week treatment, the treatment group had a mean tumor volume of $185.9 \pm 78.3\ mm^3$ versus $402.45 \pm 1:107.6\ mm^3$ for the control group ($P < 0.05$); the tumor-inhibiting rate of high-dose was 56.16% (Figure 3(d)).

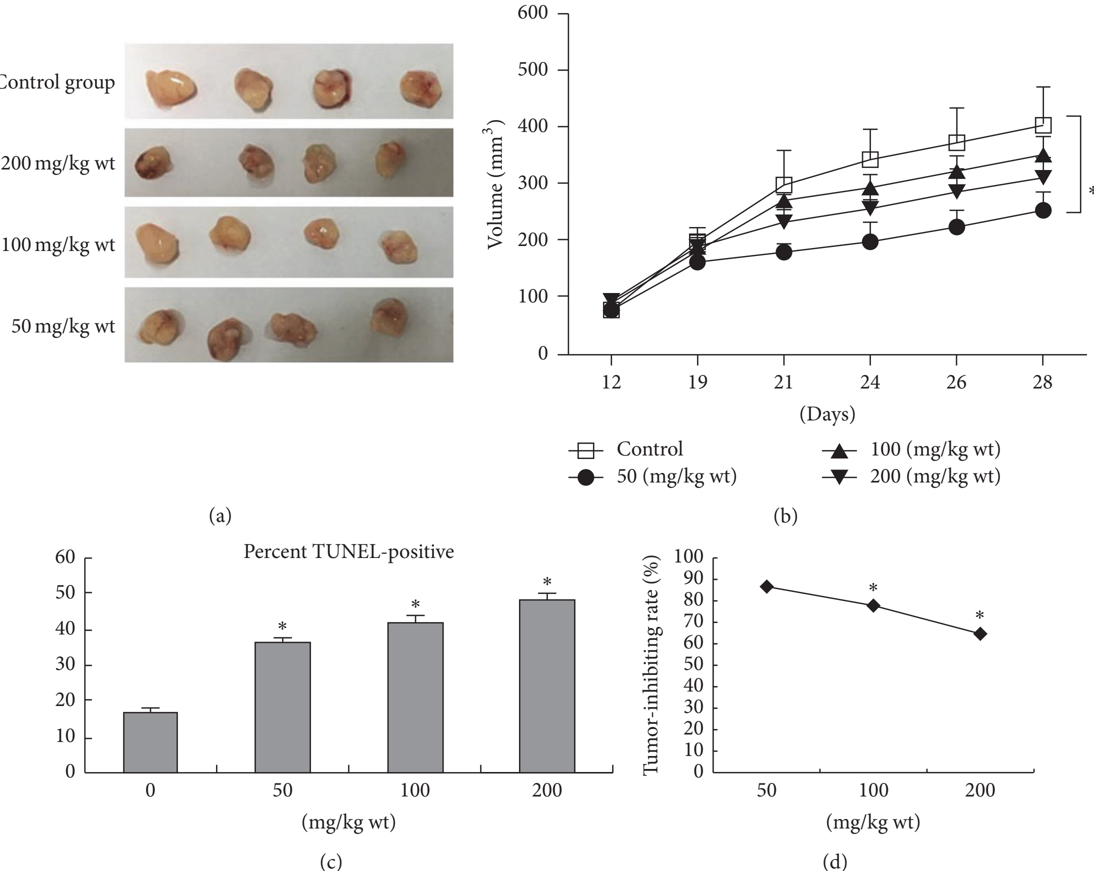


Figure 3: *In vivo* effect of NPC01 in NPC xenograft model. 5–8F cells were subcutaneously injected into the right sides of nude mice for 12 days. Then mice were daily subjected to the intragastric administration of NPC01 at the indicated doses. (a) The tumor masses were photographed at the end of the treatment. (b) The tumor volumes were measured and calculated as described in Materials and Methods. (c) Apoptotic cells were stained using a TUNEL kit and counted as described in Materials and Methods. (d) Groups showed a significant tumor-inhibiting effect. The tumor-inhibiting rate was 87.32%, 77.55%, and 56.16%, respectively. Symbol $*$ indicates $P < 0.05$ compared with the saline-injected mice.

*3.4. Effects of NPC01 on the Expression of VEGF and HIF-1α In Vivo and In Vitro.* The histological sections of xenograft mice with saline and NPC01 treatment were studied to investigate the potential mechanism underlying the antitumor effect of NPC01 *in vitro* and *in vivo*. Fewer blood vessels could be found in the NPC01-treated group compared with the saline group. Subsequently, the two representative markers, hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) and vascular endothelial growth factor (VEGF), were detected. The results showed that HIF-1$\alpha$ and VEGF significantly decreased after NPC01 treatment (Figure 4(a)). Furthermore, the protein and mRNA levels of HIF-1$\alpha$ and VEGF were also detected in 5–8F cells treated with NPC01. Parallel with the *in vivo* results, both mRNA and protein levels of HIF-1$\alpha$ and VEGF dramatically decreased on NPC01 exposure (Figures 4(b) and 4(c)).

*3.5. Effect of NPC01 on the PI3K/Akt/mTOR Signaling Pathway.* The PI3K/Akt/mTOR signaling pathway regulates various cellular activities including angiogenesis [17, 18]. 5–8F cells were treated with different concentrations of NPC01 for 24 h to investigate whether the PI3K/Akt/mTOR signaling pathway was involved in the antitumor effect of NPC01. The phosphorylation of Akt and mTOR showed a dose-dependent suppression compared with the vehicle-treated group. A significant inhibitory effect on the PI3K-Akt/mTOR signaling pathway could be detected in the 5–8F cells compared with nasopharyngeal epithelial cell line NP69. Furthermore, the expression levels of HIF-1a and VEGF, which are the downstream targets of the PI3K/Akt/mTOR signaling pathway, decreased dramatically on suppressing the aforementioned pathway (Figures 5(a) and 5(b)). We further analyzed the ratio of protein levels on p-Akt/AKT and p-mTOR/mTOR expression levels in 5–8F cells and found that was significantly and positively correlated with p-PI3K ($P < 0.01$) and p-mTO ($P < 0.05$), and no significance was found in NP69 (Figure 5(c)). 5–8F cells were treated with the inhibitor of PI3K (LY2940), Akt inhibitor (10 $\mu$M), or mTOR inhibitor rapamycin (50 nM) to further investigate the involvement of the PI3K/Akt/mTOR signaling pathway in the NPC01-induced antiangiogenic effects. The results demonstrated that the PI3K/Akt inhibitor could reduce the protein expression level of VEGF more effectively compared with the mTOR inhibitor (Figure 5(d)), indicating that NPC01 mainly exerted its antiangiogenic effect by modulating the PI3K/Akt signaling pathway.

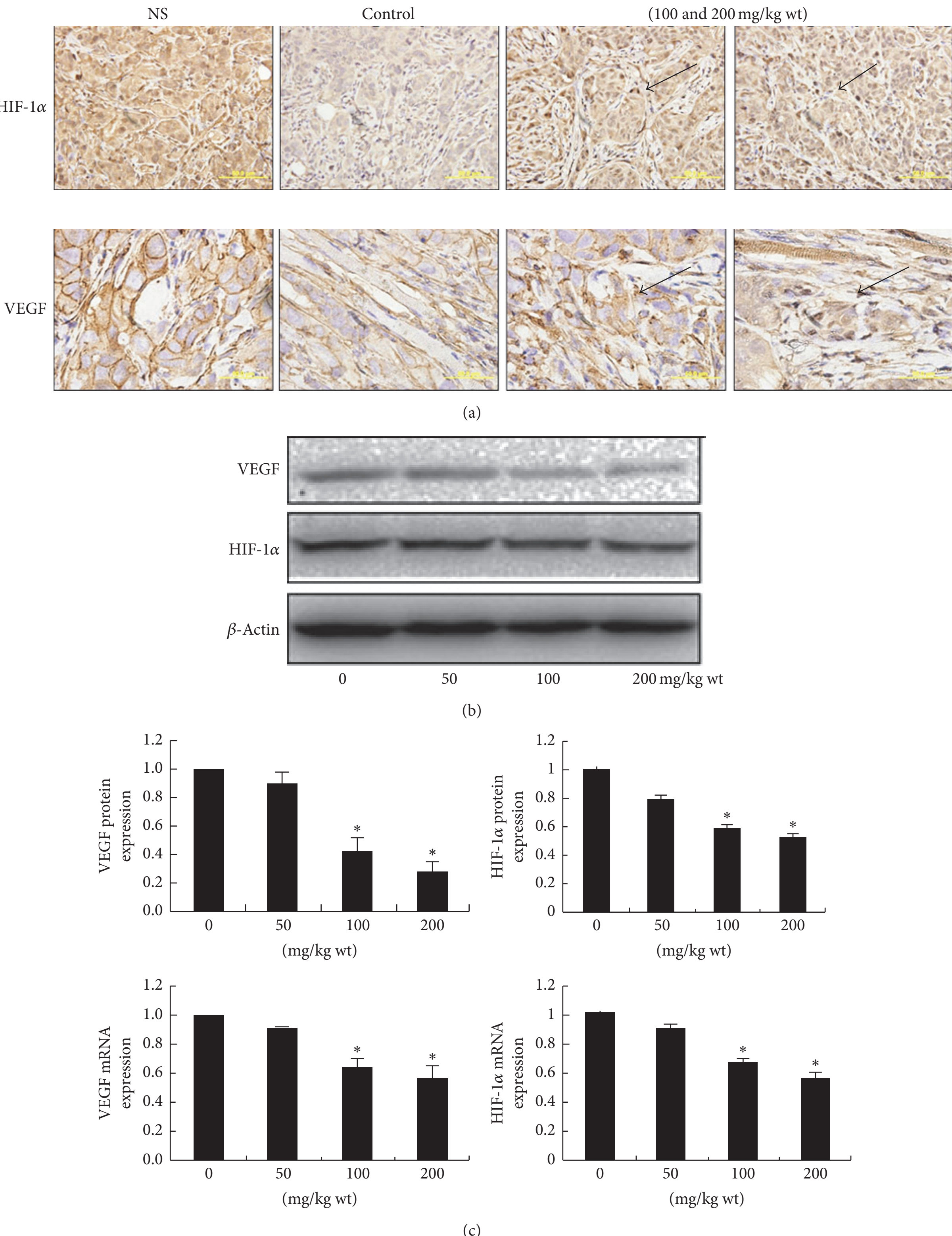


Figure 4: Effect of NPC01 on the *in vivo* and *in vitro* expression level of VEGF and HIF-1$\alpha$. (a) Right lower lobes of mice were dissected and stained with hematoxylin and eosin. (b) 5–8F cells were treated with different concentrations of NPC01 for 24 h. (c) The relative mRNA and protein expression of VEGF were represented as the ratio. Data are shown as mean ± standard error of mean. The symbol $*$ indicates $P < 0.05$ compared with the vehicle group.

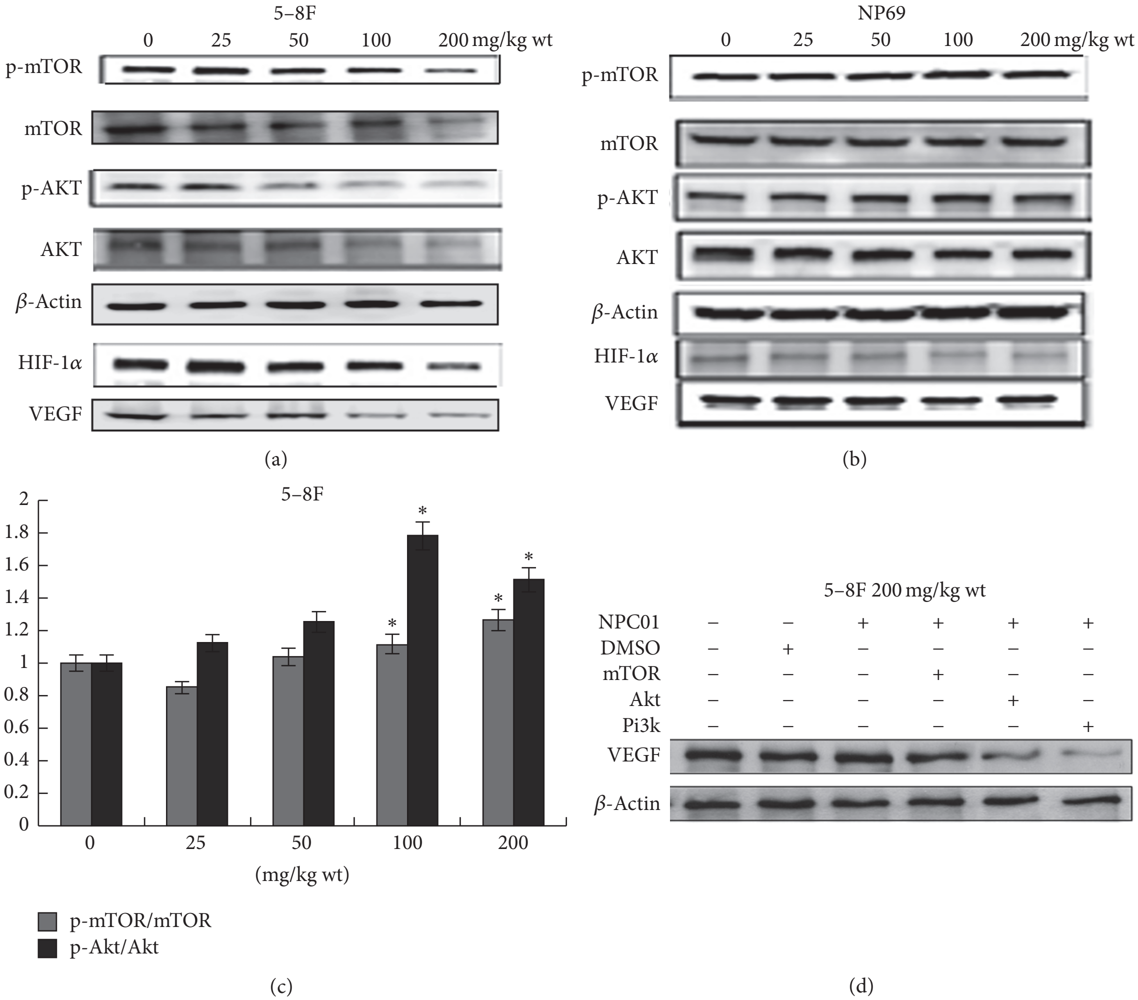


Figure 5: Effect of NPC01 on the PI3K/Akt/mTOR signaling pathway. (a, b) Representative Western blots treated with 25, 50, 100, or 200 $\mu$g/mL NPC01 for 24 h or left untreated (control). Protein levels were normalized using $\beta$-actin as the internal control. (c) Representative blots of the phosphorylated Akt and mTOR, total protein levels of Akt and mTOR, ratio of p-Akt/AKT and p-mTOR/mTOR, and the expression levels in cells. (d) The cells were pretreated with inhibitors of PI3K (LY2940), Akt inhibitor (10 $\mu$M), and mTOR inhibitor rapamycin (50 nM) and then stimulated with NPC01 [200 mg/(kg·wt)] for 24 h. Combined results from three independent experiments are shown. $^{*}P < 0.05$, compared with the control.

## 4. Discussion

Nasopharyngeal carcinoma, as a unique endemic cancer, is the most common head and neck cancer in Southern China. Despite the relatively high response rates to chemotherapy [19, 20] and emerging new drugs, survival in advanced disease cases of NPC is still poor [21, 22]. Hence, it is imperative to explore novel treatments including traditional Chinese medicine (TCM) treatment for patients with NPC. A recent meta-analysis for the clinical efficacy of TCM as a concomitant therapy for nasopharyngeal carcinoma reported significant efficacy of TCM in terms of survival, immediate tumor response, quality of life, immunostimulation, and acute adverse effects [12]. The present study reported that NPC01 could inhibit NPC cell proliferation and induce NPC cell apoptosis in a time- and dose-dependent manner.

Targeting cancer-associated angiogenesis is a promising strategy toward preventing cancer progression or treating cancer [23]. Accumulating evidence established the important role of PI3K/Akt/mTOR signaling pathway in normal and abnormal angiogenesis [17, 18]. Additionally, it has been reported that herbs can impede tumor angiogenesis by suppressing the HIF-1$\alpha$-induced expression of VEGF [24–27]. NPC01 is modified from Liang-Ge-San especially used to treat chronic head and neck diseases in China for centuries [28]. In modern pharmaceutical studies, each herb in NPC01 has different activities, including anti-inflammatory and antiangiogenic [29, 30]. This study demonstrated the important roles of NPC01 in antitumor angiogenesis by inhibiting the PI3K/Akt/mTOR signaling pathway and expression of HIF-1$\alpha$ and VEGF both *in vitro* and *in vivo*.

Collectively, the findings indicated that antiangiogenesis could be a promising antitumor strategy for NPC treatment and the PI3K/Akt/mTOR–HIF-1$\alpha$/VEGF signaling pathway axis could be a potential target for antitumor drug screening. NPC01, as a powerful therapeutic TCM formula, can serve as

the targeted therapeutic agent for patients with NPC. Further clinical investigation and a detailed understanding of the molecular mechanism underlying the effects of NPC01 would be required for further studies.

## Conflicts of Interest

No conflicts of interest exit in the submission of this manuscript, and manuscript is approved by all authors for publication.

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*Research Article*

# Autumn Royal and Ribier Grape Juice Extracts Reduced Viability and Metastatic Potential of Colon Cancer Cells

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Antioxidants are known to be beneficial to health. This paper evaluates the potential chemopreventive and anticancer properties of phenolic compounds present in grape juice extracts (GJE) from Autumn Royal and Ribier varieties. The effects of these GJE on viability (SRB day assay) and metastatic potential (migration and invasion parameters) of colon cancer cell lines HT-29 and SW-480 were evaluated. The effects of GJE on two matrix metalloproteinase gene expressions (MMP2 and MMP9) were also evaluated via qRT-PCR. In the former, GJE reduced cell viability in both cell lines in a dose-dependent manner. GJE treatment also reduced cell migration and invasion. Moreover, MMP-2 and MMP-9 gene expression diminished depending on extract and on cell type. *Conclusions*. These results provide novel information concerning anticancer properties of selected GJE by revealing selective cytotoxicity and the ability to reduce invasiveness of colon cancer cells.

## 1. Introduction

Epidemiological data and studies carried out in animal models have established that regular dietary intake of fruits and vegetables is protective against cancer [1]. Specifically, grapes (*Vitis vinifera* L.) are attributed to health-promoting effects related to elevated polyphenol contents [2]. The benefits reported include anti-inflammatory, anticarcinogenic, and immune-stimulatory effects, which are thought to be mediated by polyphenol-induced alterations in signaling pathways [3]. The most abundant polyphenols present in grapes include hydroxybenzoic acids, such as gallic acid; hydroxycinnamic acids (caffeic acid); stilbenes (resveratrol); and flavonoids, like anthocyanins, catechins, and others [4, 5]. The biological activities of these compounds have been ascribed to their antioxidant and free radical scavenging properties [6].

Colon cancer is the third most prevalent cancer in adult populations and is responsible for 9% of cancer-related deaths worldwide [7, 8]. However, colon cancer is, to some extent, preventable [9]. For instance, changes in diet and lifestyle have a significant impact on reducing the risk of developing colorectal cancer [10]. Particularly, polyphenols derived from

diverse dietary foods or beverages, such as grapes, teas, and turmeric, display chemopreventive and therapeutic properties against colon and other cancer types [11, 12]. In this respect, resveratrol (RSV) (3,5,4′-trihydroxytrans-stilbene), one of the most abundant grape polyphenols, exhibits interesting properties in terms of colon cancer prevention potential. Furthermore, RSV is a recognized antioxidant molecule [13] that reportedly intervenes in different stages of carcinogenesis, for example, initiation, promotion, and progression. This has been reported in numerous experimental models, including cancer cell lines, animal models, and clinical trials [14]. In related *in vitro* and *in vivo* studies, RSV has been shown to possess anticancer potential against several cancer types, including prostate, hepatic, breast, skin, colorectal, and pancreatic cancer [15–18]. Similar observations have been described for anthocyanins and catechins [19]. Importantly, these three phytochemical compounds constitute nearly 50% of grape polyphenols [19]

Tumor metastasis is one the major causes of morbidity in cancer patients [20]. Unfortunately, no therapy has been developed that successfully targets metastasis-associated processes in any human cancer [21]. Metastasis is a process in which cancer cells migrate from primary tumor sites to distant tissues, where secondary tumors are formed [21]. Initially, this requires detachment from the initial tumor, extracellular matrix (ECM) degradation, migration, and intravasation of cancer cells [22]. Importantly, migration and degradation of the ECM appear to represent critical steps during metastasis [22]. For instance, MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) are capable of degrading most ECM components forming the basal membrane, facilitating tumor cell escape and metastasis [23].

In this study, GJE were obtained from two widely produced grape varieties in Chile (blue grapes Autumn Royal (AR) and Ribier (RB)), and their anticancer properties were analyzed. The investigation considered *in vitro* effects of GJE on viability and metastatic parameters in SW-480 and HT-29 human colorectal carcinoma cell lines. The results show that GJE decreases cell viability in both cell lines in a dose-dependent manner. Importantly, this effect was selective, in that human dermal fibroblasts (HDF) were not affected. Moreover, GJE exposure diminishes metastatic potential of colon cancer cells by reducing migration and invasion of these cells. Additionally, GJE diminishes expression of matrix metalloproteinase genes MMP-2 and MMP-9. The conclusion discusses evidence that AR and RB GJE selectively reduce colon cancer cell proliferation and, more importantly, inhibit the metastatic potential of colon cancer cells.

## 2. Materials and Methods

*2.1. Plant Material.* Blue grapes cv. Autumn Royal and Ribier were harvested from Aconcagua Valley, Valparaíso, Chile, in summer. They were placed in polyethylene bags and transported at 4°C to the laboratory. The stems were manually removed, while damaged and poor quality fruits were eliminated. The samples were packed in polyethylene bags and stored at –32°C until being used.

*2.2. Grape Juice Extracts (GJE).* Approximately 200 grapes were defrosted, washed with distilled water, and pressed in a stainless steel manual grape crusher at room temperature. The juice was collected and homogenized by manual stirring, kept in polypropylene bottles, sealed, and stored in a cold chamber at –32°C until analysis. Subsequently, GJE were lyophilized, solubilized in 4 mL of distilled water, and filtered (pore size 0.2 $\mu$m).

*2.3. Cell Culture and Grape Juice Extract Treatment.* Human colon cancer cell lines SW-480 and HT-29 and human dermal fibroblast (HDF) were maintained in DMEM High glucose medium (Gibco, San Diego, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified 5% $CO_2$ incubator.

For GJE treatment, appropriate volumes of work solutions were added to the medium to reach indicated concentrations (0, 10, 25, 50, or 100 mg/mL) and cells were then incubated for indicated periods of time (24, 48, 72, and 144 hours).

*2.4. Cell Viability Assay.* Cell viability was determined by the Sulforhodamine B (SRB) (Sigma, St Louis, MO, USA) dye assay as previously described [24]. Cells were seeded at density $3 \times 10^3$ cells/well in a 96-well plate for 24 h and then treated with GJE as mentioned above. Following treatment, cells were fixed by adding 25 $\mu$L of cold 50% (wt/vol) trichloroacetic acid (TCA) followed by incubation for 60 min at 4°C. Plates were washed with deionized water and dried. Then, 50 $\mu$L of SRB solution (0.1% wt/vol in 1% acetic acid) was added per well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried and protein-bound dye was solubilized with 100 $\mu$L of Tris base (10 mM). Optical densities were read in an automated spectrophotometer plate reader at 540 nm.

*2.5. Cell Migration and Invasion Assays.* For cell migration assay, cells were treated with GJE (25 mg/mL) for 24 h and then seeded in a Transwell chamber without Matrigel coating. Cells ($2 \times 10^5$ cells/well in 300 $\mu$L of serum-free medium) were then seeded into the upper chamber, and 0.5 mL medium containing 20% FBS was added to the lower chamber as a chemoattractant. After incubation for 24 h at 37°C in 5% CO2, the upper surface of the porous membrane was wiped with a cotton swab. Cells that migrated to the lower surface of the porous membrane were fixed in 50% methanol and stained with 0.1% crystal violet.

The cell invasion assay was conducted in a similar fashion in a Transwell chamber with Matrigel coating (Sigma) for 48 h. Matrigel was diluted with serum-free medium to a final concentration of 2 mg/mL, and 8 $\mu$m-pore polycarbonate membrane filters were coated with 50 $\mu$L of Matrigel. In both migration and invasion assays, cell numbers were counted in twenty random fields (×100) per filter, for a total of three filters ($n = 3$).

% migration = (number of treated cells/number of control cells) × 100

% invasion = (number of treated cells/number of control cells) × 100

Table 1

| Primer | bp | Sense | Antisense |
|---|---|---|---|
| MMP-2 | 133 | 5′-CCTCCCTGCCCCTCCCTTCA-3′ | 5′-GCTTCTGGCTGGGTCTGTGGC-3′ |
| MMP-9 | 174 | 5′-GCCTTTGGACACGCACGACG-3′ | 5′-GCCAAAGCAGGACGGGAGCC-3′ |
| GAPDH | 226 | 5′-GAAGGTGAAGGTCGGAGTC-3′ | 5′-GAAGATGGTGATGGGATTTC-3′ |

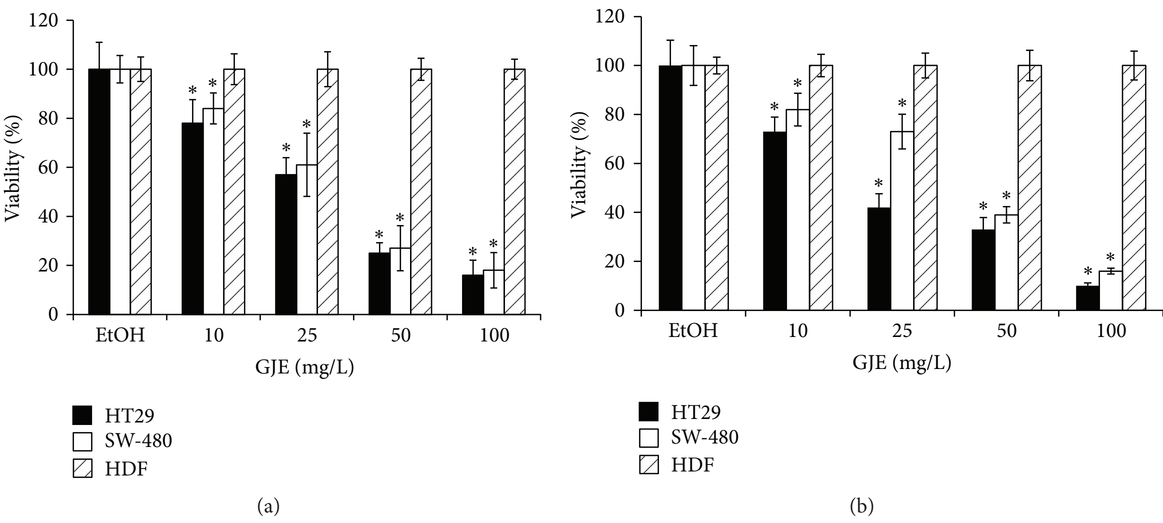


Figure 1: Effect of AR and RB GJE on cell growth of colon cancer cell lines and normal human dermal fibroblast (HDF). Following treatment with the indicated doses of GJE (0, 10, 25, 50, and 100 mg/mL), cell viability was determined by SRB dye assay (see Materials and Methods). Dose-response effects are presented as the percentage of each GJE treatment with respect to EtOH-treated cells (control 100%). The graphs show viability values for HT29 (black bars), SW-480 (white bars), and HDF (slashed bars) in response to AR and RB GJE treatment ((a) and (b), resp.). The data shown are means ± SEM of four independent experiments. $^{*}p < 0.05$ compared with control.

*2.6. Reverse Transcription-Polymerase Chain Reaction (RT-PCR).* Total RNA was isolated with Trizol reagent, following instructions provided by the manufacturer (Invitrogen, Carlsbad, CA, USA). RNA samples (2 $\mu$g) were treated with the RQ1 DNAse (Promega) and reverse-transcribed using the M-MLV reverse transcriptase (Promega, Madison, WI, USA), using the oligo (dT) 15 primers (Promega) as instructed by the manufacturer. MMP-2, MMP-9, and GADPH mRNA expressions were determined by quantitative real-time PCR conducted in a StepOne™ Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). Briefly, each amplification mixture (20 $\mu$l) consisted of 10 $\mu$l SYBR Green PCR Master Mix 2x (Applied Biosystems), 2 $\mu$l primer mix (250 nM each one), 6 $\mu$l of nanopure water, and 2 $\mu$l of cDNA. Cycling conditions were as follows: 96°C, 10 min; 40 cycles of 96°C, 5 s; 58°C, 30 s; 72°C, 25 s. Reactions were finished with extensions of 72°C for 25 s. Primers used for MMP-2, MMP-9, and GAPDH amplification were as follows:

MMP-2 (133 bp): 5′-CCTCCCTGCCCCTCCCTT-CA-3′ (sense) and 5′-GCTTCTGGCTGGGTCTGT-GGC-3′ (antisense)

MMP-9 M (174 bp): 5′-GCCTTTGGACACGCA-CGACG-3′ (sense) and 5′-GCCAAAGCAGGA-CGGGAGCC-3′ (antisense)

GAPDH (226 bp): 5′-GAAGGTGAAGGTCGG-AGTC-3′ (sense) and 5′-GAAGATGGTGATGGG-ATTTC-3′ (antisense) (see Table 1).

*2.7. Statistical Analysis.* All data are expressed as the means ± SEM taken from at least three independent experiments. Data were processed using INSTAT v3.05 (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com/). Analysis of variance (ANOVA) for multiple comparisons was used as noted. In all cases, $p < 0.05$ was considered significant. All statistical tests were performed with statistical analysis software.

## 3. Results and Discussion

Since *in vitro* cytotoxicity screening models provide important preliminary data to help select grape extracts with potential antineoplastic properties for future study, the cytotoxic effects of the Royal and Ribier grape juice extracts on Human colon cancer cell lines SW-480 and HT-29 were evaluated by the Sulforhodamine B (SRB) assay. The results demonstrate that extracts exhibit significant inhibitory effects in SW-480 and HT-29 at 48 h of treatment (Figure 1). Furthermore, the extracts examined under experimental conditions as described had no cytotoxic effects against normal human

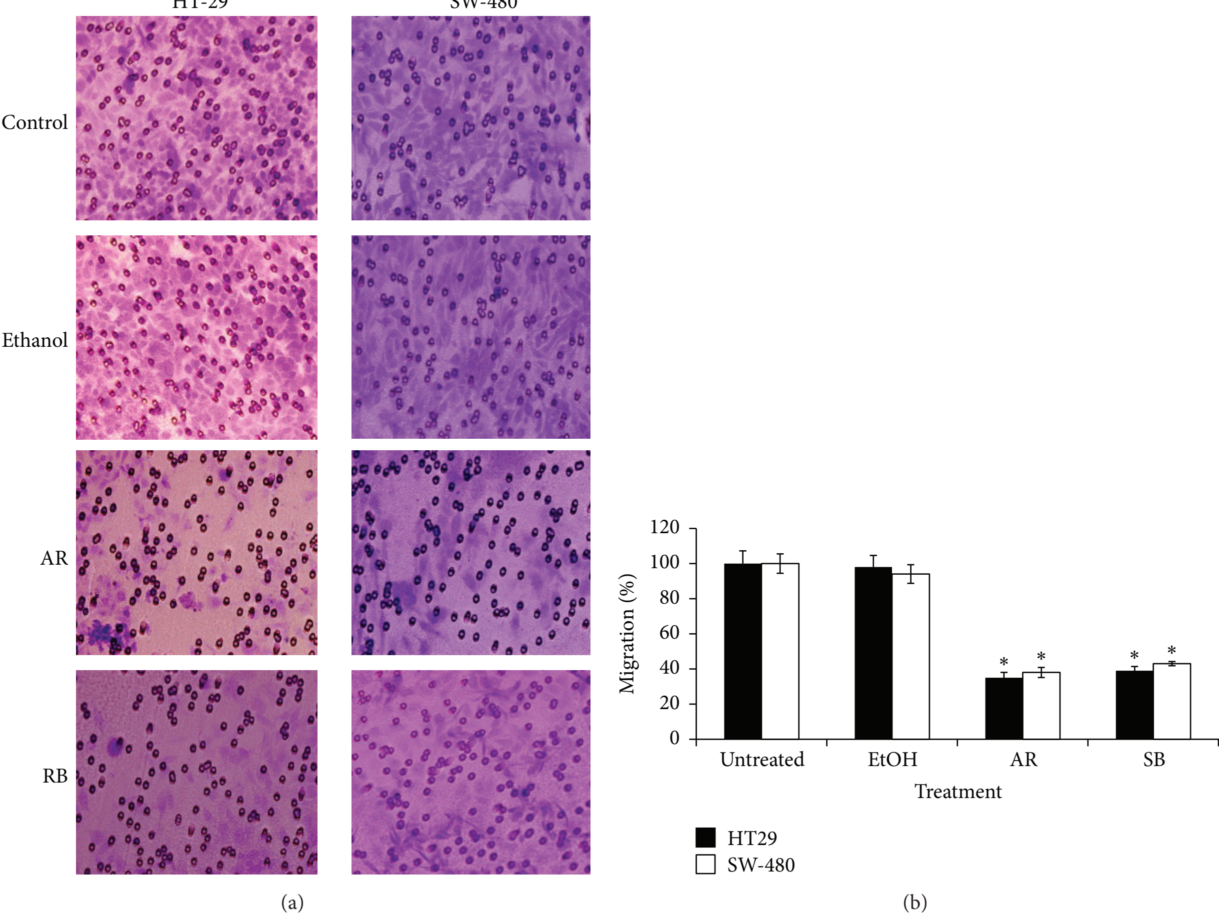


FIGURE 2: Inhibitory effect of GJE on migration of colon cancer cells. HT-29 and SW-480 cells were treated with AR or RB GJE (25 mg/ml) for 24 h. Following treatment, migration was evaluated in a Transwell chamber (see Materials and Methods). (a) Representative photos of HT-29 and SW-480 migration assays are shown for each indicated condition (magnification ×100). (b) Quantification of the migration assay of SW-480 (white column) and HT-29 (black column). Data represent percentage of migration of treated cells with respect to untreated controls (means ± SEM) from three independent experiments. $^*p < 0.001$ versus control.

dermal fibroblast cells (similar results were obtained with colon epithelial cells (CCD841 CoN), data not shown).

### 3.1. Cell Viability and Cell Migration

*3.1.1. Inhibition of Colon Cancer Cell Viability by GJE.* To assess effects of GJE treatment on cell viability, SW-480 and HT-29 colon cancer cell lines were exposed to increasing concentrations of AR and RB GJE, and viability was determined using the SRB dye assay (Figure 1). In these dose-response experiments, the lowest GJE dose (10 mg/mL) significantly reduced cell viability in SW-480 and HT-29 cells after 72 h of treatment, as compared to the ethanol control (Figure 1). GJE at a concentration of 25 mg/mL led to significant cell viability reductions in SW-480, 40% and 34% for AR (Figure 1(a)) and RB (Figure 1(b)), respectively, and in HT-29 cells, 43% and 58%, respectively ($p < 0.001$) (Figure 1). The highest GJE concentration (100 mg/mL) induced the most significant reduction in viability for colon cancer cell lines SW-480 (82% and 84% for AR and RB, resp.) and HT29 (84% and 90%, resp.) ($p < 0.001$) (Figures 1(a) and 1(b)). Interestingly, GJE at these same concentrations had no effect on nontumoral cell line derived from human dermal fibroblasts (HDF). These data indicate that AR and RB GJE inhibit the growth of HT-29 and SW-480 colon cancer cells in a dose-dependent manner and that no such detrimental effects are observed in a nontransformed control cell line (HDF).

*3.1.2. Inhibition of Colon Cancer Cell Migration by GJE.* Given that the GJE under study reduced cell viability of colon cancer cells, research turned to whether GJE might have additional effects on migration. As determined in Transwell assays, AR and RB grape juice extracts significantly reduced migration of HT-29 and SW-480 cells (Figures 2(a) and 2(b)). In Figure 2(a), representative photos of HT-29 migration after treatment with AR and RB GJE (25 mg/mL) are shown. HT-29 cell migration (white bars) was reduced to 35% and 38% of control levels after treatment with AR and RB GJE (25 mg/mL, *time*), respectively. In SW-480 cells, the percentage of migrating cells was reduced to 38% and 44% of control

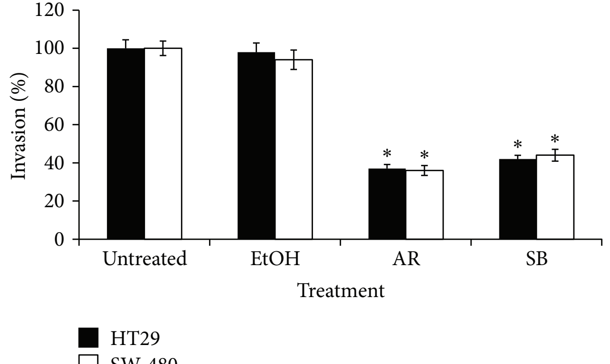


Figure 3: Inhibitory effect of GJE on the invasion capacity of colon cancer cells. HT-29 and SW-480 cells were treated with either AR or RB GJE (25 mg/mL), or EtoH (solvent control) for 48 h, and then harvested and seeded into the upper Transwell chamber coated with Matrigel. Data represent the percentage of the number of cells that invaded the lower chamber with respect to untreated control condition (100%), HT-29 (black column), and SW-480 (white column). Data represent means ± SEM obtained from three independent experiments. $^{*}p < 0.001$ versus. control.

cells following AR and RB GJE treatment (25 mg/ml, 24 h), respectively (Figure 2(b), black columns). This inhibitory effect is significant ($p < 0.001$) in all treatments when compared to controls for both SW-480 and HT-29 cells.

*3.1.3. Inhibition of Colon Cancer Cell Invasion by GJE.* Additionally, the effect of GJE on invasion was determined using a Transwell chamber coated with Matrigel. As is shown in Figure 3, AR and RB GJE significantly reduced invasion of SW-480 and HT-29 cells. In the case of HT-29 cells, AR and RB GJE significantly reduced invasion by 64% and 52%, respectively, when compared to control condition ($p < 0.001$). Similarly, treatment with AR and RB GJE significantly decreased ($p < 0.001$) invasion of SW-480 by 63% and 51%, respectively. In order to elucidate the underlying molecular mechanisms responsible for the observed anti-invasive activity of GJE, changes at the level of MMP-2 and MMP-9 mRNA levels were analyzed via qRT-PCR. As shown in Table 2, AR and RB GJE treatments significantly decreased MMP-2 and MMP-9 mRNA levels in both cell lines. Together, these findings suggest that downregulation of MMP-2 and MMP-9 might be responsible for reduced invasiveness of HT-29 and SW-480 cells observed following GJE treatment.

*3.2. Discussion.* AR and RB GJE display chemoprotective properties against colon cancer, one of the most commonly diagnosed cancers in Western countries. A number of studies have suggested that regular consumption of grapes is associated with the reduced risk of certain cancers, such as breast and colon cancers [25]. The grape juices of Autumn Royal and Ribier exhibited higher phenolics content and health benefits than other species of grape [26]. These extracts are particularly enriched in anthocyanins, with elevated antioxidant capacity compared to other polyphenols present in GJE. Here, we show for the first time that AR and RB GJE reduce viability, migration, and invasive potential of colon cancer cells. These observations are consistent with previous studies where grape seed extracts reduced viability and cell growth in colon cancer cell lines [27, 28]. Interestingly, AR and RB GJE did not modulate HDF cell viability at the same concentrations. Similar results were obtained in studies performed by Zhao et al. [29], who determined that anthocyanin-rich grape extracts were not cytotoxic to normal colon cell line NCM460. Together, these results suggest that this inhibitory effect is restricted to colon cancer cells.

Table 2: Effects of GJE on MMP-2 and MMP-9 gene expression in colon cancer cells. HT-29 and SW-480 colon cancer cells were treated with GJE for 24 h; MMP-2 and MMP-9 mRNA levels were analyzed by quantitative real-time PCR. Results are expressed as the percentage of expression versus control condition. Data represent means ± SEM from three independent experiments. $^{*}p < 0.001$ versus control.

| | HT-29 | | SW-480 | |
|---|---|---|---|---|
| | AR | RB | AR | RB |
| MMP-2 | 61.6 ± 1.1* | 56.8 ± 4.7* | 90.4 ± 3.9* | 96.3 ± 2.8* |
| MMP-9 | 94.9 ± 3.6* | 97.3 ± 2.4* | 95.3 ± 4.1* | 91.3 ± 6.4* |

Two important aspects of metastasis are the migratory and invasive ability of tumor cells, which are key characteristics of more aggressive cell phenotypes [30]. Acquisition of these properties allows cancer cells to escape from tissues or organs (affected by primary tumors) to distant sites (producing secondary tumors) [30]. In the present study, a Boyden chamber assay was used to quantify the effect of GJE on migration and invasion parameters of colon cancer cell lines. As expected, GJE inhibited migration in HT-29 and SW-480 colon cancer cell lines. Another study using grape seed proanthocyanidin extracts obtained similar results against lung cancer cell lines A549 and H129. In those cells, effects were attributed to guanylate cyclase and MAP kinase pathway inhibition [30]. Furthermore, GJE also displayed potent inhibitory effects on the invasive potential of colon cancer cells in Matrigel assays. These results are consistent with other studies that found grape seed extracts rich in polyphenols reduced invasion via MMP-2 and MMP-9 expression reduction, for instance, in prostate cancer cell lines [31]. Indeed, the observed effects on invasion and migration in colon cancer cells were correlated here with significant changes in metalloproteinase expression in response to GJE treatment. Metastasis is accompanied by various molecular alterations, including the ability to degrade ECM: this is associated with the overexpression of enzymes displaying proteolytic activity, such as MMPs [32]. Accordingly, augmented expression of MMP-2 and MMP-9 had been shown to play a critical role in degrading basement membranes during cell invasion and migration. Moreover, Bajaj et al. [33] demonstrated that overexpression of MMP-9 is related to tumor invasion and metastasis in gastric carcinoma. Sun et al. [34] reported that the expression of MMP-2 and MMP-9 has significant prognostic value in node-negative patients for predicting relapse-free survival, while Li et al. [35] determined that these MMPs are markers for metastasis in colon cancer. Despite the

above observations, to date, the possibility that AR and RB GJE could alter MMP expression in colon cancer cells had not been explored. Indeed, reduced invasive capacity following AR and RB GJE treatment correlated with decreased expression of MMP-2 and MMP-9 genes in both cell lines; however, these results do not exclude the possible participation of other metalloproteinases involved in invasion. For instance, MMP-1 and MMP-7 are also overexpressed in colon cancer [36], and urokinase-PA (u-PA) is implicated in colon cancer metastasis [37]. That said, similar studies on anthocyanins support our results, finding MMP-2 and MMP-9 activity in colon cancer cell line HT-29 [38]. Moreover, a reduction in MMP-2 expression was also observed in prostate cancer cell line DU-145 [30]. Other purified polyphenols present in either grapes or turmeric, that is, myricetin or curcumin, also reduce MMP-2 expression in colon cancer cell lines COLO 205 and HT-29 [39], as well as MMP-9 expression in rat colon cell models [40].

## 4. Conclusions

In summary, we have shown that it is possible to reduce motility-associated processes by exposing colon cancer cells to GJE. These results suggest that the anti-invasive effects of GJE are linked to the inhibition of MMP-mediated degradation processes that favor tumor metastasis. Nevertheless, the underlying molecular mechanisms leading to MMP-2 and MMP-9 inhibition remain to be determined.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Authors' Contributions

Joan Villena and Mario Párraga designed research; Manuel Valenzuela, Alejandro Madrid, Lorena Bastias, Iván Montenegro, and Joan Villena performed research and analyzed the data; Iván Montenegro and Joan Villena wrote the paper. All authors read and approved the final manuscript.

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*Research Article*

# Inhibition of Tumor Growth of Human Hepatocellular Carcinoma HepG2 Cells in a Nude Mouse Xenograft Model by the Total Flavonoids from *Arachniodes exilis*

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A tumor growth model of human hepatocellular carcinoma HepG2 cells in nude mice was employed to investigate the antitumor activity of the total flavonoids extracted from *Arachniodes exilis* (TFAE) *in vivo*. Several biochemical assays including hematoxylin-eosin (HE) staining, immunohistochemistry, and Western blot were performed to elucidate the mechanism of action of total flavonoids extracted from *Arachniodes exilis* (TFAE). The results showed that TFAE effectively inhibited the tumor growth of hepatocellular carcinoma in nude mice and had no significant effect on body weight, blood system, and functions of liver and kidney. Expression levels of proapoptotic proteins Bax and cleaved caspase-3 remarkably increased while the expressions of Bcl-2, HIF-1$\alpha$, and VEGF were suppressed by TFAE. These results suggested that the antitumor potential of TFEA was implied by the apoptosis of tumor cells and the inhibition of angiogenesis in tumor tissue.

## 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth common form of liver cancer worldwide, especially in Asia and Africa [1]. Surgical resection is the most efficient therapeutic strategy, but only 20% of patients are able to receive surgical therapy [2]. Currently some obvious problems exist in clinical therapy of HCC, such as early diagnosis, high recurrence rate, and lack of specific treatment and innovative medicines [3]. Many studies have shown that traditional Chinese medicines (TCM) may be able to retard HCC progression with multiple actions, either alone or in combination with other conventional therapies to improve life quality of HCC patients [4–6]. Moreover, TCM (including plants, animal parts, and minerals) have drawn a great deal of attention in recent years for their potential in the treatment of HCC [7].

*Arachniodes exilis (A. exilis)*, a fern belonging to *Arachniodes* species (Dryopteridaceae), is widely distributed in the tropical and subtropical moist areas of the world, especially in the eastern and southeastern Asia. In China, this plant is mainly distributed in the south areas of the Yangtze River and Shandong and Henan provinces. *A. exilis* has been used as a folk medicine for a long time to treat acute jaundice hepatitis, arthritis, lumbago, dysentery, and burn injuries and proved to exhibit antibacterial, anti-inflammatory, and sedative activities by modern pharmacological studies [8]. Studies indicate that Dryopteridaceae plants usually possess antivirus and anticancer effect, which is based on the bioactive constituents of flavonoids and phloroglucinol derivatives [9]. Mechanisms of anticancer effect of Dryopteridaceae plants are different from those of the common chemotherapeutic drugs. They possess the cytotoxicity towards tumor cells, meanwhile, without damage to the hematopoietic stem cells [10].

Our previous research suggested that TFAE was able to induce apoptosis of HepG2 cells *in vitro* [11]. In this study, the nude mice xenograft model was employed to investigate the mechanisms of antitumor effect of TFAE, such as the inhibition rate, biochemical index, HE staining, immunohistochemistry, and Western blot.

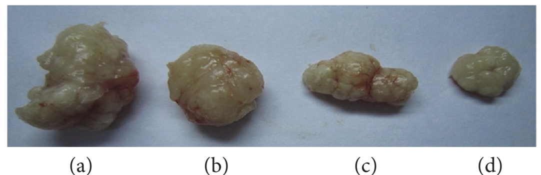


Figure 1: Effect of TFAE on the tumor growth in nude mice. (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

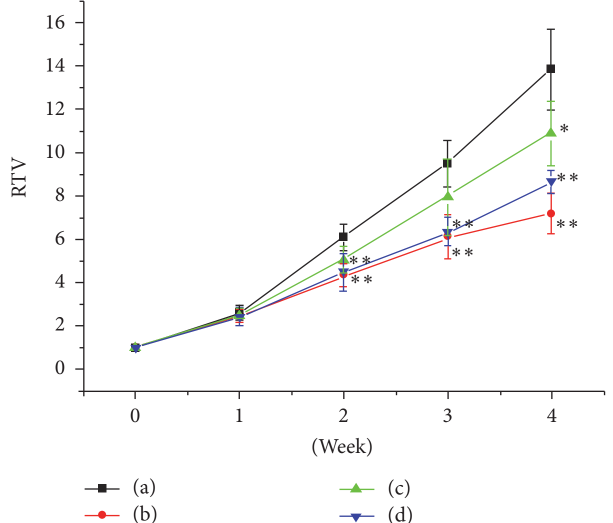


Figure 2: Changes in the relative tumor volume of tumor-bearing mice in each group. (a) Negative control group; (b) positive control group; (c) TFAE low-dose group; (d) TFAE high-dose group; $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the negative control.

## 2. Materials and Methods

*2.1. Materials.* The total flavonoids from *Arachniodes exilis* (TFAE) were extracted and purified in our laboratory. First, appropriate dried powder (20–30 mesh) of *A. Exilis* roots was extracted by using 60% ethanol (solid-liquid ratio of 1 : 30) with ultrasonication for 3 times, each time for 2 hours. The extract of *A. exilis* was obtained by concentrating the extracting solution to dryness in a rotary evaporator and a freeze dryer; then the extract was purified by the polyamide column chromatography with 70% ethanol to get TFAE. The total flavonoids in TFAE were estimated as rutin equivalent and the content was measured on the basis of the rutin calibration curve ($Y = 82.645X + 2.661$ $R^2 = 0.9998$, where $Y$ is the absorbance and $X$ is the concentration ($\mu$g/ml)) [11]. The content of the total flavonoids in TFAE was 82.86% after the polyamide purification process.

The DMEM/F-12 medium was from HyClone (Thermo Fisher Scientific, Massachusetts, USA) and fetal bovine serum was from Trans Gen Biotech Biotechnologies Co., Ltd (Beijing, China). The trypsin, DMSO, and agarose were all from Solarbio Technology Co., Ltd (Beijing, China). The total protein extraction kit was from Aidlab Biotechnologies Co., Ltd (Beijing, China). The 4% paraformaldehyde fixative solution and HE staining solution were from Biossic Biotechnology Co., Ltd (Wuhan, China). The Bcl-2, Bax, VEGF, and HIF-1$\alpha$ immunohistochemistry kit were from eBioscience (Thermo Fisher Scientific, Massachusetts, USA). The Bax, HIF-1$\alpha$, and VEGF antibodies were from Santa Cruz Biotechnology, Inc. (California, USA), and the Bcl-2, cleaved caspase-3, and $\beta$-actin antibodies were obtained from Abcam (Cambridge, UK). The PVDF membrane was from Millipore. Additional reagents and solvents used in this study were commercial products of analytical grade.

*2.2. Methods.* Human hepatoma HepG2 cells were inoculated into immunodeficient nude mice to establish the animal model of liver tumor. Several indicators related to tumor growth were evaluated. The experimental mice were also investigated by biochemical and histological detection. Furthermore, the expressions of apoptosis-related proteins in tumor tissues were determined to explore the underlying mechanisms for the antitumor activity of TFAE.

*2.2.1. Establishment of the Tumor Models in Nude Mice.* Male BALB/c-nu mice of SPF grade, 4-5 week-old, were purchased from Hunan SJA Laboratory Animal Co., Ltd, in Changsha, China [License Number SCXK (Xiang) 2014-0002]. The mice were bred in animal house (SPF degree) with barrier system assisted with apinoid laminar flow chamber in the Experimental Animal Center of Huazhong University of Science and Technology. These animals were housed under controlled conditions (temperature 22 ± 2°C, relative humidity 50 ± 10%) with a natural light-dark cycle for one week before the experiment was carried out. The animal studies were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation. The HepG2 cells of logarithmic phase were washed twice by serum-free culture solution and resuspended to a concentration of about $1 \times 10^7$/ml. The cells (0.2 ml) were inoculated subcutaneously on the back of each nude mouse.

*2.2.2. The Administration Groups of Nude Mice.* After the inoculation, the animals were randomly divided into four groups: the negative control group, the positive control group, the TFAE low-dose group, and the TFAE high-dose group. The administration was designed according to Table 1. The drug for the negative control group was prepared as follows: 1.0 g of sodium carboxymethylcellulose (CMC-Na) and 200 ml of distilled water were fully swollen and dissolved to yield a 0.5% CMC-Na solution. 5-FU was used as the drug for the positive control group and dissolved with distilled water to prepare the administrative solution. The TFAE was dissolved in the 0.5% CMC-Na solution to obtain the sample solution. All animals were given once intragastric administration (ig) every other day which lasted for 4 weeks. The mice were weighed and the tumor growth in nude mice was monitored carefully once a week.

*2.2.3. Growth Indicators of Tumors in Mice.* The long diameter ($a$), short diameter ($b$), and volume ($V$, mm$^2$) of tumors in the mice were measured; relative tumor volume (RTV)

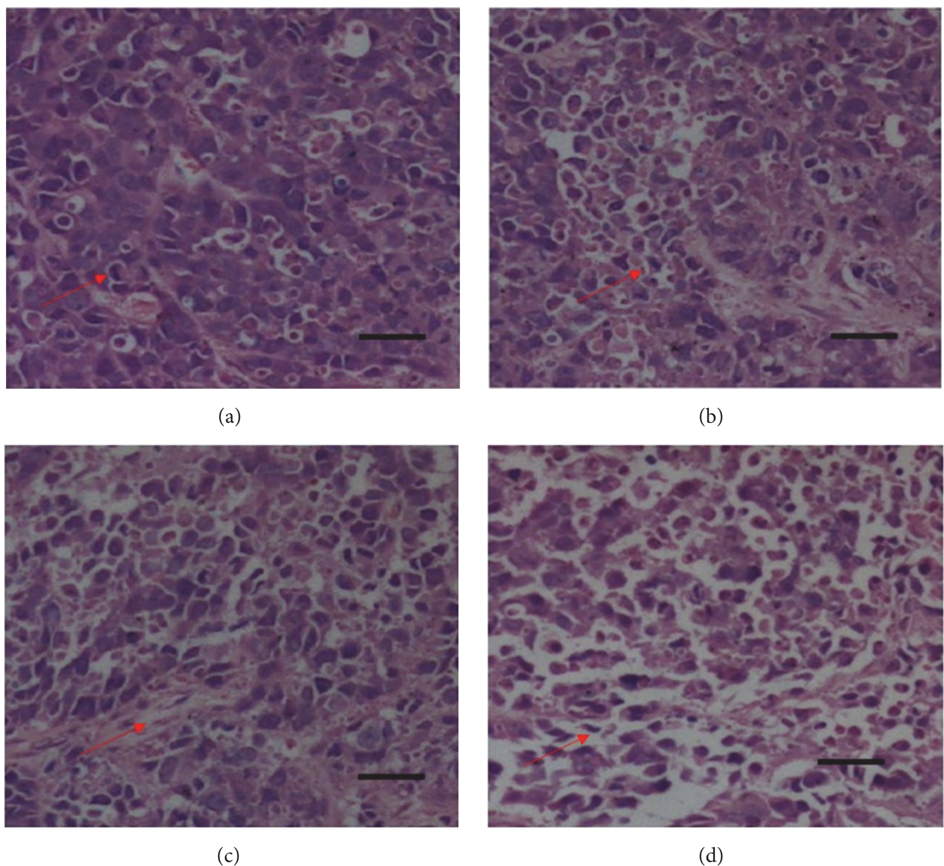

Figure 3: HE staining of tumor tissues belonged to mice of different administration groups (×100). (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

Table 1: The administration groups of nude mice.

| Groups | Administration |
|---|---|
| Negative control | 0.5% CMC-Na solution |
| TFAE high-dose | 500 mg/kg TFAE |
| TFAE low-dose | 125 mg/kg TFAE |
| Positive control | 10 mg/kg 5-FU |

and the relative rate of tumor growth rate $T/C$ (%) were calculated.

$$V = \frac{a \times b^2}{2}, \qquad \text{RTV} = \frac{Vt}{V_0}. \tag{1}$$

$V_0$ is the volume measured after the first administration; $Vt$ is the volume measured after each administration.

$$\frac{T}{C}(\%) = \frac{T_{\text{RTV}}}{C_{\text{RTV}}}. \tag{2}$$

$T_{\text{RTV}}$ is the RTV of mice in positive control group and TFAE administration groups; $C_{\text{RTV}}$ is the RTV of mice in the control group.

*2.2.4. Pathological Examination.* After 24 h of the last administration, mice were sacrificed under anaesthesia and dissected. Blood samples and tumor tissues were collected. A part of the tumor tissue was fixed by 4% paraformaldehyde for pathology detection, and the remaining part was frozen in refrigerator.

*(1) Biological Assays*. The blood analysis was performed using the whole blood samples to measure the number of red blood cells (RBC), white blood cells (WBC), blood platelet (PLT), and hemoglobin (HB) by using DxH800 automatic hematology analyzer (BECKMAN COULTER). Alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine (Cr) were measured by AU5821 automatic biochemical analyzer (BECKMAN COULTER). All of the work had been done in the hospital attached to the Tongji Medical College, Huazhong Science and Technology University.

*(2) Morphological Study of the Tumor Tissues.* Tumor tissue was stained by hematoxylin-eosin (HE) and observed under a microscope.

*(3) Immunohistochemistry Analysis*. The slice of tumor tissue was prepared by conventional process. Immunohistochemical analysis of Bcl-2, Bax, HIF-1$\alpha$, and VEGF expressions

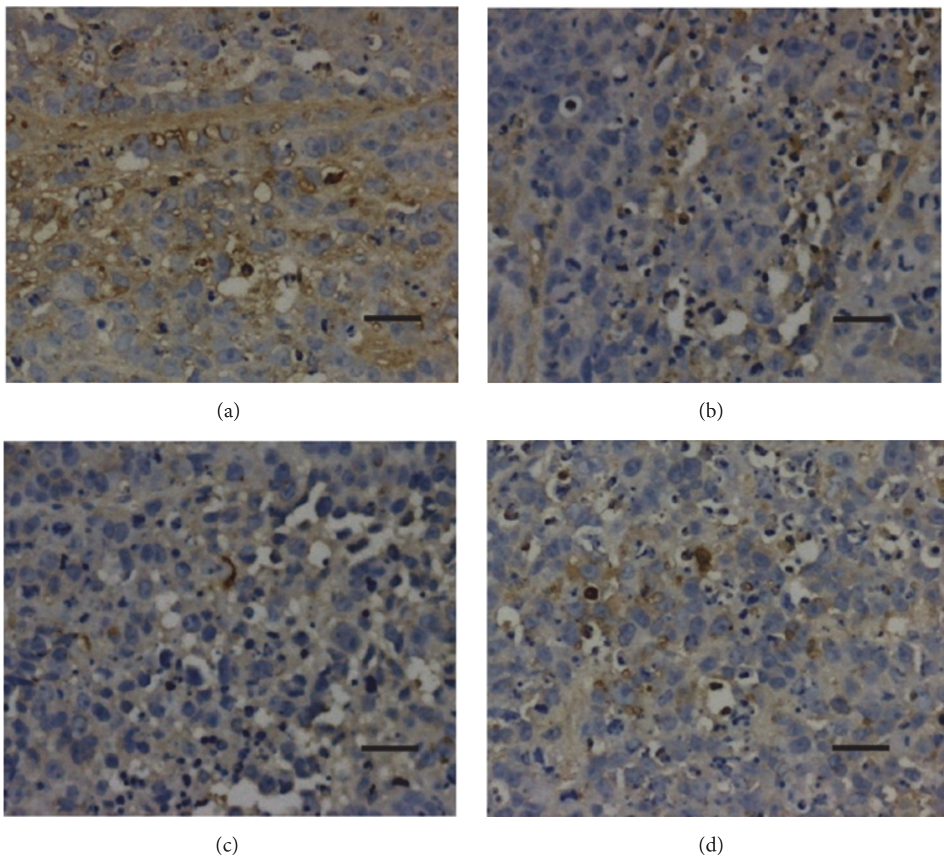

Figure 4: Expression of apoptosis-related protein Bcl-2 in tumor tissues (×100). (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

in tumor tissue was applied according the guideline of each test kit. The following antibodies were used: Bcl-2 (ab32124, Abcam, Cambridge, UK; 1 : 100), Bax (Sc-526, Santa Cruz, California, USA; 1 : 100), HIF-1$\alpha$ (Sc-13515, Santa Cruz; 1 : 100) and VEGF (SC-57496, Santa Cruz; 1 : 100).

*(4) Western Blot Assay*. Western blot assay was conducted to detect expression levels of Bax, Bcl-2, cleaved caspase-3, HIF-1$\alpha$, and VEGF. Total proteins of the tumor tissues were extracted using a total protein assay kit. After the determination of protein contents via the Bio-Rad protein assay, these protein samples (40–50 $\mu$g) were separated by 12% SDS-PAGE and electrotransferred to nitrocellulose membrane. The membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (TBST), blocked with 5% nonfat milk in TBST for 1 h at room temperature, and incubated with following specific antibodies: Bax (Sc-526, Santa Cruz, California, USA; 1 : 1000), HIF-1$\alpha$ (Sc-13515, Santa Cruz; 1 : 800), VEGF (SC-57496, Santa Cruz; 1 : 1000), Bcl-2 (ab32124, Abcam, Cambridge, UK; 1 : 1000), cleaved caspase-3 (ab2302, Abcam; 1 : 1000), and $\beta$-actin (ab115777, Abcam; 1 : 1000) overnight at 4°C. The membranes were washed three times in TBST, followed by incubation with the appropriate horseradish peroxidase- (HRP-) linked secondary antibodies (Proteintech, Wuhan, China; 1 : 5000) for 1 h at room temperature. The specific proteins on the blots were developed using enhanced chemiluminescence (ECL; Vazyme Biotech Co., Ltd., Nanjing, China) and visualized as bands on CL-XPosure film (Thermo Fisher Scientific, Inc.). The optical densities of the bands were measured on the GS710 Densitometer and analyzed using Quantity One image analysis software version 4.6 (Bio-Rad Laboratories, Inc.).

*2.2.5. Statistical Analysis.* Data were expressed as mean ± standard deviation (SD). All data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. A $p$ value of <0.05 was considered as an indication for statistical significance. The SPSS 19.0 software (IBM Corporation, Armonk, NY, USA) was used to analyze the data.

## 3. Results

*3.1. Observation of the Animals during the Experiment.* After a certain period of inoculation with HepG2 cells, obvious lumps were visible under the skin at the inoculation site, indicating the successful establishment of heterotopic transplantation model of human hepatoma HepG2 cells. Animals in each group behaved normally in eating, drinking, and excretion during the experiment. After the experiment, nude mice of each group were dissected, and no significant organ damage was observed.

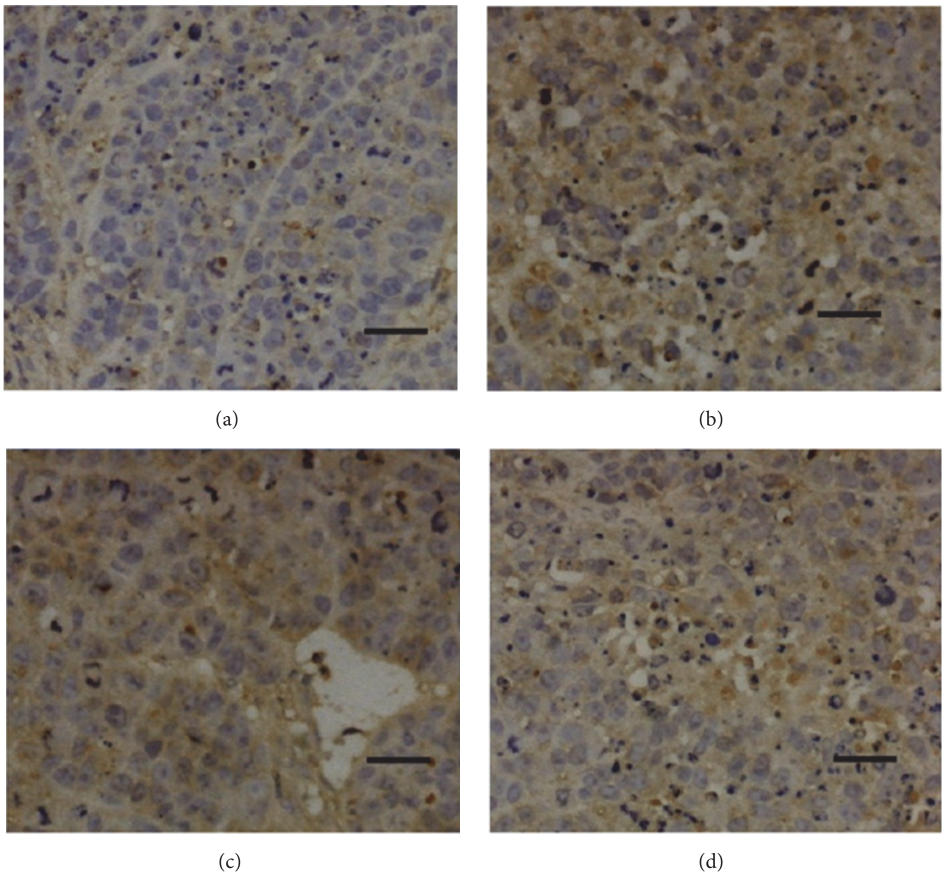

Figure 5: Expression of apoptosis-related protein Bax in tumor tissues (×100). (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

Table 2: Changes in body weight of animal in different groups.

| Groups | $n$ | Body weight (g) | |
|---|---|---|---|
| | | Before the experiment | After the experiment |
| Negative control | 6 | 18.44 ± 2.56 | 24.63 ± 3.37 |
| TFAE low-dose | 6 | 18.56 ± 1.89 | 23.78 ± 3.01 |
| TFAE high-dose | 6 | 19.17 ± 2.03 | 24.12 ± 2.56 |
| Positive control | 6 | 18.75 ± 1.77 | 20.35 ± 2.86 |

The body weight of animals in each group increased as shown in Table 2. Statistical analysis showed that there was no significant difference between the average weights of animals in each group, suggesting that the TFAE had no effect on body weight of the hepatocellular carcinoma-bearing nude mice.

*3.2. Effect of TFAE on the Tumor Growth in Nude Mice.* As shown in Figure 1, the animals treated with 5-FU and TFAE showed significantly lower tumor growth rate than the mice in negative control group, revealing that administration with TFAE effectively inhibited the tumor growth in the nude mice.

Changes on the relative tumor volume of mice in every experimental group were analyzed and shown in Figure 2.

*3.3. The Impact of TFAE on the Blood System and Functions of Liver and Kidney of the HCC Animal Model.* Results are listed in Table 3. WBC in the positive control group (5-FU treatment group) were significantly lower than those in the negative control group while the content of ALT, AST, and BUN was significantly higher on the contrary ($p < 0.05$). It was shown that mice in the positive control group (5-FU treatment group) showed significant differences in the indicators of blood system as well as liver and kidney functions when compared with animals in the negative control group, proving that 5-FU had toxicity to the blood system, liver, and kidney. Meanwhile, the results also suggested that mice in TFAE administration groups had no significant difference in these indicators compared with mice in negative control group. This indicated that TFAE have no obvious damage on the blood system, liver, and kidney.

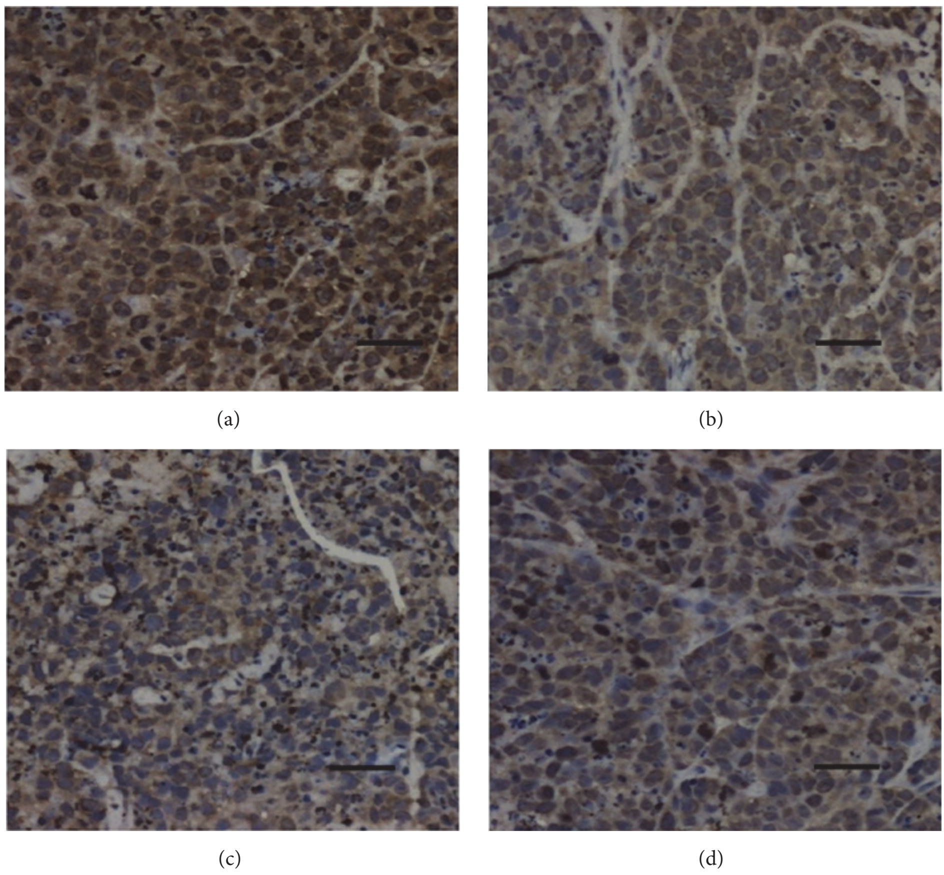


Figure 6: Expression of apoptosis-related protein HIF-1$\alpha$ in tumor tissues (×100). (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

Table 3: Assays about the blood system and functions of liver and kidney of the HCC animal model ($X \pm$ SD, $n = 6$).

| Groups | Negative control group | TFAE low-dose group | TFAE high-dose group | Positive control group |
|---|---|---|---|---|
| RBC ($\times 10^{12}$/l) | 9.60 ± 1.62 | 9.54 ± 1.75 | 9.18 ± 1.34 | 9.12 ± 1.31 |
| WBC ($\times 10^{9}$/l) | 12.26 ± 2.43 | 12.14 ± 2.39 | 11.66 ± 1.34 | 7.56 ± 1.44* |
| PLT ($\times 10^{9}$/l) | 686.20 ± 105.99 | 678.02 ± 128.52 | 669.42 ± 95.46 | 618.78 ± 93.81 |
| HB (g/l) | 163.18 ± 24.01 | 165.60 ± 15.92 | 158.49 ± 27.73 | 154.74 ± 26.53 |
| ALT (U/l) | 73.73 ± 17.36 | 80.27 ± 24.72 | 81.57 ± 31.28 | 168.37 ± 42.83* |
| AST (U/l) | 377.27 ± 65.37 | 407.82 ± 88.31 | 383.60 ± 80.16 | 702.63 ± 106.45* |
| BUN (mmol/l) | 12.56 ± 2.38 | 13.71 ± 2.74 | 13.18 ± 1.77 | 16.84 ± 3.76* |
| Cr ($\mu$mol/l) | 12.30 ± 3.01 | 12.04 ± 2.06 | 13.17 ± 2.65 | 15.45 ± 3.54 |

* $p < 0.05$, compared with negative control.

*3.4. Morphology of Tumor Tissue.* HE stained samples of tumor tissues of tumor-bearing mice were shown in Figure 3. The tumor cells in the tissues of nude mice belonging to negative control group were closely aligned and possessed bigger cell nucleus and higher nuclear/cytoplasm ratio than those in the other three groups. After the treatment with TFAE or positive control drug 5-FU, however, cancer cells exhibited phenomena such as sparse arrangement, cell shrinkage, fragmentation, and chromatin disappearance, indicating necrosis at varying degrees happened in the cancer cells.

*3.5. Immunohistochemical Assay.* Immunohistochemical tests were applied to detect the expressions of Bcl-2, Bax, HIF-1$\alpha$, and VEGF in tumor tissues. Results are shown in Figure 4.

As shown in Figure 4, administration with different concentrations of TFAE significantly inhibited the expression of Bcl-2 in tumor tissues when compared with tissues belonging to negative control mice.

As shown in Figure 5, TFAE at different concentrations effectively regulated the expression of Bax in tumor tissues

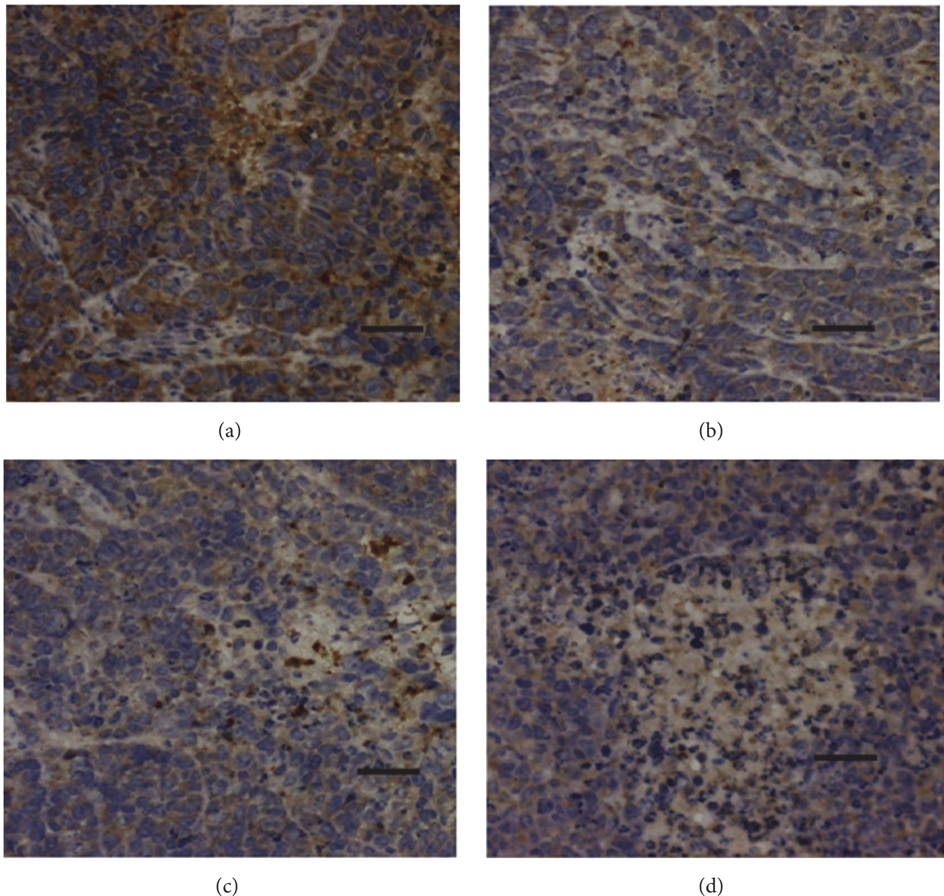

FIGURE 7: Expression of apoptosis-related protein VEGF in tumor tissues (×100). (a) Negative control group; (b) TFAE low-dose group; (c) TFAE high-dose group; (d) positive control group.

compared with tissues of negative control mice. These findings well indicated that treatment with TFAE might induce the apoptosis in the tumor tissues.

The protein HIF-1$\alpha$ is expressed in the cytoplasm or nucleus. The HIF-1$\alpha$ high-expressed cells show brown particles in nucleus and cytoplasm, while the HIF-1$\alpha$ low-expressed cells appear to be blue in cytoplasm. As shown in Figure 6, the expression level of HIF-1$\alpha$ in tissues of TFAE treated groups remarkably decreased compared with mice in negative control group, indicating that TFAE inhibited HIF-1$\alpha$ expression level in hepatoma cells, leading to the decreased ability to tolerate hypoxia and proliferation.

As shown in Figure 7, the expression of VEGF effectively decreased in the tissues of mice of TFAE high-dose group when compared with that of negative control group, suggesting that TFAE was able to inhibit the expression of VEGF in the tumor tissue, which resulted in the apoptosis of the cancer cells.

*3.6. Expression Assay for Apoptosis-Related Proteins by Western Blot.* The results are shown in Figures 8 and 9. Western blot assays showed that, when compared with mice of negative control group, the expression level of proapoptotic protein Bax in the mice significantly increased after the treatment with TFAE; meanwhile the expression of anti-apoptotic protein Bcl-2 reduced remarkably in the tumor tissue, resulting in a higher ratio of Bax/Bcl-2. Furthermore, expression level of cleaved caspase-3 protein was effectively upregulated. The reducing expression of HIF-1$\alpha$ and VEGF was also shown in the Western blot assay.

## 4. Discussion

The current study investigated the potent antitumor activity of TFAE using a heterotopic liver cancer xenograft model *in vivo*. By detecting the changes of body weights and the tumor growth of the nude mice as well as determining the corresponding biochemical indicators involving blood system and functions of liver and kidney, it was confirmed that TFAE effectively inhibited tumor growth in the liver cancer ectopic xenograft models and, furthermore, had no significant effect on body weights, blood system, and functions of liver and kidney.

In order to further elucidate the mechanisms of the TFAE antitumor effects, immunohistochemical assay and Western blot assay were employed to explore the expression of proliferation-related proteins. Results showed that the expression level of proapoptotic protein Bax in tumor tissue

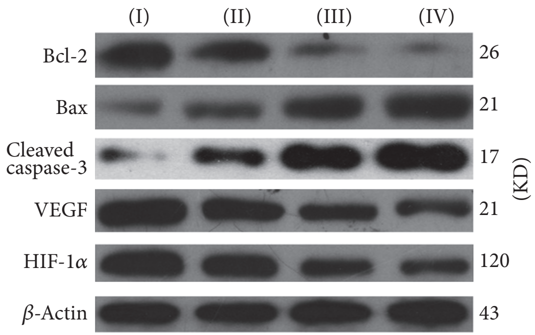


Figure 8: Expression assay for apoptosis-related proteins by Western blot. (I) Negative control group; (II) TFAE low-dose group; (III) TFAE high-dose group; (IV) positive control group.

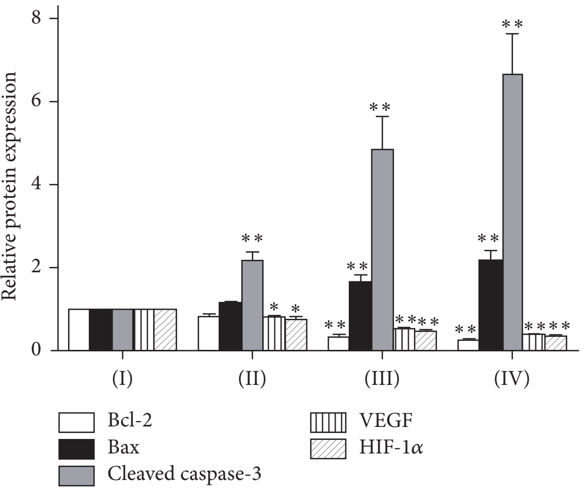


Figure 9: Relative protein expression assay for apoptosis-related proteins by Western blot. Values were presented as mean ± SD, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with negative control group.

was significantly elevated while expression of antiapoptotic Bcl-2 protein was effectively reduced, resulting in an increased Bax/Bcl-2 ratio. In addition, expression level of cleaved caspase-3 protein was increased and the reduced levels of HIF-1*α* and VEGF expressions were observed after the treatment of TFAE, suggesting the antitumor effect of TFAE by trigging apoptosis and inhibition of tumor angiogenesis.

Apoptosis is a process of programmed cell death, controlled by multiple signaling pathways, including the proteins of Bcl-2 family [12, 13] and caspase proteases [14, 15]. It is believed that the ratio of Bax/Bcl-2 activity level is a critical determinant of cell susceptibility to apoptosis, rather than the levels of individual proteins [16]. Increased Bax/Bcl-2 ratio and caspase-3 protein expression level induced by TFAE in this study are fully consistent with our previous study on HepG2 cells, confirming that the antitumor effect of TFAE is related to apoptosis *in vivo* [11].

Moreover, we also investigated the expression of HIF-1*α* protein in tumor tissue of nude mice with a liver cancer xenograft model. HIF-1*α* is a hypoxia-inducible factor-1*α* (hypoxia-inducible factor-1*α*), consisting of the heterodimer of HIF-1 protein with HIF-1*β*. The expression of HIF-1 was closely associated with the hepatocellular proliferation under hypoxic conditions. The physiological activity of HIF-1 mainly depends on the activity of HIF-1*α* subunit. Hypoxia is one of the key characteristics of the solid tumor microenvironment [17, 18], which leads to a series of changes in gene expression [19, 20]. Thus, hypoxia is closely related to tumor occurrence and development. Besides, the hypoxic environment can cause the tolerance of tumor cells to chemotherapy and radiation [21] and accelerate the tumor invasion and metastasis [22]. HIF-1 also plays a regulatory role in tumor cell apoptosis. Antiapoptosis genes of Bcl-2 family and survivin interact with the proapoptosis genes of Fas and p53 to regulate the apoptosis of liver cancer cells. Previous study indicated that HIF-1 possesses the control over expressions of Bcl-2, Bax, and survivin [23]. Expression of HIF-1*α* in tumor tissues was inhibited remarkably after the administration of TFAE, suggesting that the regulatory effect of TFAE on apoptosis of tumor cells is associated with the downregulated level of HIF-1*α*. However, the relationships between the expression of HIF-1*α* and the expression of Bcl-2 or between the expression of Bax and the expression of caspase need to be further confirmed by future studies.

HIF-1*α* has been proved to promote the expression of vascular endothelial growth factor (VEGF) [24]. VEGF is an important tumor vascular endothelial growth factor which can specifically induce the growth and proliferation of endothelial cells and accelerate angiogenesis occurrence and tumor growth [25, 26]. Additionally, VEGF inhibits tumor cell apoptosis [27]. Our results showed that TFAE could significantly reduce the expression level of VEGF in tumor tissue of nude mice and exert obvious effect on the inhibition of proliferation of tumor cells.

## 5. Conclusion

In this study, a heterotopic liver cancer xenograft model was adopted in nude mice to evaluate the anti-liver cancer effects of TFAE *in vivo* and explore the possible mechanisms. The heterotopic cancer xenograft model in nude mice has provided a convenient approach to be conducted and observed and, moreover, has short incubation period and high rate of tumor occurrence. It would greatly shorten the experimental period and would be suitable for screening of anticancer drugs.

In future studies, an orthotopic HCC animal model will be needed to make the results more accurate, reliable, and convincing. In addition, other animal models of liver cancer will be used to clarify the antihepatoma effect of TFAE *in vivo*.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

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*Research Article*

# MMP-Inhibitory Effects of Flavonoid Glycosides from Edible Medicinal Halophyte *Limonium tetragonum*

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*Limonium tetragonum* has been well-known for its antioxidative properties as a halophyte. This study investigated the antimetastasis effect of solvent-partitioned *L. tetragonum* extracts (LTEs) and isolated compounds on HT1080 mouse melanoma cell model with a focus on matrix metalloproteinase (MMP) activity and TIMP and MAPK pathways. Upregulation and stimulation of MMPs result in elevated degradation of extracellular matrix which is part of several complications such as metastasis, cirrhosis, and arthritis. The anti-MMP capacity of LTEs was confirmed by their MMP-inhibitory effects, regulation of MMP and TIMP expression, and suppression of MAPK pathway. Among all tested LTEs, 85% aq. MeOH and n-BuOH were found to be most active fractions which later yielded two known flavonoid glycosides, myricetin 3-galactoside and quercetin 3-o-beta-galactopyranoside. Anti-MMP potential of the compounds was confirmed by their ability to regulate MMP expression through inhibited MAPK pathway activation. These results suggested that *L. tetragonum* might serve as a potential source of bioactive substances with effective anti-MMP properties.

## 1. Introduction

Spread and growth of malignant tumorous cells are conducted through invasive and metastatic activities of cancer cells. Evolution of a primary abnormal tissue growth from a neoplasm to invasive tumor cells includes several-step biological processes. Degradation of extracellular matrix with proteolytic enzymes is one of the main pathways regulating the aforementioned evolution [1]. Matrix metalloproteinases (MMPs), zinc-containing calcium-dependent endopeptidases, are responsible for the proteolytic degradation of extracellular matrix and hence closely linked with tumor invasion, angiogenesis, and metastasis of cancer. In invasive tumor cells, two MMPs, MMP-2 and MMP-9, are predominantly upregulated and take crucial roles in invasion and metastatic spread of tumor [2, 3]. Regulation of MMP activities is carried out by intracellular inhibitors called tissue inhibitor of metalloproteinases (TIMPs) of which TIMP-1 and TIMP-2 are studied intensely in cancer studies. In this context, balance between MMPs and TIMPs was suggested to be deteriorated during cancer spread and growth which promotes a new target for therapeutic action against malignant tumors. Inhibition of MMPs coupled with increase in TIMP activity is also considered a promising path against tumor cell growth [4]. In addition, therapeutic intervention in metastasis and growth of tumor cells is aimed to be achieved by natural agents which lately are sourced from medicinal plants and traditional folk medicine [5–7]. The scientific world witnessed discovery and development of novel and potential bioactive substances against cancer from natural sources, especially

both terrestrial and marine plants [8–10]. Recent developments in natural product research suggests that plants are important sources that exhibit anticancer properties through MMP/TIMP regulation tumor cells.

Salt marshes and muddy seashores of South Korea contain a commonly known edible halophyte species, *Limonium tetragonum*, which grows in places that come into contact with waters of high salinity [11]. However, apart from antioxidants which are known to be present in abundance in plants of harsh environments, current studies do not contain any detailed report on health beneficial effect of *L. tetragonum* [12]. Considering the potential of halophytes, as a part of prominent research trend to develop novel substances from plants for nutraceutical purposes, solvent-partitioned *L. tetragonum* extracts has been tested. *L. tetragonum* was assayed for its effect on MMP-linked pathways *in vitro* using a human fibrosarcoma cell model. Isolated bioactive substances that have potential use against MMPs were characterized and screened.

## 2. Materials and Methods

*2.1. Plant Material. L. tetragonum* was provided by Korea Maritime and Ocean University (Yeongdo, Busan, Korea). Crude extracts were prepared as described by Bae et al. [13]. Solvent fractions were obtained from the organic and aqueous layers. First, $CH_2Cl_2$ layer was fractionated with n-hexane and 85% aqueous MeOH. The aqueous layer was further fractionated with n-BuOH and $H_2O$, resulting in *L. tetragonum* solvent-partitioned fractions (LTEs) of n-hexane (2.64 g), 85% aqueous (aq.) MeOH (1.42 g), n-BuOH (1.53 g), and $H_2O$ (26.47 g). Fractions were dissolved in 10% DMSO in order to be used in experiments. Further isolation of the active compounds was carried out as described earlier [14].

*2.2. Cell Culture and Cytotoxicity Determination.* HT1080 human fibrosarcoma cell line was used for *in vitro* assays. Cells were grown in flasks (T-75, Nunc, Roskilde, Denmark) at 37°C humidified atmosphere of 5% $CO_2$ and fed with Dulbecco's modified Eagle medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 $\mu$g/mL penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD, USA). Culture medium was replaced with fresh one every 3 days unless otherwise stated.

For cell viability assessment, cells were cultured in 96-well plates at $5 \times 10^3$ cells/well density. Following 24 h incubation, cell culture medium was removed and the cells were washed with fresh medium prior to treatment with samples (5, 10, 20, 50, and 100 $\mu$g/mL). Cells were rewashed with fresh medium after 24 and 48 h of incubation and 100 $\mu$L of MTT solution (1 mg/mL) was introduced to the wells, followed by a 4 h incubation. Finally, 100 $\mu$L of DMSO was used for each well in order to solubilize the formazan crystals for the determination of absorbance values at 540 nm using a GENios® microplate reader (Tecan Austria GmbH, Grödig, Austria). Cell viability was defined by the absorbance value as a way to indicate the amount of MTT converted into formazan crystal. Viability of cells was determined by comparing untreated control against sample treated wells and dose-response curves were established as percentage.

*2.3. Determination of MMP Activity by Gelatin Zymography.* Enzymatic activities of MMP-2 and MMP-9 from HT1080 cells that were treated with or without samples were detected by gelatin zymography. Method of Bae et al. [13] was followed with small modifications. Briefly, phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) was used to enhance the MMP expression of cells and conditioned cell culture media were used to determine the activity of MMPs following treatment with LTEs or isolated compounds at the stated concentrations. Following substrate-gel electrophoresis of cell culture medium, gels were kept in a buffer solution containing 10 mM $CaCl_2$, 50 mM Tris–HCl, and 150 mM NaCl at 37°C for 48 h in order to promote the gelatin digestion of MMPs. Activities of MMP were observed as digested clear zones following subjection to Coomassie Blue staining under a CAS-400SM Davinch-Chemi imager™ (Davinch-K, Seoul, Korea).

*2.4. RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis.* Effects of samples on mRNA expression of MMPs and TIMPs were analyzed by RT-PCR. Common protocols were followed using the sense and antisense primers that were stated previously [13]. Briefly, 2 $\mu$g of total RNA from treated or untreated cells was converted to single stranded cDNA using a reverse transcription system (Promega, Madison, WI, USA) and later amplified using specific primers according to manufacturer's protocol. Final products were visualized under UV light using a CAS-400SM Davinch-Chemi imager™ (Davinch-K, Seoul, Korea) and AlphaEase® gel image analysis software (Alpha Innotech, San Leandro, CA, USA) was used for quantification.

*2.5. Western Blot Analysis.* Immunoblotting was performed according to common standard procedures described earlier [13]. To explain briefly, HT1080 cells were agitated in RIPA lysis buffer (Sigma-Aldrich Corp., St. Louis, USA) at 4°C for 30 min. Cell lysates (35 $\mu$g) were subjected to electrophoresis using 12% SDS-polyacrylamide gel for separation which was followed by transfer onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biosciences, England, UK). Detection of immunoreactive proteins was carried out with an electrochemiluminescence kit (Amersham Pharmacia Biosciences, England, UK). Protein bands on membranes were observed using a CAS-400SM Davinch-Chemi imager™ (Davinch-K, Seoul, Korea).

*2.6. Statistical Analysis.* All plotted data were presented as a mean of three different experiments ± SD. Differences between the calculated means of each individual group were determined by one-way ANOVA coupled with Duncan's multiple range tests using the statistical software SAS v9.1 (SAS Institute, Inc., Cary, NC, USA). Any difference was considered statistically significant at $P < 0.05$.

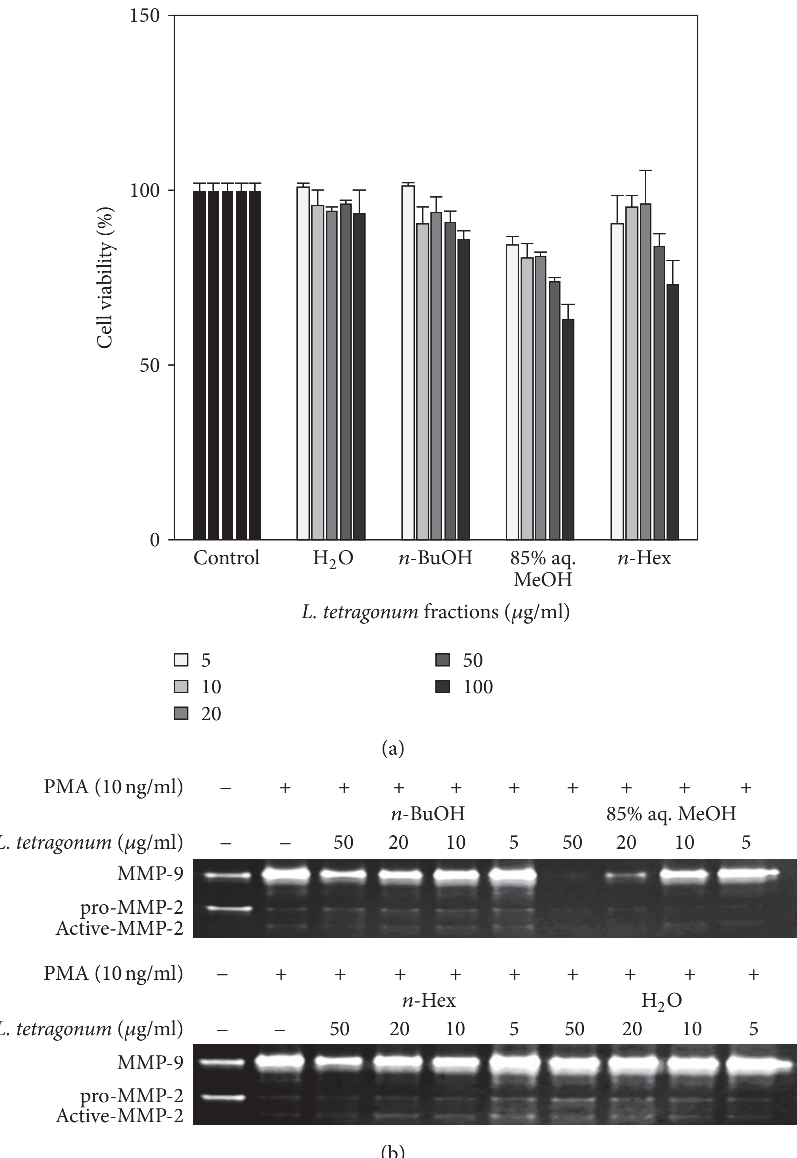


Figure 1: (a) Effect of solvent-partitioned *L. tetragonum* extracts (LTEs) on cell viability of HT1080 human fibrosarcoma cells. (b) Effect of LTEs on enzymatic activity of MMP-2 and MMP-9 tested by gelatin zymography. Values are mean ± SD ($n = 3$).

## 3. Results and Discussion

MMPs are known to influence and intervene with several particular important pathways for metastasis, oxidative stress, and fibrosis [15]. Hence, successful inhibition of MMP activity steadily gains high interest to develop potent pharmaceuticals against metastasis-linked complications and tumor growth. MMP inhibitors of natural origin are being intensively studied nowadays and marine organisms hold a great deal of potential in this context due to their ability to survive in unique and challenging environments. Various organisms, especially marine plants, are promoted to contain bioactive metabolites some of which were credited as potent MMP inhibitors with suggested mechanism of actions [16–18]. In order to provide valuable insights on that matter, *L. tetragonum* was studied to evaluate its MMP-inhibition efficiency along with MMP inhibiting constituents. In this regard, extract of *L. tetragonum* was fractioned with organic solvents and solvent-partitioned extracts were tested separately. As a part of ongoing research, previously reported MMP-inhibitory activities of crude extract of *L. tetragonum* [13] were further detailed in order to provide the action mechanism and rationale behind the findings by characterization of active constituents.

First, the LTE samples were tested for their cytotoxic presence in human fibrosarcoma cell line HT1080 for 48 h at five different concentrations (5, 10, 20, 50, and 100 $\mu$g/mL) (Figure 1(a)). The cytotoxicity test revealed that these concentrations were cytocompatible up to 50 $\mu$g/mL and any observed inhibition of MMP-2 and MMP-9 activity was not caused by any cytotoxic influence at the concentrations below that.

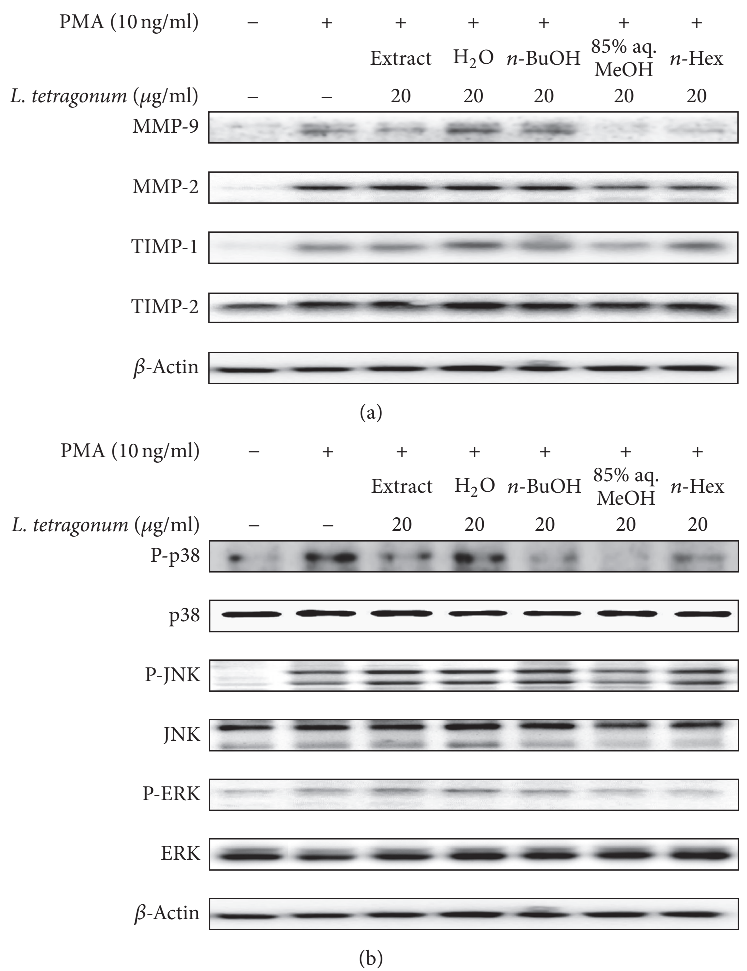


Figure 2: Effect of solvent-partitioned *L. tetragonum* extracts (LTEs) on protein levels of MMP-2 and MMP-9, TIMP-1 and TIMP-2, p38, JNK, and ERK observed by immunoblotting. $\beta$-Actin was used as an internal standard.

LTEs were analyzed for their possible activity to inhibit MMP-2 and MMP-9 enzymes following a PMA stimulation. Gelatinolytic activity of MMP-2 and MMP-9 secreted from fibrosarcoma cell line HT1080 was evaluated with gelatin zymography which was carried out using conditioned medium of LTE treated cells after PMA stimulation (Figure 1(b)). Introduction of PMA (10 ng/mL) to cells resulted in enhanced activation of MMP-2 and MMP-9; hence gelatinolytic activity in gelatin zymography was elevated. Among tested samples, 85% aq. MeOH LTE decreased the most of MMP-2 and MMP-9 activity in a dose-dependent manner relevantly higher than that of other samples. Remaining LTEs were observed to inhibit both MMP activity in an order of n-Hex, n-BuOH, and $H_2O$ fractions in regard to their efficiency. Inhibition of MMP enzymatic activities indicated that active samples possess bioactive substances that could have inhibit the MMP-2 and MMP-9 activity in the extracellular matrix dependent or independent from their intracellular secretion pathways. However, as seen in cell viability assay results (Figure 1(a)), the 85% aq. MeOH and n-Hex fractions exhibit increased cytotoxicity as the concentration increases. Although the enzymatic activity inhibition of LTEs was carried out using 50 ug/mL concentration, any possible inhibition may be caused by small amounts of cytotoxic substances in samples. However, at lower concentrations such as 10 and 20 ug/mL which at LTEs did not exhibit any cytotoxicity, an inhibition of both MMP-2 and MMP-9 activity still could be observed. In this context, further cell-based experiments were carried out with normalization of the results against a housekeeping gene in order to eliminate the any possible interference of cytotoxic presence.

Further, protein levels of MMP-2 and MMP-9 were determined by immunoblotting along with the levels of TIMP-1 and TIMP-2. TIMPs are known inhibitors of MMPs and also reported to elevate the activity of MMP-2 in some situations as a part of regulation [19]. Immunoblotting results suggested the treatment with 20 $\mu$g/ml LTEs was able to suppress the protein levels of MMP-2 and MMP-9 (Figure 2(a)). Presence of TIMPs is considered to imply inhibited MMP activity as a part of cellular response to extracellular stimuli [4]. Hence, the PMA stimulation caused TIMP levels to decrease and MMP expression to increase. However, treatment with LTEs was observed to produce mixed results regarding the effect on the TIMP levels following the PMA stimulation. Expected

results were to inhibit MMP and enhance TIMP expressions in order to regulate the extracellular matrix degradation. According to results, only n-BuOH and n-Hex LTE were able to regulate the MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in an expected manner where suppression of MMP expression is coupled with elevated TIMP levels. On the other hand, protein levels of MMP-2 and MMP-9 were slightly elevated after $H_2O$ and n-BuOH LTE treatment with elevated TIMP-1 and TIMP-2 levels. Elevated levels of TIMP-2 were observed, in addition to an increase in MMP-2 protein levels after n-Hex LTE treatment. Nonetheless, LTEs were shown to have effect on both activity and expression of MMP pathways but with suggested different mechanism of actions. In cases of $H_2O$ and n-BuOH LTEs, a possible intervention for the activation of MMP-2 and MMP-9 enzymes was suggested following the elevated protein levels of MMPs which would explain the inhibited enzyme activity despite the elevated protein levels. In terms of remaining LTEs, a mechanism where there isTIMP-linked regulation of MMP activity was suggested.

In order to grasp the suggested intervention on MMP-related intracellular pathways, levels of MAPK-related proteins, namely, p38, p-ERK, and p-JNK, were also evaluated (Figure 2(b)). Expectedly, PMA stimulation resulted in an elevated expression of ERK, JNK, and p-38 as well as their phosphorylation in regard to raised cellular activity. Reports showed that the inhibition of MAPK pathways is closely related to downregulation of MMP secretion [20, 21]. The activation of MAPK pathway induced by PMA stimulation was seen in Figure 2. However, treatment with LTEs at the concentration of 20 $\mu$g/mL did not show any consistent change in aforementioned increment in protein expressions. Among tested samples, 85% aq. MeOH and n-BuOH were able to regulate the MAPK pathways besides the MMP and TIMP levels. Also, n-Hex and $H_2O$ LTE were not able show any notable effect on MAPK pathway. These results implied the suggested action mechanisms of LTEs. On the other hand, 85% aq. MeOH and n-BuOH LTEs were able to present a regulatory effect on MMP activation through suppression of MAPK pathway shown as lowered p-ERK, p-JNK, and p-38 levels. However, among all tested MAPK proteins, phosphorylated p38 levels were inhibited by treatment of all LTEs. Kim et al. [22] reported that upregulation of MMP-2 and MMP-9 was induced by TGF-$\beta$-linked p38 expression but not ERK and JNK signaling in human breast epithelial cells. Likewise, current data suggested that LTEs treatment-induced downregulation of MMP-2 and MMP-9 activity and mRNA expression were not linked to phosphorylation of ERK and JNK mainly but only p38. Therefore, it could be suggested that possible inhibition of MMP-2 and MMP-9 by main components of *L. tetragonum* was exerted through TGF-$\beta$ pathways as well as enzymatic inhibition. The LTEs were able to downregulate the MAPK pathway and suggested to possess potential compounds that can inhibit MMP enzymatic activity as well. All data were in accordance with previous findings [13], stating the suggested anti-MMP effects of crude *L. tetragonum* extracts were rooted from the possible bioactive substances from the most active fractions, n-BuOH and 85% aq. MeOH.

Comparing the ability of n-BuOH and 85% aq. MeOH LTEs to inhibit MMP activity and intracellular regulation, two known compounds myricetin 3-galactoside (A) and quercetin 3-o-beta-galactopyranoside (B) were isolated through an activity-guided isolation (Figure 3) as described earlier [14]. These two compounds are known to be derivatives of several antioxidant and anti-MMP compounds which were previously reported [23, 24]. Possible antioxidant properties of these compounds due to their flavonoid backbone were suggested to affect their inhibitory effects on MMPs. Different sizes and chemical structures of the compounds were suggested to be main reason for selective inhibition of MMP activity.

In order to verify the compounds as active constituents of the LTEs, their inhibitory effects on MMP activity were tested with gelatin zymography assay (Figure 4). While compound A was able to inhibit MMP-2 activity and fails to affect MMP-9, opposite results were obtained from compound B. Following compound B treatment, MMP-9 activity was lowered up to 63% in a dose-dependent manner while MMP-2 activity was further increased. Further, both compounds were tested for their possible effect on the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNAs (Figure 5(a)) and proteins (Figure 5(b)). Results confirmed their involvement in regulation of the MMP activity through TIMP-linked pathways. Unlike their effect on enzymatic activity, both compounds were able to lower the PMA-stimulated overexpression of MMP-2 and MMP-9 in a non-dose-dependent manner. Compounds A and B were also able to elevate the PMA-linked downregulation of TIMP-1 and TIMP-2 in terms of mRNA and protein levels. In order to elucidate their action mechanism, compounds were tested for their effect on the levels of MAPK pathway proteins in their preactivation and phosphorylated states. As seen on Figure 6, compound B was able to lower the phosphorylation of ERK and p-38 while compound A showed similar results but not as statistically significant as compound B. Possible intervention of MAPK pathway was hence suggested to be the step in which compound B exerted its anti-MMP activity, confirmed by lowered MMP expression as opposed to slightly affected TIMP levels. Involvement of JNK pathways was suggested to have elevating effects on MMP-2 activity through TIMP-2 regulation. Enhanced MMP-2 activity after compound B treatment can be suggestively linked to its intervention of TIMP-2 and JNK pathways.

## 4. Conclusions

In conclusion, *L. tetragonum* was suggested to contain anti-MMP substances, including but not limited to myricetin 3-galactoside and quercetin 3-o-beta-galactopyranoside that can inhibit MMP activity and suppress MAPK-linked MMP upregulation. Detailed action mechanism of isolated compounds and their efficiency *in vivo* remain unclear. Nonetheless, potential of *L. tetragonum* as a source of anti-MMP compounds for further utilization in nutraceutical and cosmetic fields was verified by current study and paved the way for detailed analysis in order to facilitate the potential of flavonoid glycosides as anti-MMP compounds.

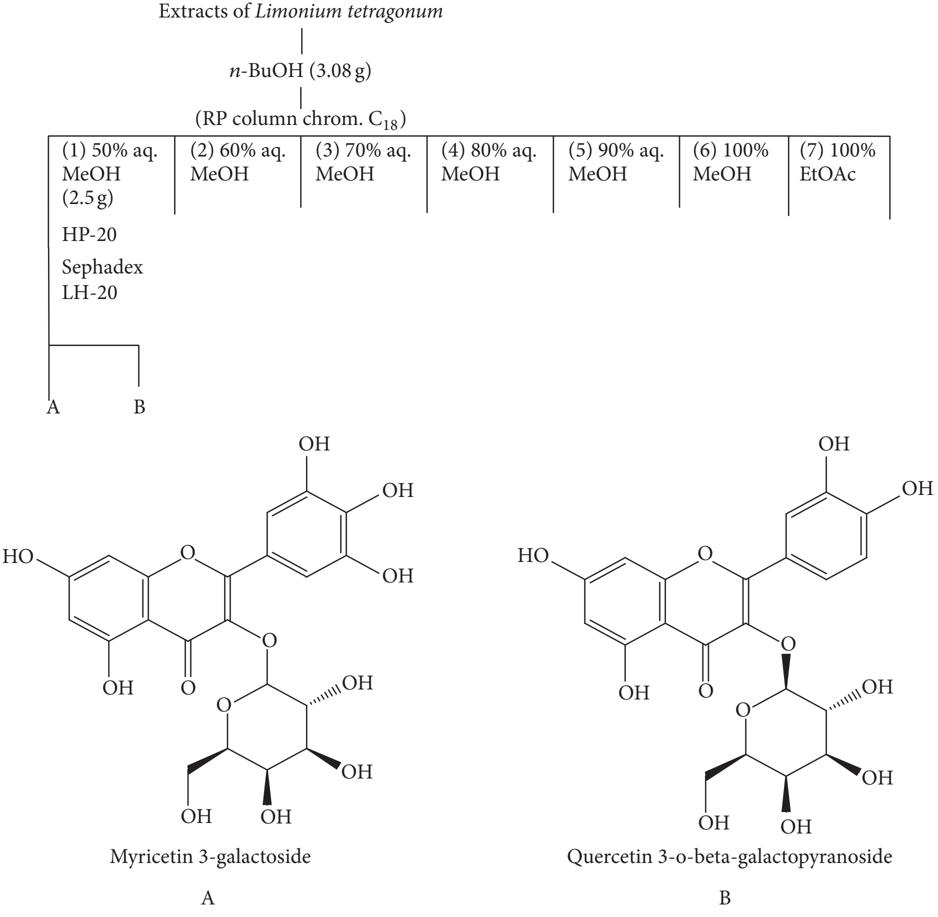


Figure 3: Basic isolation scheme and structural elucidation of two isolated flavonoid glycosides. A: myricetin 3-galactoside; B: quercetin 3-o-beta-galactopyranoside.

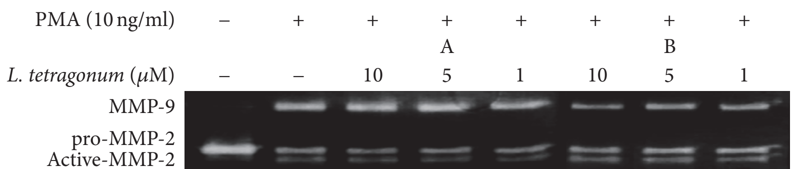


Figure 4: Effects of compounds A and B on enzymatic activity of MMP-2 and MMP-9 tested by gelatin zymography with cell lysates of treated HT1080 fibrosarcoma cells. A: myricetin 3-galactoside; B: quercetin 3-o-beta-galactopyranoside.

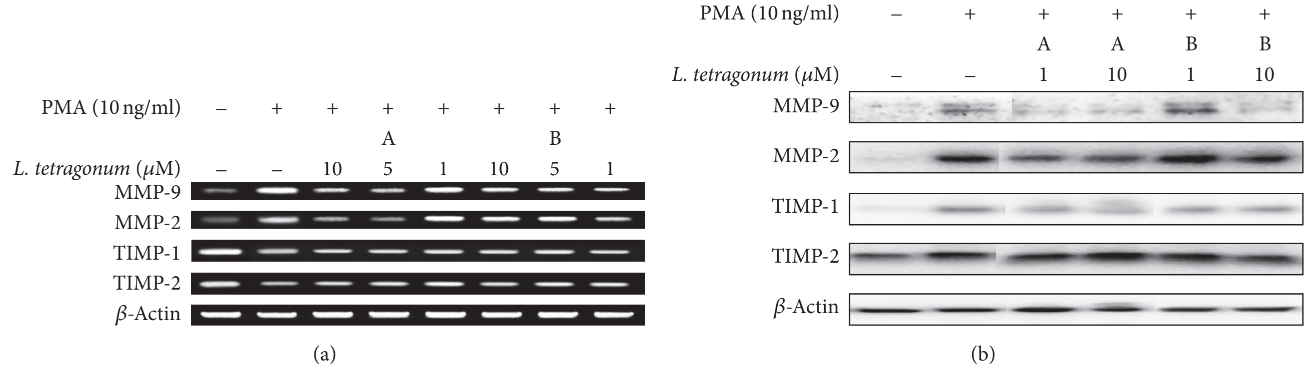


Figure 5: Effects of compounds A and B on mRNA (a) and protein (b) levels of MMP-2 and MMP-9 and TIMP-1 and TIMP-2 observed by RT-PCR and immunoblotting, respectively, in HT1080 human fibrosarcoma cells. $\beta$-Actin was used as an internal standard. A: myricetin 3-galactoside; B: quercetin 3-o-beta-galactopyranoside.

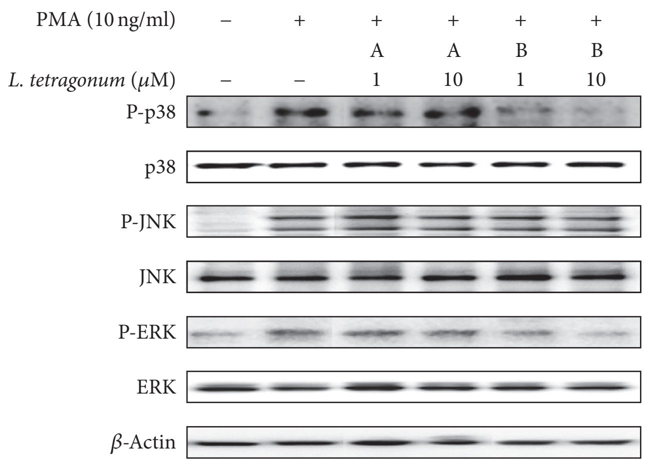


Figure 6: Effect of compounds A and B on phosphorylated (P-) and native protein levels of p38, JNK, and ERK in HT1080 human fibrosarcoma cells. $\beta$-Actin was used as an internal standard.

## Conflicts of Interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

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*Research Article*

# Curcumin Induces Autophagy, Apoptosis, and Cell Cycle Arrest in Human Pancreatic Cancer Cells

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*Objective*. Curcumin is an active extract from turmeric. The aim of this study was to identify the underlying mechanism of curcumin on PCa cells and the role of autophagy in this process. *Methods*. The inhibitory effect of curcumin on the growth of PANC1 and BxPC3 cell lines was detected by CCK-8 assay. Cell cycle distribution and apoptosis were tested by flow cytometry. Autophagosomes were tested by cell immunofluorescence assay. The protein expression was detected by Western blot. The correlation between LC3II/Bax and cell viability was analyzed. *Results*. Curcumin inhibited the cell proliferation in a dose- and time-dependent manner. Curcumin could induce cell cycle arrest at G2/M phase and apoptosis of PCa cells. The autophagosomes were detected in the dosing groups. Protein expression of Bax and LC3II was upregulated, while Bcl2 was downregulated in the high dosing groups of curcumin. There was a significant negative correlation between LC3II/Bax and cell viability. *Conclusions*. Autophagy could be triggered by curcumin in the treatment of PCa. Apoptosis and cell cycle arrest also participated in this process. These findings imply that curcumin is a multitargeted agent for PCa cells. In addition, autophagic cell death may predominate in the high concentration groups of curcumin.

## 1. Introduction

Pancreatic cancer (PCa) is the third leading cause of cancer-related death in the United States with a 5-year survival rate of 7.7% [1] and ranks 12th of all cancer incidences. Nearly 81% PCa patients were diagnosed at a terminal stage, which determines a poor prognosis. According to some statistical data, both morbidity and mortality of PCa continue to rise, while those of most other cancers have declined [2]. Surgery is applicable only to a few early-stage patients, and chemotherapy is the most important remedy for patients with metastatic cancer. Pre- and postoperative chemotherapy can also benefit the patient. According to a randomized study [3], the median survival increased from 4.41 months of treatment with 5-FU to 5.65 months of gemcitabine, and the 1-year survival rate improved from 2% in 5-FU-treated patients to 18% in gemcitabine-treated patients. Gemcitabine has therefore become the first-line chemotherapy regimen. However, owing to multidrug resistance and the intolerable adverse effects of the drug, searching for new alternative and adjuvant chemotherapy drugs has become an urgent mission.

The notion of autophagy was put forward several decades ago to describe the "self-eating" phenomenon extensively existing in many organisms [4]. It is a process of degrading cytoplasmic ingredients especially protein reacting against harsh conditions like nutrition deficiency and stress. Recent evidence suggests that autophagy is a double-edged sword in tumorigenesis and metastasis, because it can suppress tumor formation but on the other hand it promotes tumor growth once the tumor is formed [5]. Some studies [6, 7] demonstrated that autophagy inhibition could attenuate PCa activity markedly. mTOR (mechanistic target of rapamycin) possesses serine/threonine kinase and acts as an important regulator of cellular growth and metabolism [8]. In normal conditions, mTOR always represses the ULK1-Atg13-FIP200 complex and blocks autophagy, while autophagy ensued when mTOR activity was suppressed in the state of nutritional scarcity or stress [5, 9]. This pathway can trigger the

formation of autophagosomes. At the same time, LC3-I is formed by the removal of the C-terminal 22 amino acids from LC3, followed by a conversion of some LC3-I into LC3-II, leading to the maturity and isolation of autophagosomes. The amount of LC3-II has a positive correlation with the extent of autophagosome formation and thus can be regarded as a good marker of autophagy [10].

Curcumin, as an ingredient of turmeric, has attracted increasing attention for decades due to its various biological effects, including anti-inflammatory [11], antioxidant [12], and anticancer [13] properties. Curcumin is extracted from the rhizome of curcuma longa belonging to the ginger family and chemically known as 1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione, with the chemical formula of $C_{21}H_{20}O_6$ [13, 14]. Numerous experiments in vitro and vivo have demonstrated that curcumin could inhibit the growth of various cancers including gastric cancer, ovarian cancer, and colorectal cancer by inducing apoptosis [15–17] or curbing cell proliferation individually [18]. In addition, some studies in recent years have shown that autophagy plays a certain role in the anticancer process of curcumin [19–21]. However, the underlying mechanism remains elusive and controversial. The aim of the present study was to determine whether autophagy plays a role in the treatment of PCa with curcumin and explore the underlying mechanism.

## 2. Materials and Methods

*2.1. Cell Lines and Reagents.* PANC1 and BxPC3 cell lines were the subjects of this study as representatives of human pancreatic cancer cell lines, where PANC1 cells were derived from ductal epithelial cells and BxPC3 cells were derived from acinous adenocarcinoma. On the other hand, human-derived cell lines are closer to clinical drug efficacy than animal sources. Both of them were purchased from Xiangya Cell Center of the Central South University (Changsha, China). PANC1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and BxPC3 cells in RPMI 1640 medium at 37 °C in 5% $CO_2$. Both of them were supplied with 10% fetal bovine serum (FBS). Curcumin (P0206, purity > 98%, purchased from PureOne Biotechnology, Shanghai, China) was formulated into liquid at 100 mg/ml with DMSO, followed by being diluted into different required concentrations with culture medium as follows.

*2.2. Cell Growth Inhibition Test.* Cells were seeded in 96-well plates with $10^4$ cells per well. Curcumin with a concentration gradient was added in different groups after cells adhered to the bottom. At first, PANC1 cells were treated with curcumin at concentrations of 0, 0.2, 2, 10, 20, 40, 80, and 200 $\mu$g/ml for 24 h. It was found that there were no differences in cell morphology and proliferative status at the concentration of 0, 0.2, 2, and 10 $\mu$g/ml, but when the concentration was 20 $\mu$g/ml, the cell proliferation began to be inhibited and the cell morphology changed from the regular spindle and polygonal shape to round shape, and the intercellular antennae began to be reduced. With the increase of culture concentration, cell debris gradually increased and cell proliferation was inhibited obviously. When the incubated concentration reached 200 $\mu$g/ml, there was almost only cell debris left. So the concentrations of 0, 10, 20, 40, and 80 $\mu$g/ml were selected to study the cell proliferation rate for PANC1 cells, and similarly, BxPC3 cells were treated with 0, 0.4, 0.8, 1, 4, 8, 10, and 20 $\mu$g/ml of curcumin. The incubation time was set at 24, 48, and 72 h. Cell viability was tested via Cell Counting Kit-8 (CCK-8, DOJINDO, Japan) with absorbance at 450 nm.

According to the results, the groups were set as follows: 0, 10, 20, 40, and 80 $\mu$g/ml of curcumin for PANC1 cells and 0, 0.1, 0.5, 1, 5, and 10 $\mu$g/ml for BxPC3 cells in the following experiments, among which 0 $\mu$g/ml group was the control group. The incubation time was set to 24 hours.

*2.3. Cell Cycle Analysis.* Cells of different groups as previously described were harvested and washed with PBS and fixed with 75% ethanol at 4°C overnight. After being stained with PI/RNase solution (BD Pharmingen, 550825) for 15 min in the dark, cells cycle distribution of different groups was detected by flow cytometry (Becton Dickinson, USA).

*2.4. Apoptosis Quantitative Detection.* The two cell lines were trypsinized after being treated with different concentrations of curcumin as previously described and washed three times with PBS. The operating procedures were performed referring to the instruction. Cells were double stained with PI (Propidium Iodide)/Annexin V-FITC kit (DOJINDO, Japan). All the samples were detected using a BD flow cytometer. The apoptosis quantity was defined as the sum of the Q2 and Q3 quadrant. Data and diagrams were treated with FlowJo 7.6 software.

*2.5. Immunofluorescent Assay.* In this study, we used LC3 to detect autophagy. After being treated with different concentrations of curcumin in 24-well plates, cells were fixed with paraformaldehyde for 15 min and incubated with LC3 antibody (Cell Signaling Technology, #12741) overnight. The next day, the second antibody conjugating with FITC (a fluorescent dye) was used to detect LC3 with a fluorescent microscope.

*2.6. Western Blotting Test.* Protein obtained by lysis of cells, sonication, and centrifugation procedurally [22] was loaded to each well, electrophoresed in polyacrylamide gels, and transferred onto the polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% nonfat milk for 1 h, membrane was incubated with primary antibodies overnight at 4°C, including anti-LC3 (1 : 1000), anti-caspase 3 (1 : 1000, CST, #9663), anti-mTOR (1 : 1000, CST, #2983), anti-Bax (1 : 1000, CST, #2772S), anti-Bcl2 (1 : 1000, CST, #2870S), anti-$\beta$-actin (1 : 5000, KeyGEN BioTECH, KGAA001, Jiangsu, China), and anti-$\alpha$-Tubulin (1 : 1000, CST, #2144S). Then, the membrane was incubated with second antibodies (goat anti-rabbit IgG-HRP) for 1 h at room temperature on a shaker. Finally, bands were visualized via ECL assay (KeyGEN BioTECH, Jiangsu, China) by the Tannon 5200 automatic imaging system (Shanghai, China), and optical density (OD) was measured by Tannon GIS image analysis system.

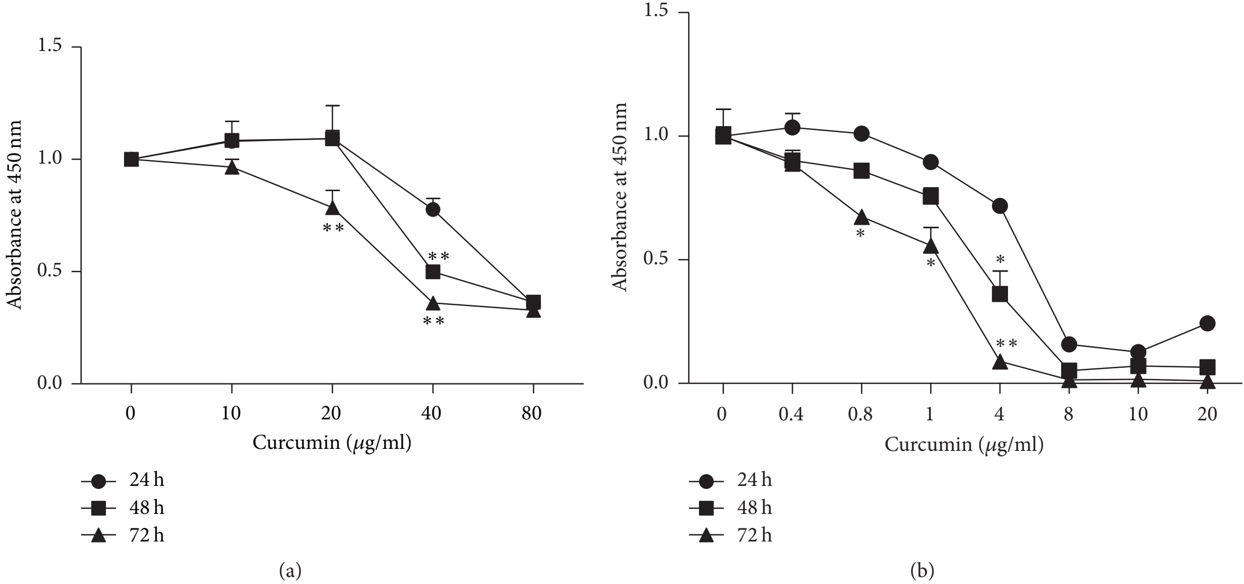


FIGURE 1: Curcumin inhibits the proliferation of PCa cells in a time- and dose-dependent manner. (a) As cultivate time and concentration increase, the cell viability deteriorates for PANC1 cell line. (b) Similar inhibitory effect was found in the BxPC3 cell line. Data are representative of thrice independent experiments. Error bar, SEM. ∗ and ∗∗, compared with 24 h group. $^{*}p < 0.05$. $^{**}p < 0.01$.

*2.7. Statistical Analysis.* Correlation analysis between cell viability and LC3II/Bax was calculated by SPSS 22.0. Other statistical analysis results were completed by GraphPad Prism 5 software. One-way analysis of variance and Bonferroni's multiple comparison test were used to determine differences between groups. $p$ value < 0.05 was considered statistically significant.

## 3. Results

*3.1. Curcumin Inhibits the Proliferation of PCa Cells.* The inhibitory effect of curcumin on the PCa cell lines showed a dose- and time-dependent trend. As shown in Figure 1, the absorbance at 450 nm of the ordinate represents cell viability. As for PANC1 cells (Figure 1(a)), cell viability of 40 μg/ml in 48 h and 72 h groups was significantly lower than 24 h group, and cell proliferation of 20 μg/ml in 72 h group was inhibited more than 24 h group. What is more, with the increase of drug concentration, cell viability decreased gradually at each incubation time group. Curcumin exerted the similar dose- and time-dependent inhibitory effect on BxPC3 cells (Figure 1(b)). They were 4 μg/ml group at 48 h and 0.8, 1, and 4 μg/ml groups at 72 h that showed significant differences compared with 24 h groups, respectively. The higher the concentration of curcumin, the lower the cell viability.

*3.2. Curcumin Induces Cell Cycle Arrest in PCa.* As previously described, PANC1 cells were treated with curcumin of 0, 10, 20, 40, and 80 μg/ml for 24 hours, and BxPC3 cells were cultured with 0, 0.1, 0.5, 1, 5, and 10 μg/ml of curcumin for 24 h. PCa cells cycle was arrested at G2/M stage (Figures 2 and 3). The average proportion of G2/M stage in 0, 10, 20, 40, and 80 μg/ml groups was 18.1%, 19.6%, 28.8%, 39.1%, and 37.6% for PANC1 cells orderly (Figure 2). Compared with control group, cell cycle in 40 and 80 μg/ml groups was significantly blocked (Figure 2(b)). The average proportion of G2/M stage for BxPC3 cells in 0, 0.1, 0.5, 1, 5, and 10 μg/ml groups was 14.9%, 15.6%, 15.2%, 12.7%, 15%, and 29.9%, successively (Figure 3). 10 μg/ml group had a significantly difference compared with the control group (Figure 3(b)). The results suggested that curcumin blocked PCa cells in G2/M phase.

*3.3. Curcumin Induces Apoptosis of PCa Cells*

*3.3.1. Effect of Curcumin on Cell Apoptosis Detected by Flow Cytometer.* The apoptosis ratio of 0, 10, 20, 40, and 80 μg/ml groups for PANC1 cells was 2.3%, 2.8%, 9.9%, 55.6%, and 90.3%, orderly (Figure 4). The total ratio of lower right (Annexin $V^+/PI^-$) and upper (Annexin $V^+/PI^+$) quadrants which revealed that apoptosis level was higher in 80 μg/ml group than control group (Figure 4(b)). As for BxPC3 cells, the apoptosis ratio of 0, 0.1, 0.5, 1, 5, and 10 μg/ml groups was 4.7%, 5.1%, 4.8%, 6.5%, 36.6%, and 74.6%, respectively (Figure 5). And it is 10 μg/ml group that had significant difference from control group (Figure 5(b)). Consistent with cell cycle distribution image, we can see the apoptotic peak in 40 and 80 μg/ml groups of PANC1 cells and 5 and 10 μg/ml groups of BxPC3 cells.

*3.3.2. Effect of Curcumin on Bax/Bcl2 Protein Expression.* Bax is one of proapoptosis regulators [23], and Bcl2 belongs to the antiapoptosis molecular family [23, 24], both of which have long been used to detect the level of apoptosis. In this study, protein expression of Bax was significantly increased in 80 μg/ml group for PANC1 cells (Figure 6(a)) and 10 μg/ml group for BxPC3 cells (Figure 6(b)) compared to untreated control groups. In addition, Bcl2 protein expression was significantly decreased in 80 μg/ml group for PANC1 cells (Figure 6(a)) and 10 μg/ml group for BxPC3 cells (Figure 6(b)).

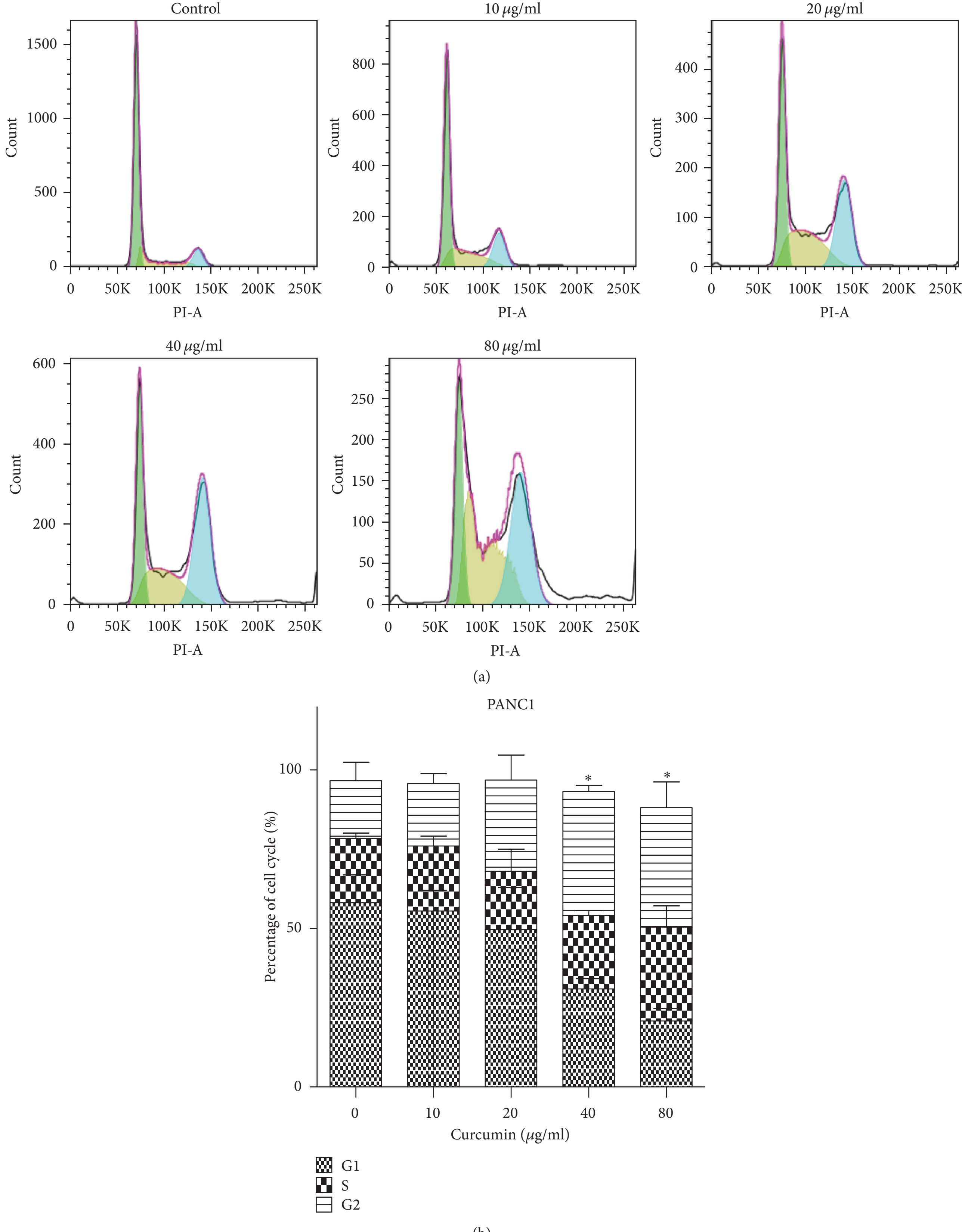


Figure 2: Curcumin induced G2/M arrest in PANC1 cell line. (a) Cell cycle distribution was detected by flow cytometer. (b) The proportion of G2/M stage obviously increased in 40 and 80 μg/ml groups. Each dataset represents three independent experiments. Error bar, SEM. ∗, significantly different from control group ($p < 0.05$).

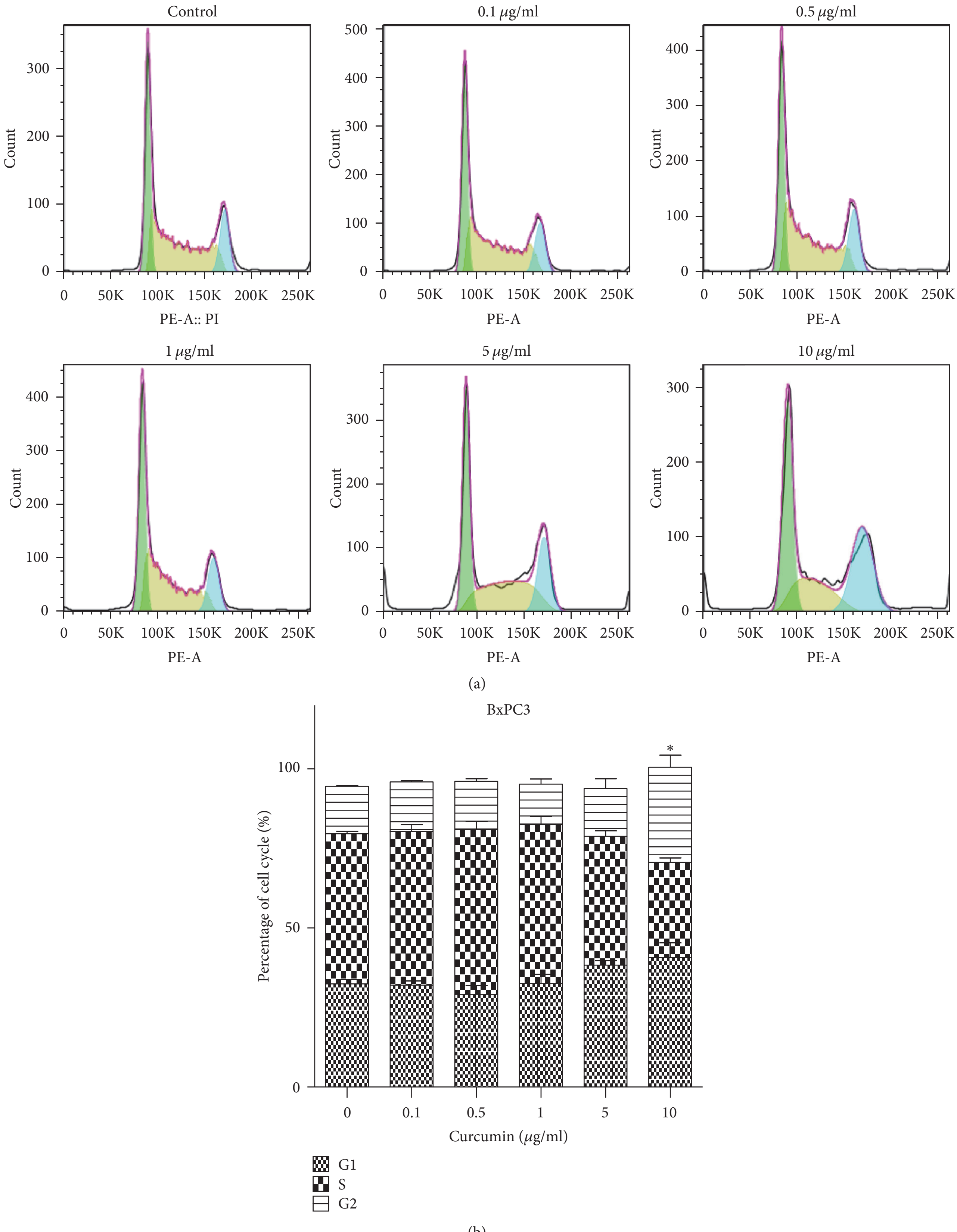


Figure 3: Curcumin induced G2/M arrested in BxPC3 cells. (a) Cell cycle distribution of BxPC3 cells after being treated with different concentration of curcumin. (b) The proportion of G2/M stage significantly increased in 10 $\mu$g/ml group. Data are representative of three independent experiments. Error bar, SEM. $*$, significantly different from control group ($p < 0.05$).

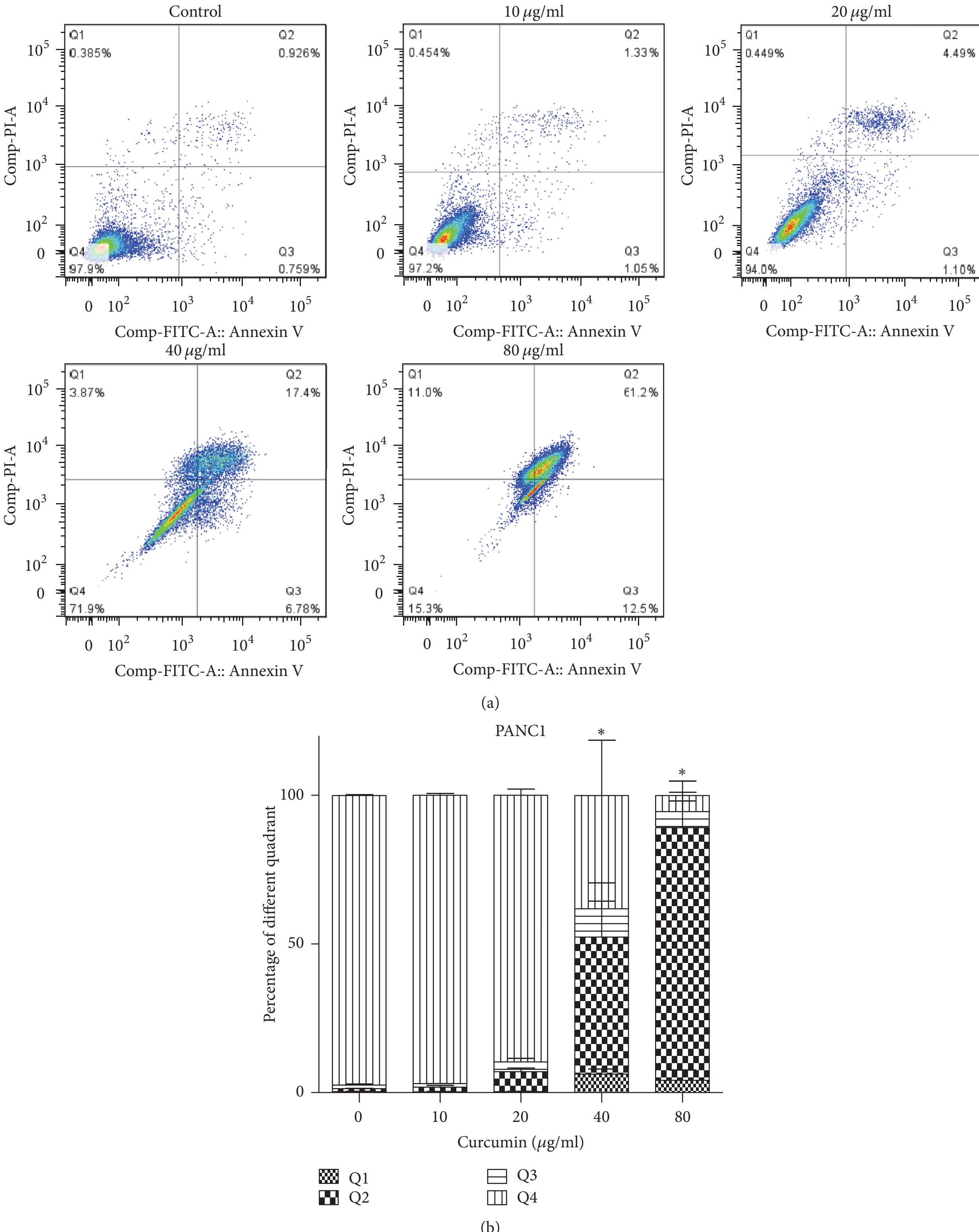


Figure 4: Curcumin induced apoptosis in PANC1 cells, measured by flow cytometry. (a) Each diagram was composed of four parts on behalf of different living state of cells. The total ratio of lower right (Annexin $V^+/PI^-$) and upper (Annexin $V^+/PI^+$) quadrants stands for apoptosis level. (b) The percentage of apoptosis was significantly higher in 80 $\mu$g/ml groups. Data are representative of three independent experiments. Error bar, SEM. $*$, significantly different from control group ($p < 0.05$).

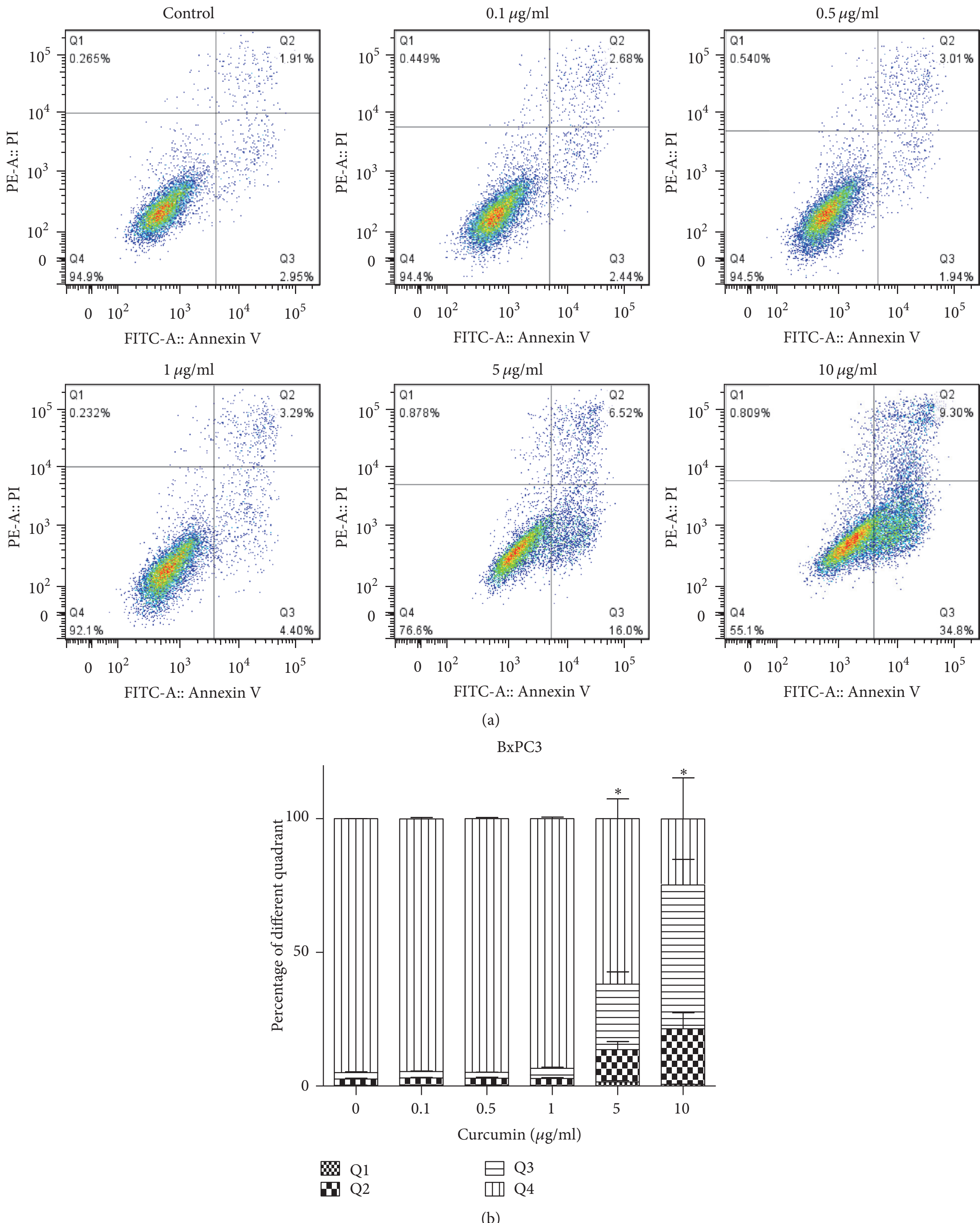


FIGURE 5: Curcumin induced apoptosis in BxPC3 cell lines. (a) Percentage of vitality, apoptosis, and necrosis was detected by flow cytometer. (b) The total ratio of apoptosis (Q2 and Q3 quadrants) was higher in 10 ug/ml groups. Data are representative of three independent experiments. Error bar, SEM. ∗, significantly different from control group ($p < 0.05$).

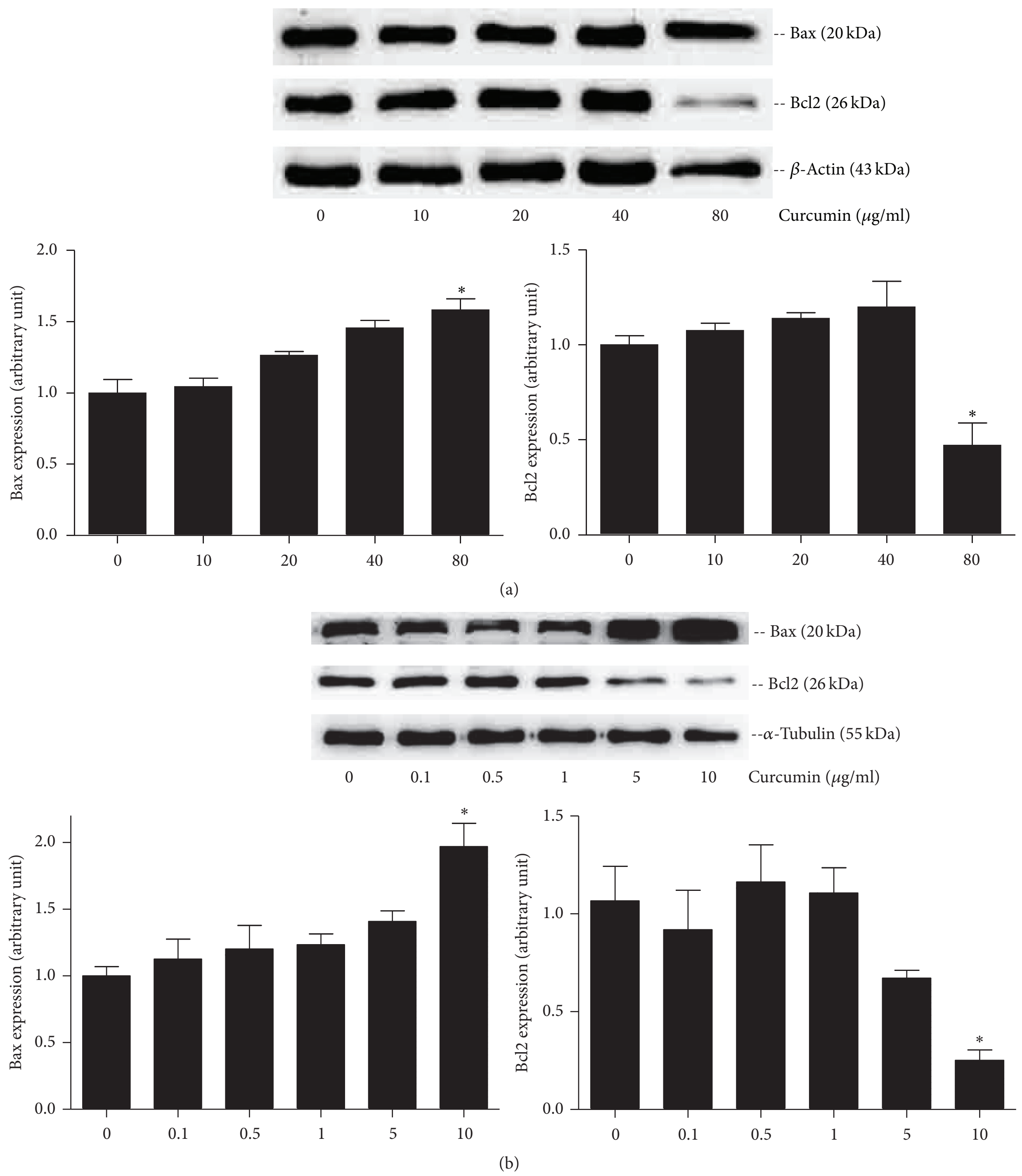


Figure 6: Expression of Bax and Bcl2 protein in PCa cells, detected by Western blot assay. (a) PANC-1 cells were treated with curcumin of different concentration. The protein expression of Bax was significantly increased in 80 $\mu$g/ml group, while Bcl2 protein expression was decreased in 80 $\mu$g/ml group. (b) The protein expression of Bax was significantly increased in 10 $\mu$g/ml group of BxPC3 cells, while Bcl2 protein expression was decreased in 10 $\mu$g/ml group. Error bar, SEM. $*$, significantly different from control group ($p < 0.05$).

### *3.4. Curcumin Induces Autophagy of PCa Cells*

*3.4.1. Curcumin Increases Autophagosomes in PCa Cells.* In this study, luminescent autophagosomes were probed by tracking LC3 protein in cell immunofluorescence way (Figures 7 and 8). It was found that the expression level of punctuate autophagosomes was the highest in 40 $\mu$g/ml group for PANC1 cells (Figure 7), and control group did not present much green autophagosomes. Similarly, positive green-light puncta were also detected in BxPC3 cells incubated with curcumin at a dose of 1 $\mu$g/ml for 24 h versus negative in the blank control group (Figure 8).

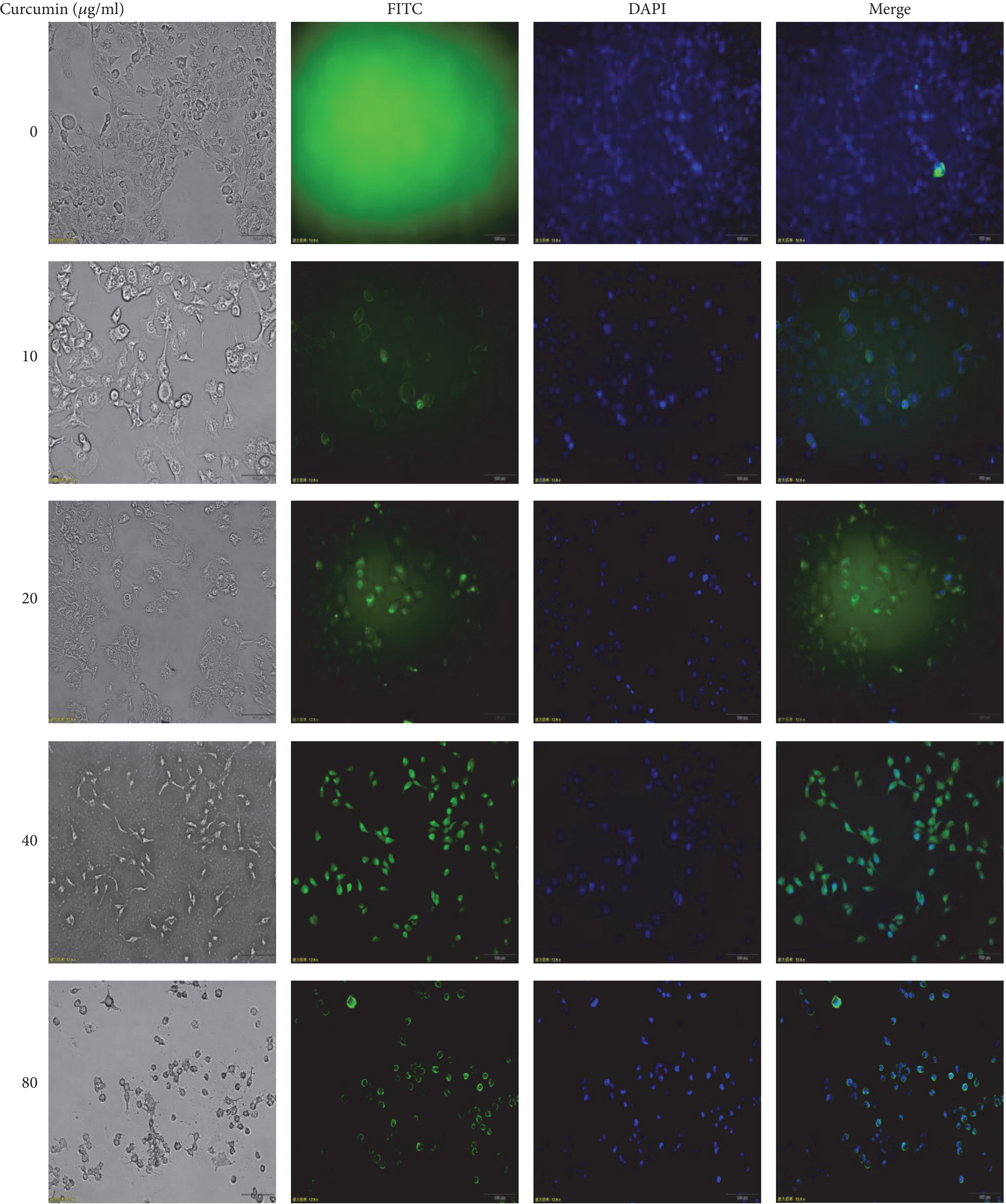


Figure 7: Autophagosomes in PANC1 cells were detected by immunofluorescence method. Cells were stained with FITC dye connecting second antibody after being incubated with LC3 antibody for an hour. Positive green dots in dosing groups show more when compared with the control group.

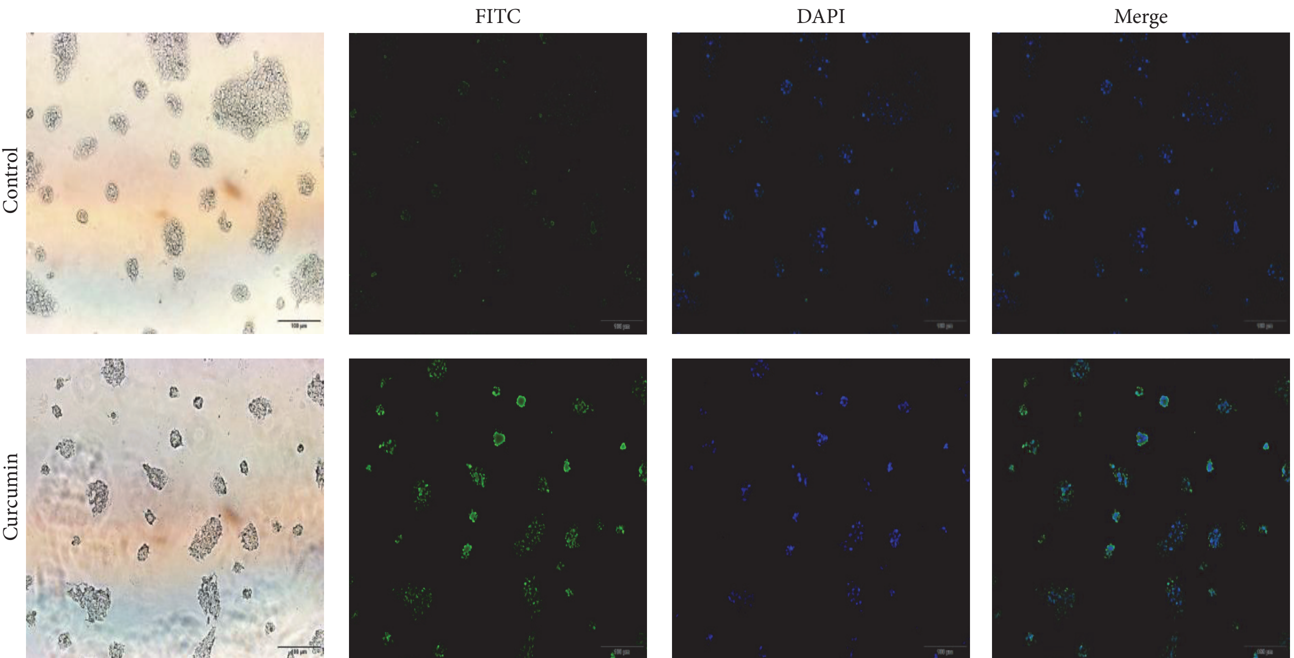


Figure 8: Immunofluorescence micrographs of BxPC3 cell lines by staining LC3 protein. Positive green-light puncta were detected in BxPC3 cells incubated with curcumin at a dose of 1 $\mu$g/ml for 24 h versus negative in the blank control group.

*3.4.2. Effect of Curcumin on LC3II and mTOR Protein Expression.* The protein expression of LC3II was significantly increased in 40 and 80 $\mu$g/ml groups for PANC1 cells (Figure 9(a)). Similarly, the protein expression of LC3II was significantly increased in 5 and 10 $\mu$g/ml groups of BxPC3 cells. In addition, the result of Western blot showed that the expression of protein mTOR was reduced significantly when cells were exposed to curcumin, especially at a dose of 20, 40, and 80 $\mu$g/ml for PANC1 cells and 10 $\mu$g/ml for BxPC3 cells (Figure 9). These results are consistent with the previous conclusion that the downregulation of mTOR could trigger the appearance of autophagosomes accompanied with the formation and flow of LC3II [25, 26].

*3.4.3. Comparison between Apoptosis and Autophagy.* In this study, we calculated the relative ratio of Bax and LC3II (Table 1) and did the correlation analysis between cell viability and LC3II/Bax (Figure 10). With Pearson method, we got the results that $r$ value equaled −0.979 and $p$ value was less than 0.05. It meant that the greater the ratio, the lower the cell viability. In addition, when the incubation concentration of curcumin was high, like 80 $\mu$g/ml group, the ratio of LC3II protein was far much higher than the ratio of Bax protein.

## 4. Discussion

Pancreatic cancer is a malignant tumor with a poor prognosis despite surgical intervention, and the standard chemotherapy is based on gemcitabine. However, the mild efficacy of gemcitabine against the drug-resistant pancreatic cancer limits its application. In this study, we found that curcumin had a diverse range of targets for PCa cells, including cell cycle arrest, apoptosis, and autophagy pathways. The multitargeted characteristics can make curcumin act synergistically in combination with standard chemotherapy drugs [27], especially for drug-resistant cancers.

Curcumin, a spice commonly used in curries and other south Asian cooking, has been found to possess anticancer effects, including breast cancer, colorectal cancer, lung cancer, and PCa [28–30], which is consistent with the results obtained in this study. Curcumin was found to inhibit the growth of two PCa cell lines in a dose- and time-dependent manner (Figure 1), and BxPC3 cells were more sensitive to curcumin compared with PANC1 cells (Figure 1). In this study, we found that curcumin could induce cell cycle arrest at G2/M stage in high concentrations (40 and 80 $\mu$g/ml for PANC1 cells and 10 $\mu$g/ml for BxPC3 cells) (Figures 2 and 3). It has been previously described that gemcitabine can induce cell cycle arrest in the S phase [31, 32]. Curcumin may produce synergistic effects when combined with gemcitabine by total arrest of cell cycle in most phases. Further studies are needed to confirm this presumption.

Apoptosis, also called programmed cell death, is a complex process of metabolic mediation involving numerous molecules in the organism. The amplified apoptosis results in damage or death of cells and tissues. While tumors will take place as apoptosis is weakened, in some sense, the antitumor mechanism of many chemotherapy drugs is to promote apoptosis. Similar results were obtained with curcumin in PCa cells. Previous experiments reported that treatment of BxPC3 cells with curcumin caused significant cell arrest in the G2/M phase and induced significant apoptosis [33]. In this study, curcumin was found to induce apoptosis of PANC1 and BxPC3 cell lines with the culture concentration

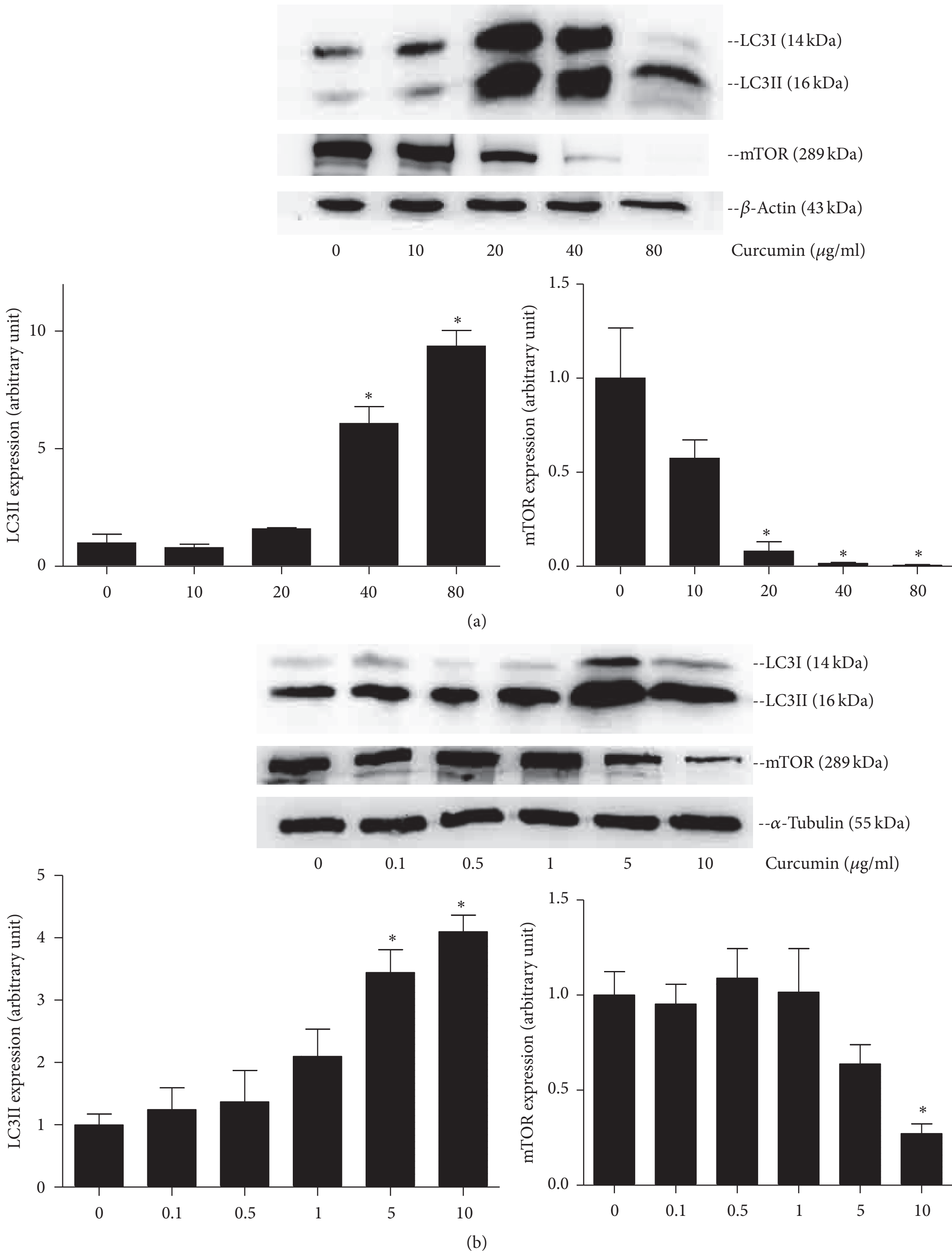


Figure 9: Expression of LC3II and mTOR protein in PCa cells, detected by Western blot assay. (a) PANC-1 cells were treated with curcumin of different concentration. The protein expression of LC3II was significantly increased in 40 and 80 $\mu$g/ml groups, while mTOR protein expression was decreased in 20, 40, and 80 $\mu$g/ml group. (b) The protein expression of LC3II was significantly increased in 5 and 10 $\mu$g/ml groups of BxPC3 cells, while mTOR protein expression was decreased in 10 $\mu$g/ml group. Error bar, SEM. $*$, significantly different with control group ($p < 0.05$).

Table 1: Relative ratio of Bax and LC3II.

| | C10/control | C20/control | C40/control | C80/control |
|---|---|---|---|---|
| Relative ratio of protein Bax | 1.04480417 | 1.26475652 | 1.455598125 | 1.582404383 |
| Relative ratio of protein LC3II | 0.79957309 | 1.60000026 | 5.737708117 | 9.365680576 |
| LC3II/Bax | 0.76528512 | 1.26506584 | 3.941821591 | 5.918639178 |

C10, C20, C40, and C80: dosing group with different concentration of curcumin. LC3II/Bax: relative ratio of two above.

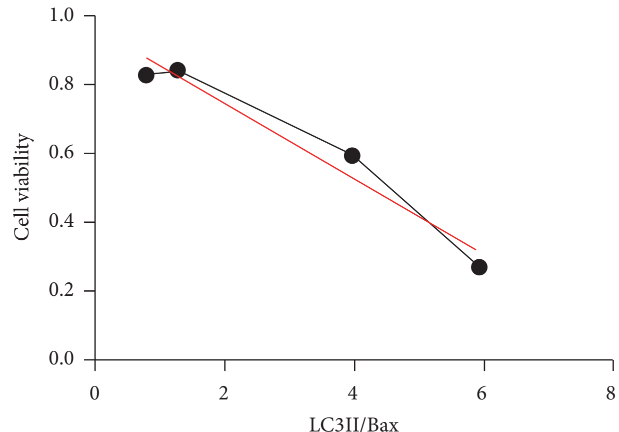


Figure 10: Correlation analysis between cell viability and LC3II/Bax. $r = -0.979$, $p < 0.05$. The greater the ratio, the lower the cell viability.

increasing (Figures 4(a) and 5(a)), and the apoptosis fraction in high concentration groups (80 $\mu$g/ml curcumin for PANC1 cells and 10 $\mu$g/ml for BxPC3 cells) was significantly higher than that in the control groups (Figures 4(b) and 5(b)). Furthermore, the expression of proapoptosis protein Bax was significantly upregulated in high concentration groups (80 $\mu$g/ml for PANC1 cells and 10 $\mu$g/ml for BxPC3 cells), and the expression of antiapoptosis protein Bcl2 was downregulated in the same groups (Figure 6), demonstrating that curcumin promoted apoptosis of PCa cells.

Autophagy is a self-help process that provides cells with necessary amino acids by degrading damaged organelles or proteins against harsh conditions, stress, or hypoxia conditions [34]. Recent findings suggest that curcumin can induce autophagy to suppress proliferation of cancer cells as a prodeath or inhibitory signal [19, 35, 36]. Few studies have focused on the autophagy of PCa cells by curcumin. In this study, we demonstrated that curcumin could promote autophagosome formation in PCa cells (Figures 7 and 8). Then, curcumin was found to obviously upregulate the expression of LC3II protein (Figure 9) and downregulate the expression of mTOR protein (Figure 9). These results demonstrate that curcumin could induce the autophagy of PCa cells.

Finally, we compared the relative intensities of apoptosis and autophagy in different concentrations of curcumin by quantifying the Bax and LC3II protein expression (Table 1). We found that there was a negative correlation between cell viability and the LC3II/Bax ratio (Figure 10). We therefore speculated that autophagy-related cell death may play a key role in the high concentration of curcumin, and further experiments need to be carried out to test it. The signaling pathways and molecules of apoptosis and autophagy are widely interconnected, and many efforts have also been made to illustrate the interplay between them [37, 38]. When the level of stimulation is low, the process of apoptosis is instructed to occur and autophagy is also induced to prosurvive at the same time. From this point, there may be a balance between them, and we can regulate their interactions to achieve the desired results someday.

In conclusion, we discovered that curcumin effectively inhibited the proliferation of PCa cells by acting on different molecular mechanisms, including arresting PCa cells at G2/M phase, and inducing apoptosis and autophagy. Although curcumin is a monomer, it has a very wide range of effects, which may be one of the reasons for its high anticancer efficacy. Curcumin, therefore, is a potent multi-targeted suppressor of PCa cell viability and may become a novel therapeutic candidate for PCa. Based on the finding of the present study that curcumin could induce apoptosis and autophagy, our next work will focus on the underlying mechanisms.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

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