# Natural Bioactives in Cancer Treatment and Prevention

Guest Editors: Yih-Shou Hsieh, Shun-Fa Yang, Gautam Sethi, and Dan-Ning Hu

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Contents

Editorial

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Natural bioactives are generally referred to the compounds exclusive of essential nutrients that have specific biological activity to human. From several decades ago to now, cancer continues to be the leading lethal cause worldwide. Studies have shown that natural phytochemicals derived from certain plants have the capability to prevent carcinogenesis. In this special issue, we collected numerous studies which provide novel evidence to support the opinion. For instance, epigallocatechin gallate inhibits migration of human uveal melanoma cells; marine sponge *Hyrtios* sp. extract induces apoptosis in human colorectal carcinoma RKO cells with different p53 status; Andrographolide induces apoptosis of C6 glioma cells via the ERK-p53-caspase 7-PARP pathway; and osthole induces human colon cancer cell death and inhibits migratory activity.

We also collected some review articles in this special issue. A paper evaluated the cancer therapeutic potential of cardiac glycosides. A paper proposed vitamin A as the potent anticancer agent on targeting cellular retinol binding proteins. Three other papers addressed the anticancer molecular mechanisms of betulin, Goniothalamin, and Zerumbone. In summary, it is therefore believed that the appropriate application of natural bioactives should be a supplementary and safe way that enhances the efficacy of cancer therapy.

Yih-Shou Hsieh
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Review Article

Vitamin A, Cancer Treatment and Prevention: The New Role of Cellular Retinol Binding Proteins

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Retinol and vitamin A derivatives influence cell differentiation, proliferation, and apoptosis and play an important physiologic role in a wide range of biological processes. Retinol is obtained from foods of animal origin. Retinol derivatives are fundamental for vision, while retinoic acid is essential for skin and bone growth. Intracellular retinoid bioavailability is regulated by the presence of specific cytoplasmic retinol and retinoic acid binding proteins (CRBPs and CRABPs). CRBP-1, the most diffuse CRBP isoform, is a small 15 KDa cytosolic protein widely expressed and evolutionarily conserved in many tissues. CRBP-1 acts as chaperone and regulates the uptake, subsequent esterification, and bioavailability of retinol. CRBP-1 plays a major role in wound healing and arterial tissue remodelling processes. In the last years, the role of CRBP-1-related retinoid signalling during cancer progression became object of several studies. CRBP-1 downregulation associates with a more malignant phenotype in breast, ovarian, and nasopharyngeal cancers. Reexpression of CRBP-1 increased retinol sensitivity and reduced viability of ovarian cancer cells *in vitro*. Further studies are needed to explore new therapeutic strategies aimed at restoring CRBP-1-mediated intracellular retinol trafficking and the meaning of CRBP-1 expression in cancer patients' screening for a more personalized and efficacy retinoid therapy.

1. Retinol and Derivatives

1.1. Metabolism of Retinol and Its Derivatives. Vitamin A can be acquired from the diet either as preformed vitamin A (primarily as retinyl ester, retinol, and in much smaller amount as retinoic acid) or provitamin A carotenoids (Figure 1). Dietary retinyl esters are converted to retinol within the lumen of the small intestine or the intestinal mucosa and then reesterified to form retinyl ester (RE) within the enterocyte [1]. Provitamin A carotenoids, absorbed by the mucosal cells, are converted first to retinaldehyde and then to retinol [1]. After secretion of the nascent chylomicrons into the lymphatic system, the bulk of dietary vitamin A is taken up by hepatocytes and hydrolyzed again. The free retinol binds the epididymal retinoic acid binding protein (ERABP) and the retinol binding protein (RBP) [2] and into plasma transthyretin. Free retinol can be transferred to hepatic stellate cells for storage. Hepatocytes and hepatic stellate cells are very rich in retinyl ester hydrolases and in cellular retinol binding protein type 1 (CRBP-1). CRBP-1 is necessary to sovlubilize retinol in the aqveous environment of the cell [1].

1.2. Intracellular Trafficking of Retinoids. A cell-surface receptor named *stimulated by* retinoic acid 6 (STRA6) mediates vitamin A uptake from RBP [3]. Intracellular retinoid bioavailability is regulated by the presence of specific cytoplasmic retinol and retinoic acid binding proteins, CRBPs and CRABPs (Figure 2). In the cytoplasm vitamin A and derivatives are bound to cytoplasmic proteins: cellular retinol binding proteins (CRBPs) which comprised four isoforms, CRBP-1 and CRBP-2 and CRBP-3 and CRBP-4. CRBP-1, are the most represented isoform in many tissues. Cellular retinoic acid binding proteins (CRABPs) comprised two isoforms, CRABP-1 and CRABP-2. CRBPs specifically bind retinol, while CRABPs and well-characterized members of the fatty acid binding proteins (FABPs) bind retinoic acid
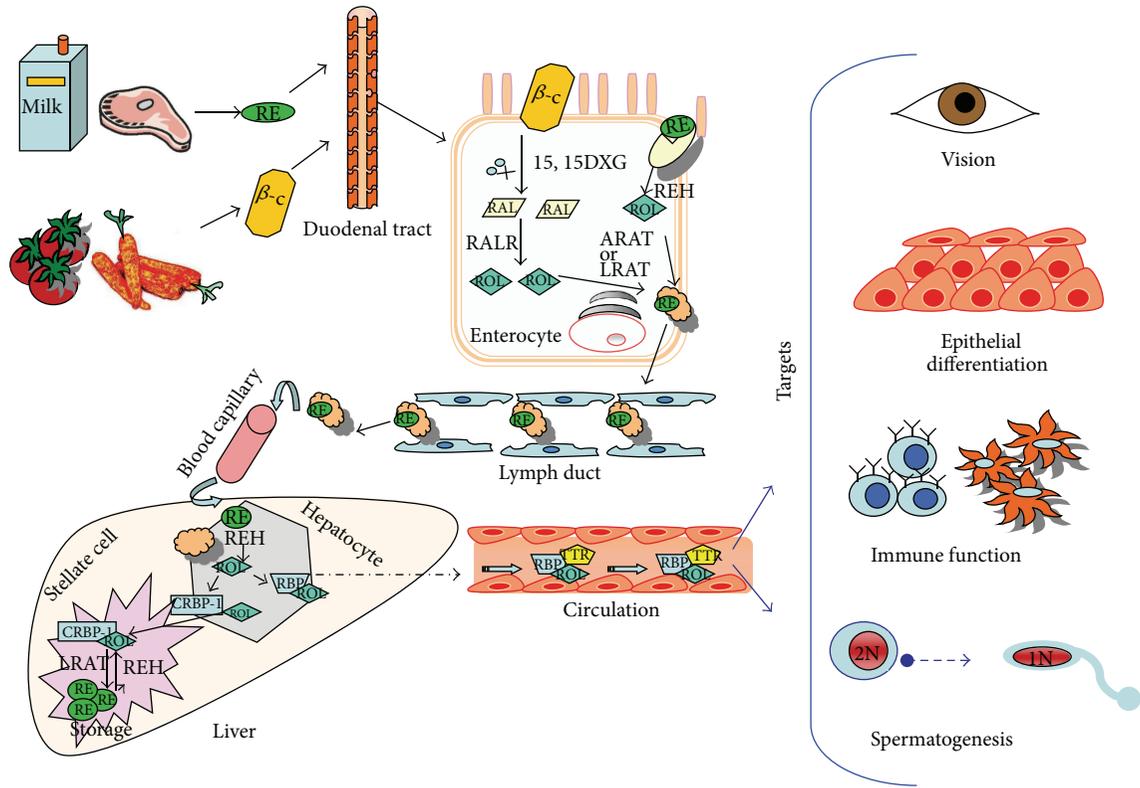
Figure 1: Absorption, transport and distribution of dietary retinoids.
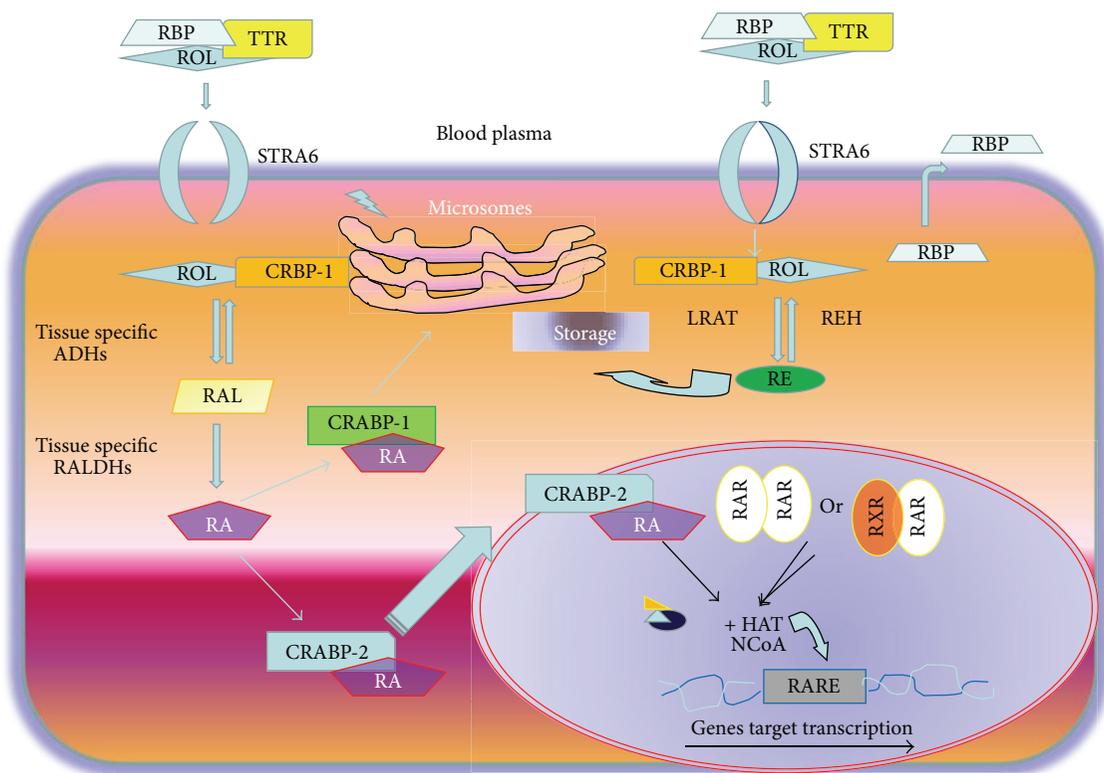
Figure 2: Intracellular retinoid pathways.

(RA). These proteins control the availability of ligands and determine the physiological response of cells and tissues to vitamin A [4]. Cellular retinoic acid binding proteins may regulate the interactions between retinoic acids and their nuclear receptors by regulating the concentration of present retinoic acids [5]. Retinoids can activate gene expression by specific nuclear retinoid acid receptors. Two distinct classes of nuclear proteins, the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs) have been identified. Each class consists of α, β, and γ subtypes. RARs and RXRs form either homodimers or heterodimers and function as transacting nuclear transcriptional factors [6]. RAR can be activated by both all-trans and 9-cis RA, whereas RXR is only activated by 9-cis-RA. Heterodimerization of retinoid receptors is essential for the biological activity, although it should be noted that RXRs can heterodimerize with numerous other non-RA associated nuclear receptors to mediate alternative signaling pathways. In the presence of retinoids, nuclear receptors bind to their respective response elements RAREs and RXREs in regulatory regions of target genes and modulate gene transcription [7].

1.3. Retinoids, Tissue Development, and Differentiation.

Vitamin A and its derivatives are essential for biological processes such as vision, immune function, reproduction, maintenance of epithelial tissue, and differentiation. Vitamin A deficiency causes different pathological consequences such as night blindness, loss of vision, retardation, shortening and thickening of bones, atrophy of the testes, foetal reabsorption, and immunodeficiency [8]. Instead, an excess of vitamin A can cause teratogenic effects including major alterations in organogenesis [9]. So vitamin A is very important during embryonic development and adult tissue regeneration [10]. The best characterized bioactive metabolites of vitamin A are 11-cis retinal and all-trans retinoic acid (ATRA). The 11-cis retinal metabolite mediates photoreception by acting as the visual chromophore. Most of the nonvisual functions of vitamin A are mediated by ATRA, which regulates the expression of specific subsets of genes within target tissues via nuclear receptors. ATRA can be easily detected in many adult and embryonic tissues [11, 12]. A peculiar kind of retinol-storing cell can be found also in the lung [13]. These cells designated lipid interstitial cells are located in the alveolar wall and release ATRA synthesized from retinol into serum-free [13]. The release of ATRA induces changes in gene expression that initiate the formation of alveoli. It is noteworthy that, during alveolar formation, an increased expression of CRBP-1 is found in the pulmonary microvascular endothelial cells [13]. RA is not synthesized during all stages of development, but his production is restricted in a unique spatiotemporal pattern [14]. RA is expressed in a specific anterior-posterior pattern and its expression becomes more restricted during organogenesis [14]. In the adult RA levels are kept by retinol esterification through CRBP-1 in hepatic stellate cells [15]. Moreover, studies with RAR knockout mice indicate that RAR-α contributes to the regulation of alveolus formation after, but not during, the perinatal period [16], whereas RAR-β is an inhibitor of alveolus formation during,

but not after, the perinatal period [17]. These receptors are important as developmental period-specific regulators of alveolus formation [13]. It has been known for more than 60 years that retinoids are involved in regulating the differentiation of various epithelia, promoting the formation of secretory epithelia and inhibiting the formation of highly keratinized, cornified epithelia. They also play a significant role in the control of endometrial growth and differentiation [18]. Given the dramatic shifts in differentiation that occurs in the rodent cervical epithelium from an early secretory phase to a later keratinizing phase, it is likely that retinoids play an important role in modulating the hormonal regulation of cellular differentiation in this tissue; the expression of retinoid binding proteins and nuclear receptors in a given tissue or cell type should be predictive of retinoid responsiveness itself. During pregnancy, in addition, spatial variations in the presence of cellular retinoid binding proteins have been observed, together with differences in patterns of expression between the functionally distinct upper and lower uterine segments [19]. Temporal variations were found with patterns of expression evolving through the three trimesters of pregnancy. This could be important for the regulation of myometrial proliferation *in vivo*. Moreover, RA is known to inhibit the binding of activator protein 1 to the cyclic AMP response element in the COX-2 promoter [20], so inhibiting phorbol-ester mediated induction of COX-2 gene transcription, whose intracellular levels consequently fall, reducing also prostaglandin production [20]. This process in human myometrium during pregnancy means that RA could contribute to the maintenance of uterine quiescence [19].

1.4. Retinoids and Vascular Pathology.

Retinoids target numerous genes implicated in the pathological processes after vascular injury. The latter involves proliferation, differentiation, and migration of medial smooth muscle cells (SMCs) as well as matrix deposition [21–23]. Vascular postinjury, dedifferentiation, growth, and migration result in the formation of a neointima [24]. SMCs migrate to the luminal layer of the arterial wall after endothelial injury. Here growth factors and cytokines induce SMCs to enter into active phases of cell cycle [25]. SMCs proliferation plays also a critical role in the development of restenosis after coronary angioplasty and in the development of fatty streaks to the atherosclerotic plaques [23, 26]. A study shows two kinds of retinol metabolism in vascular SMCs of different phenotypes [24] and, more specifically, an increased uptake of retinol in intimal SMCs compared to medial SMCs, together with an increased expression of the retinoid metabolizing enzymes, such as retinol and retinal dehydrogenase. Consequently, an increased production of RA is found in intimal cells while a higher level of CYP26A1, the retinoic acid catabolizing enzyme, is observed in medial SMCs. Intimal SMCs show a dose- and time-dependent growth inhibition when treated with retinol in contrast to medial SMCs, in which retinol has a mitogenic effect. Moreover, intimal SMCs are more sensitive to RA than medial SMCs [22] and propyonil-L-carnitine increased apoptosis [27, 28]. In fact, systemic administration of RA significantly reduces arterial intimal thickening (IT)

after endothelial injury *in vivo* [29] and induces apoptosis of CRBP-1 expressing intimal cells *in vitro*, but not of normal media SMCs [22]. Both *in vivo* and *in vitro* studies show that neointimal SMCs also display proinflammatory properties, such as higher expression of tumour necrosis factor-α (TNF-α) [30], so contributing to atherosclerotic process and restenosis. The different retinol metabolism in the two SMCs phenotypes supports the role of retinoids in preventing vascular proliferative disorders [31].

1.5. Retinoids and Cancer Therapy. Epidemiological studies have suggested an inverse correlation between cancer development and dietary consumption of vitamin A. Pharmacological concentrations of vitamin A decrease the incidence of chemically induced experimental tumours [32]. Natural and synthetic retinoids have been demonstrated to inhibit the growth and the development of different types of tumours, including skin, breast, oral cavity, lung, hepatic, gastrointestinal, prostatic, and bladder cancers [33–37]. Moreover, the addition of RA or synthetic retinoids to human cancer cell lines or human tumour xenografts in nude mice result in growth arrest, apoptosis, or differentiation [32]. It is noteworthy that natural retinoids act as chemotherapeutic agents for the treatment of acute promyelocytic leukemia (APL). APL is a subset of acute myeloid leukaemia characterized by uncontrolled expansion of leukemia blast cells, blocked at promyelocytic stage of hematopoiesis in the bone marrow [38]. It is characterized by a reciprocal translocation between the long arms of chromosomes 15 and 17 [$t(15; 17)$] [39–42]. This aberration leads to the fusion between the promyelocytic leukemia (PML) gene located on chromosome 15q21, normally responsible for the formation of the nuclear bodies and regulation of the stem cells self-renewal [43, 44] and the (RAR-α) gene from the chromosome 17q21, with the formation of a chimeric oncoprotein PML-RARA [41, 42, 45]. The fusion transcript is detectable in more than 95% of APL patients with $t(15; 17)$ and acts as an oncogene, causing an enhanced DNA hypermethylation, proliferation, and inhibition of terminal differentiation of hematopoietic cells [46, 47]. ATRA-induced degradation of the fusion product is a basic therapeutic mechanism in APL cells [41, 48]. Recent results reveal several mechanisms leading to the destruction of the fusion oncogene, such as ubiquitination [49], sumoylation [50], or autophagy [51]. High concentrations of ATRA induce postmaturation apoptosis of APL-blasts through the induction of the tumour-selective death ligand TNF-related apoptosis-inducing ligand [52]. Effectiveness of ATRA in the treatment of APL observed in more than 90% of the patients leading to the complete remission [53–55]. Moreover, in animal models several studies establish an inhibitory role of retinoids in breast cancer. It was reported a 52% reduction in the incidence of mammary cancer in animals treated with retinyl acetate [56]. *In vitro* studies show that retinoids, in particular 9-cis-RA, inhibit the growth of oestrogen receptor- (ER-) positive through blocking cell cycle [57], but not ER-negative human breast cancer cells [25, 58]; ER-negative cells have been demonstrated to express lower RAR-β levels compared to their ER-positive matched cells [59] and they

exhibit retinol-induced growth inhibition when transfected with RAR-β [60]. Preclinical studies demonstrate that ATRA induces cell cycle and proliferation arrest in breast cancer cells [61] through the modulation of cyclin-dependent kinase inhibitors p21^{WAF1} and p27^{KIP1}, with dephosphorylation of retinoblastoma protein [62]. Primary brain tumours are among the top ten causes of cancer-related deaths in the USA. Gliomas, in particular, may result from an imbalance in retinoid receptor expression initiated by environmental factors that increase the endogenous production of RA in the glial cells [63]. It is proposed that this imbalance is characterized by excessive expression of RAR-α and reduced expression of RAR-β. The combined use of RAR-α antagonist and RAR-β agonist is suggested as potential new treatment strategy for gliomas, possibly even at a late stage of the disease. According to this hypothesis, the RAR-α antagonist would be expected to inhibit RAR-α-induced gliomas, while the RAR-β agonist would suppress tumour growth and possibly contribute to the regeneration of normal glia [63]. Moreover, vitamin A reduces the induction of carcinoma of the stomach by polycyclic hydrocarbons [64] and vitamin A-deficient rats are more susceptible to induction of colon tumours by aflatoxin B than normal animals [65]. The combination treatment of histone deacetylase inhibitors SL142 or SL325 with retinoic acids exerts a significant antitumour activity and is a promising therapeutic candidate to treat human lung cancer and show antitumour effect in neuroblastoma [66]. While synthetic retinoids are generally promising for cancer treatment, only few of them are FDA-approved or currently undergoing clinical trials for cancer therapy. Preclinical studies show that synthetic retinoids inhibit human cancer growth. Fenretinide (4-HPR) is one of the most promising clinically tested retinoids. 4-HPR demonstrated a significant cytotoxic activity of tumour cells through the induction of apoptotic and nonapoptotic cell death [67] in breast [68, 69], prostate, bladder, skin [52, 70–74], colon-rectal [75], head and neck [76], ovarian cancers [77, 78], both small cell and non-small cell lung cancer [79–81], neuroblastoma [82–84], and leukaemia cell lines [85, 86]. 4-HPR has activity against tumours by generating reactive oxygen species [76, 87, 88], increasing dihydroceramide production [87, 89–91] and natural killer cell activity [92, 93] and inhibiting angiogenesis [89, 94]. TAC-101 has shown efficacy in inhibiting liver tumour growth in particular of hepatocellular carcinoma [95, 96]. Synthetic retinoids, approved from FDA for dermatological purposes, have a potential antitumour activity. Bexarotene is a retinoid that specifically binds retinoid X receptors and has numerous effects on cellular growth and differentiation. It is approved for the treatment of cutaneous T-cell lymphoma both topically and systemically [97, 98]. A preliminary clinical experience suggests tazarotene, a new acetylenic retinoid, as an effective alternative topical treatment of basal cell carcinomas. Tazarotene demonstrated a good efficacy in the therapy of basal cell carcinoma [99, 100].

2. Cellular Retinol Binding Proteins

2.1. The Specific Role of CRBP-1 in Retinol Metabolism. CRBP-1 is a small cytosolic protein of 15 KDa. CRBP-1 is widely

expressed and evolutionarily conserved in many tissues and acts as chaperone protein that regulates the uptake and subsequent esterification of retinol and its bioavailability. CRBP-1 gene is located on 3q21 chromosome. CRBP-1 is required for the efficient synthesis of ATRA [101]. CRBP-1 binds retinol and interacts with enzymes involved in esterification of retinol with long chain fatty acids and in hydrolysis of retinyl esters into retinol. *In vitro*, CRBP-1 also channels either retinol towards microsomal dehydrogenases that catalyze the oxidation of retinol into RAL or its addressing towards cytosolic dehydrogenases for oxidation into RA. Several microsomal enzymes, termed retinol dehydrogenases types I, II, and III, have high specificity for CRBP-1-bound retinol. Retinal dehydrogenases oxidize CRBP-1-bound retinal [102]. Retinal-CRBP-1 formed through oxidation of retinol-CRBP-1 by the microsomal retinol dehydrogenase might be directly oxidized to RA by retinal dehydrogenases. CRBP-1 may also delivery retinol to newly synthesized RBP for secretion from the liver into the circulation. In the mouse, CRBP-1 deficiency decreases the capacity of hepatic stellate cells to take up incoming retinol and to maintain RE stores, because of an accelerated rate of RE turnover, indicating that CRBP-1 is essential for efficient retinol storage during postnatal life [4].

2.2. Role of CRBP-1 during Development. It has been shown that a lack or an excess of RA during embryonic development results in congenital abnormalities. As a matter of fact, RA regulates genes that specify body axis pattern and may help to program limb formation in the developing embryo. In mature vertebrates, RA maintains epithelial tissues, contributes to bone remodelling and sustains reproductive processes, such as the oestrous cycle, spermatogenesis, and placental growth [103]. As RA synthesis previously seemed to involve intricate metabolic pathways, carried out by multiple enzymes recognizing both liganded (holo-CRBP-1) and nonliganded forms (apo-CRBP-1) of CRBP-1, the role of this protein in organ development has deserved to be deepened. So in mice, the absence of CRBP-1 does not show to be life-threatening during development, at least under conditions of maternal vitamin A dietary sufficiency [4]. However, retinol and RE contents were lower in CRBP-1−/− embryos and foetuses than in WT [4]. Researchers hypothesized that this depends on a reduced transfer of retinol from the maternal circulation to the foetus through the placenta, where it is normally expressed, as CRBP-1 enhances retinol uptake by placental membranes *in vitro* [104]. It could also depend on an altered retinol uptake by the foetal cells themselves [4]. As a matter of fact, tritiated retinol, administered to pregnant WT mice, specifically accumulates in regions of the embryo expressing CRBP-1 [105]. These lower ER contents could finally depend on a reduced RE half-life, as in adult liver [106] or to an impaired delivery of retinol to specific enzymes for esterification. The normal embryonic development of CRBP-1 null mice suggests that, even though involved in RA homeostasis, CRBP-1 is not critically required for development as its ablation does not change, at least under conditions of maternal vitamin A sufficiency, the expression of RA-target genes during prenatal life. Even if no embryonic,

foetal, or placental abnormality could be detected in CRBP-1-null mice (indicating the dispensability of this protein during intrauterine development), CRBP-1-null mice, fully exhausted their RE stores within 5 months and developed abnormalities characteristic of postnatal hypovitaminosis A, as also described in rats [107], and, namely, consisted in vision defects, testicular degeneration, and squamous keratinizing metaplasia. The decrease capacity to store incoming retinol in CRBP-1 null liver could depend on a higher hydrolysis of hepatic stores, due to a decrease of RE half-life and higher amounts of retinol in blood. This hydrolysis could be attributed to an impaired delivery of retinol to esterifying enzymes, above all LRAT, that requires CRBP-1-bound retinol as a substrate [108]. The morphological appearance of CRBP-1 null testes after 14 weeks under the vitamin A deficient diet represents a clear phenocopy of the RAR-α-null mutation, while the squamous keratinizing metaplasia observed in CRBP-1-null mice fed with the vitamin A deficient diet is similar to those observed in old adult mice lacking RAR-γ [109].

2.3. Expression of CRBP-1 in Normal and Adult Tissues. CRBP-1 expression has been studied at histological distinct stages of the rat oestrus cycle. CRBP-1 was detected during the early proliferative phase and after the formation of a mucinous cell layer during proestrus [18]. CRBP-1 expression closely follows the state of differentiation of the cervical epithelial cells. High levels were found in both columnar cells and incompletely differentiated epithelial cells [110]. Decreased CRBP-1 expression coincides with the loss of retinol responsiveness in rat cervical epithelial cells [18], whileCRBP-1 expression was higher in the atrophied epithelium and both CRBP-1 mRNA and protein levels decreased when animals were treated with oestrogen. During proestrus high CRBP-1 expression indicates that high local levels of retinol or retinoic acid are either directly required for the formation of these secretory cells or indirectly required to inhibit keratinization. CRBP-1 levels subsequently decline and the epithelium extensively keratinizes, this might limit retinol uptake and metabolism [18]. It is worth noting that the pattern of CRBP-1 expression in the human endocervical epithelium is identical to that reported for the rat [111]. Moreover, in human, nonpregnant myometrium CRBP-1 is easily available, together with the CRABP proteins, thus suggesting a role for ATRA in the control of myometrial proliferation *in vivo*, but CRBP-1 is downregulated in both the upper and the lower uterine segments during the first and the second trimester of pregnancy [19]. By the end of the third trimester, CRBP-1 is upregulated in upper segment myometrium, together with CRABP-2 [19]. CRBP-1 expression characterizes endometrial stromal cells at eutopic and ectopic sites and appears to be more specific than CD10 [112]. The level of CRBP-1 varies in intensity according to hormonal variations, with an increase during the secretory phase compared with those of proliferative endometrium. The highest CRBP-1 immunodetection was observed in predecidual and decidual tissue. CRBP-1 expression in the endometrial stroma was similar to that of CD10. Thus, immunodetection of CRBP-1 may help to elucidate

the physiopathological changes which occur in endometrial stroma and can also be applied as an adjuvant stromal marker [112].

2.4. Role of CRBP-1 in Tissue Remodelling.

One of the key events in the wound repair process is the infiltration of fibroblasts into the damaged area where they proliferate and differentiate into myofibroblasts expressing features of SMCs. These fibroblasts present in the granulation tissue originate from subcutaneous tissue fibroblasts [113]. CRBP-1 expression strongly increased during wound healing in rat skin, suggesting that it plays a role in the evolution of granulation tissue [114]. Liver myofibroblasts derived from hepatic stellate cells or from portal fibroblasts express CRBP-1. HSC express CRBP-1 both in normal liver and during liver fibrosis, as these cells are involved in myofibroblast differentiation [115]. Aberrant CRBP-1 expression has shown to be accompanied by parallel variations of α-smooth muscle actin. The α-smooth muscle actin is mainly controlled by TGF-β1, a fibrogenic mediator involved in both myofibroblast differentiation and extracellular matrix deposition. Surprisingly, TGF-β1 induces CRBP-1 gene and protein expression in primary cultures of HSC and in portal fibroblasts. The myofibroblast differentiation of HSC is associated with a decrease in total hepatic levels of retinyl palmitate [116], the predominant storage form of vitamin A in rat HSC, whereas levels of retinol are increased together with CRBP-1 levels [117]. So, it is possible that the stable expression of CRBP-1 in HSC during liver fibrosis is under the control of free retinol level [115]. The pattern of CRBP-1 expression in portal fibroblasts is similar to that observed in subcutaneous fibroblasts or in arterial SMCs that, after injury, rapidly acquire CRBP-1 expression [118]. The expression of CRBP-1 in arterial SMC during arterial repair, in myofibroblasts during skin wound healing and in portal fibroblasts during liver reaction to bile duct ligation, strongly supports that CRBP-1 plays a role in multiple tissue repair phenomena [118]. As a matter of fact, distinct rat aortic SMC subpopulations and clones express CRBP-1 and a CRBP-1-containing SMC subpopulation appears transiently *in vivo* during the evolution of the experimental aortic IT produced by endothelial injury. CRBP-1 expression in cultured SMCs has shown to be regulated by ATRA and retinol. Moreover, the presence of CRBP-1 in cultured SMCs appears to be correlated with the expression of cytokeratin 8 [119]. CRBP-1 has also shown to be constitutively expressed in aortic endothelial cells and, occasionally, in SMCs of the normal media of adult and old rats but not in newborn rats [118]. It is conceivable that the scattered CRBP-1-positive SMCs of the media participate in the formation of IT after endothelial injury and a proportion of CRBP-1-negative SMCs becomes positive, as the large majority of the IT SMCs express CRBP-1 at 7 days. These results suggest that a subset of medial SMCs becomes rapidly CRBP-1 positive after injury, undergoes replication during the early phases of IT development, and then disappears, possibly through apoptosis, when reendothelialization takes place. So, CRBP-1 is not simply a marker of SMC activation but participates in this process [118].

2.5. CRBP-1 and Cancer Progression: New Therapeutic Perspectives.

Because of the complexity of vitamin A metabolism, the precise role of CRBP-1 in retinoid signaling remains controversial, despite numerous studies conducted over the past three decades on its binding properties, three-dimensional structure, tissue localization, regulation of expression, involvement in retinal metabolism, and null mutation [120]. The function of the CRBP-1 gene in controlling the cell bioavailability of vitamin A suggests that it can have a special relevance in the inhibition of early steps of cancer transformation. Nevertheless, in human cancer, the presence and role of the specific binding proteins for retinol and RA have not been extensively investigated. Downregulation or loss of CRBP-1 expression occurs in a series of tumors: breast, ovarian, endometrial, prostate, renal cancer, astrocytic gliomas [112, 121, 122], cervical cancer, larynx cancer, nasopharyngeal carcinoma, lymphoma, and gastrointestinal carcinomas [123]. Furthermore, the CpG island hypermethylation of CRBP-1 is responsible for its inactivation in some cancer cell [124–127]. So, epigenetic disruption of CRBP-1 is a common event in human cancer and may have important implications for cancer prevention and retinoid therapy. What are the biological consequences of the methylation-mediated silencing of CRBP-1? The loss of CRBP-1 may compromise retinoic acid metabolism by reducing retinol transport and blocking the formation of retinyl esters and RAR activity, leading to loss of cellular differentiation and tumor progression [123, 128–130]. CRBP-1 downregulation was observed in stage I as well as in stage II and III patients and also reported in ovarian cancer precursors, suggesting that interruption of CRBP-1 signaling may occur at all stages of cancer progression [120]. Several studies highlighted the role of CRBP-1 signaling in cancer progression during the last years [131], but the mechanisms by which it affects carcinogenesis are far from being fully elucidated. Uterine and gastrointestinal leiomyosarcomas express high levels of CRBP-1, whereas its expression is weak in leiomyoma, symplastic leiomyoma, borderline tumours, and nontumour smooth muscle tissue [27]. Accumulation of CRBP-1 in leiomyosarcoma likely supports the conversion of retinol into RA and its biological effects through RAREs induction, which influence the expression of many genes through the interaction with RAREs regions, inducing the increase of CRBP-1 expression in epithelioid SMCs as well as fibroblasts [29]. These results support the possibility that the expression of CRBP-1 represents a target for pharmacological strategies aimed at influencing sarcoma growth through the control of RA availability. About 30% of colorectal cancers did not present any apparent lesion in the retinoid pathway and this subset of tumors may be extremely sensitive to treatment with retinoids [123]. With regard to liver pathologies, CRBP-1 expression is downregulated in almost all hepatocellular carcinoma specimens [132]. Some studies show that CRBP-1 is strongly expressed in the cytoplasm of hepatic stellate cells in normal liver and in myofibroblasts, with only low CRBP-1 levels in hepatocytes. Patients with high CRBP-1 expression in myofibroblasts show a significantly higher 2-year survival as compared with patients with low CRBP-1 expression [132]. The loss of CRBP-1 expression in intratumoral myofibroblasts likely disturbs retinoid homeostasis, thereby potentially
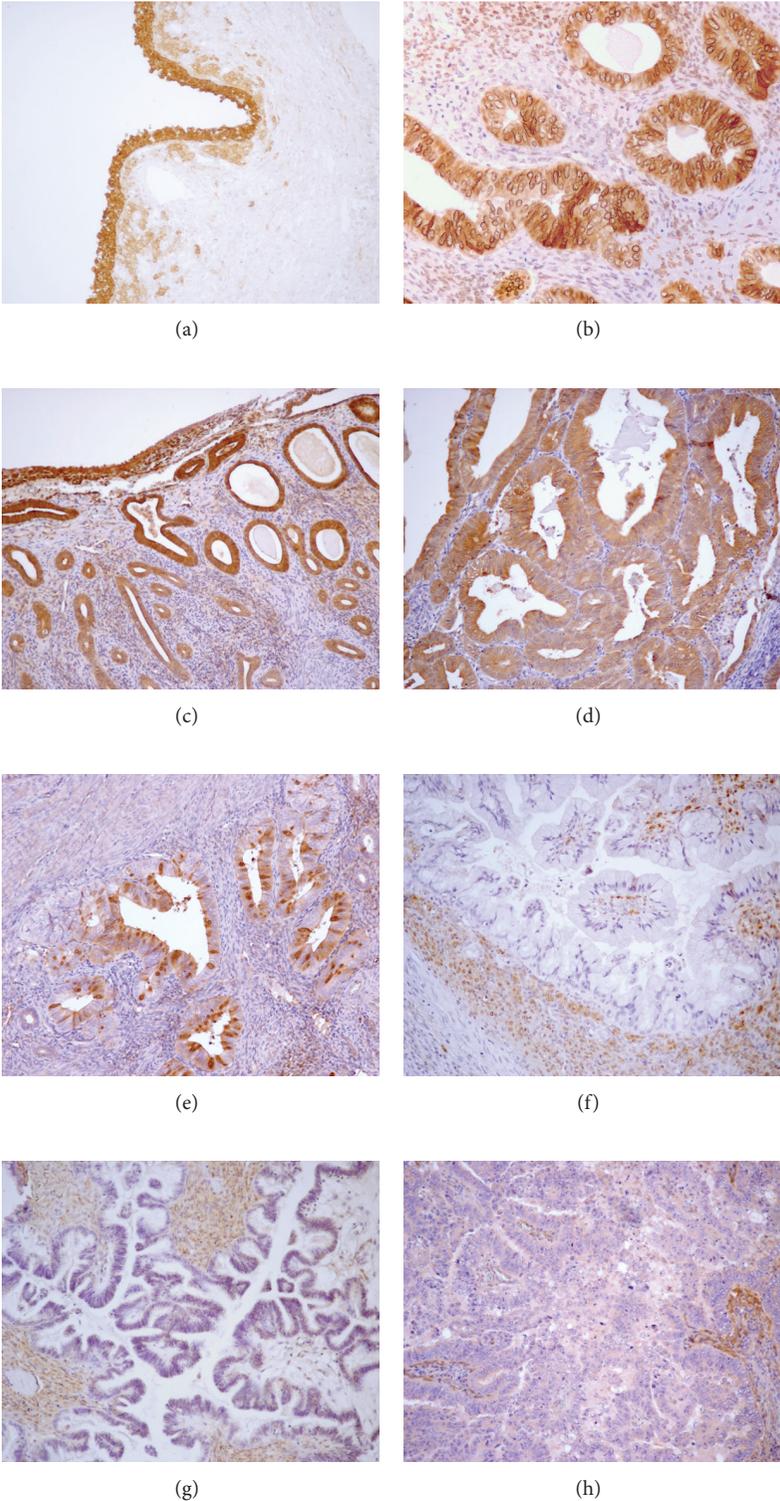
FIGURE 3: Immunohistochemical expression of CRBP-1 in normal and neoplastic female reproductive system tissues. (a) CRBP-1 is strongly expressed in normal endocervical epithelial cells, (b) proliferative and (c) atrophic endometrial glandular cells. (d) Well-differentiated (G1) endometrial carcinoma cells show strong and diffuse CRBP-1 positive cells compared to (e) lower and focal CRBP-1 expression in moderately (G2) carcinoma. In the ovary, CRBP-1 is not expressed in (f) mucinous, (g) serous borderline, and (h) well-differentiated (G3) serous carcinoma. Original magnification: (a), (c)–(h) 100x; (b) 200x.

(a)

(b)

(c)
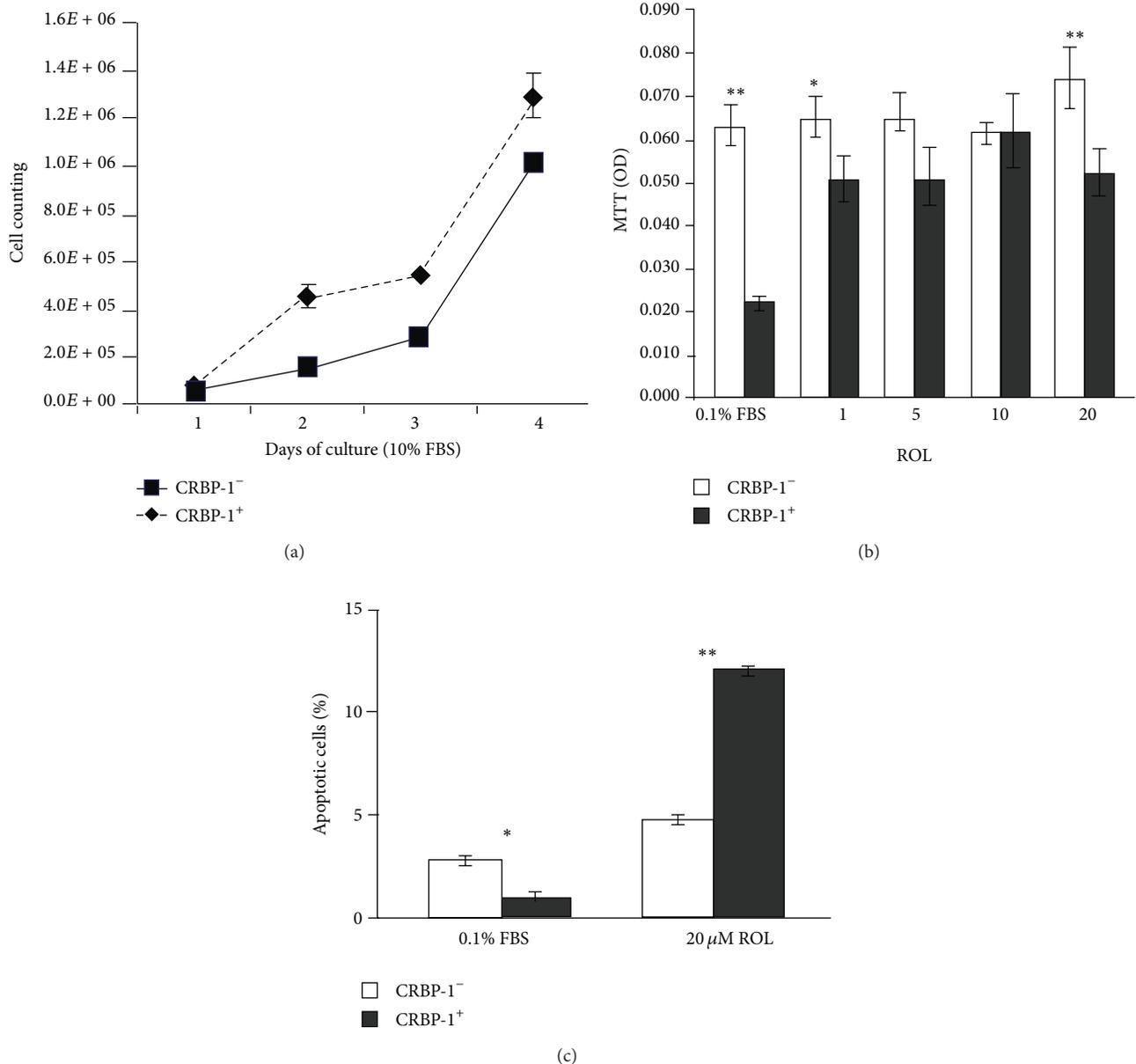
FIGURE 4: CRBP-1$^+$ expression influences retinoid-induced A2780 cancer cell viability. (a) Viability of transfected CRBP-1 (CRBP-1$^+$) A2780 cells increased after 4 days in the presence of 10% FBS compared to empty transfected (CRBP-1$^-$) A2780 cells. (b) Retinol induces a reduction of viability in CRBP-1 A2780 cells after 24 h of different ATRA treatments (c) Flow cytometry analysis of Annexin V/PI apoptotic assay shows a higher percentage of dying cells in CRBP-1$^+$ after 2 h of 20 μM retinol treatment compared to CRBP-1$^-$ A2780 cells. Values are expressed as means ± SEM of three different experiments; $^*P < 0.05$, $^{**}P < 0.01$.

leading to more aggressive liver tumour growth. These studies also highlight a strong positive association between nuclear CRBP-1 expression and nuclear β-catenin accumulation [132, 133]. This cotransactivation complex exerts several regulatory effects on a growing number of downstream target genes, many of which have been implicated in tumorigenesis [134]. In a case-controlled CRBP-1 study in gynaecological cancers, significant differences were found between the concentrations of CRBP-1 in the dysplastic cervical lesions and the normal cervix [122]. CRBP-1 was detected at a reduced

level in the carcinoma poorly differentiated of endometrium, ovary (Figure 3), and breast compared with normal tissue aliquots. CRBP-1 immunodetection in simple hyperplasia was weakly and similar to that of proliferative endometrial cells and increased in atypical hyperplasia and in G1 endometrioid carcinomas [122]. Importantly, a progressive decrease of CRBP-1 immunoreactivity was observed in less differentiated endometrial tumours [122]. The striking overall difference in the expression of CRBP-1 in type I and type II endometrial carcinomas reflects the differences in their risk factors and

molecular pathogenesis [122]. The low or absent CRBP-1 expression in serous and clear cell carcinomas further supports the presence of distinct molecular carcinogenetic pathways [122].

It has been documented that CRBP-1 upregulation exerts a direct antitranscriptional effect through the binding of CRBP-1 promoter to RAR-α [135]. *In vitro* studies confirmed the downregulation of RAR-α and RAR-γ mRNA levels in CRBP-1-stable-transfected ovarian cancer cells [127]. Abnormal RAR-α upregulation characterizes other malignancies, such as lung cancer and metastatic melanoma [121]. Similarly, RAR-γ upregulation is reported to be prooncogenic and to support the growth/progression of mammary tumours [136]. So, CRBP-1-mediated reactivation of retinol pathways could improve the efficacy of adjuvant retinoid chemotherapy, likely increasing apoptotic susceptibility [127, 137–139] (Figure 4).

Furthermore, CRBP-1 induces a reduced transcription level and activity of PI3K/Akt pathway, in particular, Akt1 [127]. Although Akt contributes to maintain differentiation of stem cells [140], aberrant pAkt activation sustains cancer progression [127]. CRBP-1 signaling restoration in A2780 ovarian cells induces the downregulation of specific cell proliferation and survival genes STAT1, STAT5, and JUN [127, 141] and downregulation of pErk. The latter is critically involved in the regulation of cell proliferation and survival and its upregulation promotes cancer proliferation, survival, and metastasis [142]. CRBP-1 can have a function independent of its retinol binding ability; in this regard, CRBP-1 also functions in mammary epithelial cell inhibition of the phosphatidylinositol 3-kinase/Akt survival pathway and suppresses anchorage independent growth [121]. Interestingly, somatic CRBP-1 silencing, induced by inoculation of athymic mice with MTSV cells, an SV40-immortalized human mammary epithelial cell line, transfected with empty vector (MTSV^{vector}) or CRBP-1 (MTSV^{CRBP-1}), has shown to prevent tumor cells from taking up, storing, and using retinol *in vivo* [137]. These *in vivo* data suggest that somatic CRBP-1 loss results in a local deficit in vitamin A storage and metabolism, with important consequences for the affected tissues.

Reintroduction of CRBP-1 signaling reduced tumorigenicity both *in vivo* and *in vitro*, inducing downregulation of survival and proliferative gene pathways and increases retinoid-mediated apoptosis [127, 137, 143]. So, CRBP-1 can represent a potential target for therapeutic strategies aimed at arresting cancer cell growth and tumour progression by increasing intracellular retinol bioavailability [127, 137]. In addition, the presence of detectable CRBP-1 level in a subset of ovarian cancers [127] suggests screening of its expression for a more efficacy and personalized adjuvant retinoid-mediated therapy.

3. Conclusions

Vitamin A and derivatives comprise a group of natural and synthetic molecules, which regulate a variety of essential biological processes during normal development, maintained tissue homeostasis, and also mediate protection from diseases. Retinoids have many important and diverse functions throughout the body including roles in vision, regulation of cell proliferation and differentiation, growth of bone tissue, immune function, and activation of tumour suppressor genes. Genomic functions of the retinoids are mediated via their nuclear DNA-binding receptors, RARs, and RXRs, which regulate gene transcription through recruitment of corepressors and coactivators. Natural and synthetic retinoids have been used as potential chemotherapeutic or chemopreventive agents because of their differentiation, antiproliferative, proapoptotic, and antioxidant effects. The function of the CRBP-1 gene in controlling the availability of retinol to cells suggests that its product has special relevance to inhibition of early steps in transformation. CRBP-1 downregulation occurs in breast and ovarian tumors and compromises RAR activity, leading to loss of cellular differentiation and tumor progression. Furthermore, the CpG island hypermethylation of CRBP-1 is responsible for its inactivation in some cancer cell lines such as cervical, larynx, nasopharyngeal, and gastrointestinal carcinomas and lymphoma [123, 128–130]. So, the loss of CRBP-1 expression is a common event in human cancer that may have important implications for cancer prevention and treatment using retinoids. The possibility to reintroduce CRBP-1-mediated intracellular retinol trafficking in cancer cells can represent a potential tool in strategies aimed at counteracting cancer cell dedifferentiation and minor aggressiveness. Moreover, variability of CRBP-1 expression in some cancers also suggests screening of tumours in order to select patients potentially sensitive to adjuvant retinoid therapy.

Abbreviations

STRA6:	Stimulated by retinoic acid 6
RE:	Retinyl ester
RBP:	Retinol binding protein
REH:	Retinyl ester hydrolases
CRBP-1:	Cellular retinol binding protein 1
RARs:	Retinoic acid receptors
RXRs:	Retinoid X receptors
RAREs:	Retinoic acid response elements
RXREs:	Retinoid X receptor response elements
RA:	Retinoic acid
ATRA:	All-trans retinoic acid
CRABP-1 and CRABP-2:	Cellular retinoic acid binding proteins 1 and 2
FABPs:	Fatty acid binding proteins
TNF-α:	Tumour necrosis factor-α
APL:	Acute promyelocytic leukemia
PML:	Promyelocytic leukaemia
ER:	Oestrogen receptor
RAL:	Retinaldehyde
SMC:	Smooth muscle cells
TGF-β1:	Transforming growth factor-β1
IT:	Intimal thickening
LEF:	Lymphoid enhancer factor
TCF:	T-cell factor
4-HPR:	Fenretinide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Comprehensive Review on Betulin as a Potent Anticancer Agent

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Numerous plant-derived substances, and their derivatives, are effective antitumour and chemopreventive agents. Yet, there are also a plethora of tumour types that do not respond, or become resistant, to these natural substances. This requires the discovery of new active compounds. Betulin (BE) is a pentacyclic triterpene and secondary metabolite of plants abundantly found in the outer bark of the birch tree *Betulaceae* sp. BE displays a broad spectrum of biological and pharmacological properties, among which the anticancer and chemopreventive activity attract most of the attention. In this vein, BE and its natural and synthetic derivatives act specifically on cancer cells with low cytotoxicity towards normal cells. Although the antineoplastic mechanism of action of BE is not well understood yet, several interesting aspects of BE's interactions are coming to light. This review will summarize the anticancer and chemopreventive potential of BE *in vitro* and *in vivo* by carefully dissecting and comparing the doses and tumour lines used in previous studies, as well as focusing on mechanisms underlying its activity at cellular and molecular level, and discuss future prospects.

1. Introduction

Epidemiological data indicated an increase in the cancer incidence and mortality. According to the GLOBOCAN 2008 estimations, there have approximately been 12.7 million new cancer cases diagnosed and 7.6 million deaths worldwide in 2008 [1]. Furthermore, it has been also prognosed that cancer will exceed heart diseases as the leading cause of death in the world, entailing serious social and economic consequences [2]. Despite the significant development of new surgical techniques, radio-, chemo-, and targeted therapy, failures in tumour treatment are still the most important challenges to oncology [3]. The current radio- and chemotherapy procedures also result in the damage of normal cells and consequently cause a number of serious side effects. Additionally, the acquired drug resistance by tumour cells is considered to be responsible for the failure of conventional types of oncological therapy, including cytostatic drugs and radiation [4]. A novel approach to the cancer treatment

has appreciated the key components of specifically altered signalling pathways in neoplastic cells or targeting of the tumour microenvironment without affecting noncancerous cells.

The use of natural plant-derived compounds has been considered to be an interesting aspect for the treatment of human neoplastic diseases. Natural plant-derived substances, relatively easily available due to their commonly occurrence in the nature, seem to constitute a promising group of anticancer or chemopreventive agents and have played a key role in the development of drugs or supplements for the treatment of several human cancers. Of all commercially offered anticancer drugs between 1981 and 2006, no more than 22.2% of the total number have been categorized as synthetic ones [5–7].

The most applicable anticancer drugs derived from plants being in clinical use are taxanes (including paclitaxel isolated from *Taxus brevifolia* Nutt., *Taxaceae*) [8] and vinca alkaloids (*Catharanthus* alkaloids) (including vinblastine and
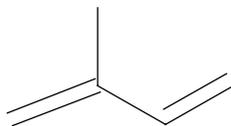
FIGURE 1: Chemical structure of isoprene.
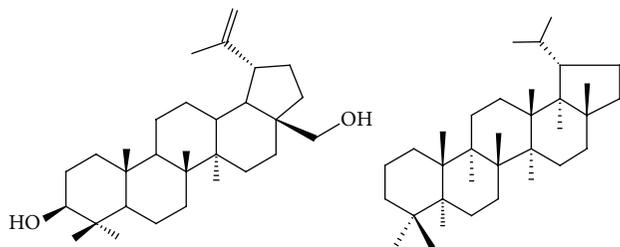
FIGURE 2: Chemical structure of betulin and lupane.

vincristine, isolated from *Catharanthus roseus* (L.) G. Don, *Apocynaceae*) [9]. Moreover, many derivatives of these substances have been synthesized.

Terpenes are a large group of widespread secondary metabolites of plants and are considered as potentially useful in cancer pharmacotherapy, because of their selective cytotoxicity towards numerous human cancer cells, as demonstrated *in vitro* and *in vivo* studies. Triterpenes, one of terpenes classes, are formed from six isoprene units (Figure 1) and occur as complex cyclic structures called triterpenoids [10].

Betulin (BE, 3-lup-20(29)-ene-3β,28-diol), also known as betulinol, betuline, or betulinic alcohol [11], is a pentacyclic lupane-type triterpenoid (Figure 2) naturally distributed in many plants [12, 13]. BE was one of the first natural substances isolated from plants, by Lowitz in 1788, and its chemical structure was finally determined in 1952. Later, BE has been found in other plant species of the *Betulaceae* family, as a component of the outer bark of the birch species, *Betula alba*, *B. pendula*, *B. pubescent*, and *B. platyphylla*. BE has also been found in *Diospyros leucomelas*, *Zizyphus mauritiana*, *Nelumbo nucifera*, seeds of *Ziziphus vulgaris* var. *spinosus*, and in the bark of *Trochodendron aralioides*. BE is predominantly found in a content between 10 and 30% [14], through 34% of dry weight of bark from white birch [15] or even over 50% in the birch bark extract from *B. pendula* Roth [16] and yellow birch (*B. alleghaniensis Britton*) in the Quebec region in Canada [17]. Chemical composition of the birch bark extracts is strongly linked to preparation and purification methods used and influences the percentage of BE which can vary from 54% to 82% of dry weight [16].

Numerous studies have demonstrated that BE elicits a broad range of biological and pharmacological properties, including antibacterial, antifungal, and antiviral activities. However, the anticancer and chemopreventive potential of BE are the focus of most attention [11].

2. BE Inhibits Proliferation and Invasion of Different Types of Cancer

BE has been shown to elicit anticancer properties by inhibiting cancer cells growth. Cytotoxicity and antiproliferative potential of BE have been studied in several established cancer cell lines, as well as primary tumour cell cultures (Tables 1 and 2 and references therein) and cancer xenograft models.

Furthermore, there are some data reporting antitumour potential of tropical plants-derived BE, suggesting that *Betulaceae* species may not be the only origin of biologically and pharmacologically active BE. It is considered that BE elicits antiproliferative and cytotoxic activity irrespectively of the natural source. BE isolated from *Chaenomeles sinensis* KOEHNE has had an inhibitory effect (with the IC$_{50}$ 20.9 μM) on soft agar colony formation induced by TPA (12-*O*-tetradecanoyl phorbol-13 acetate) in mouse epidermal cells (JB6 Cl 22, Cl 41 cells) [18], whereas BE from the twigs of *Celtis philippinensis* inhibited proliferation of lung cancer cells [19], and BE from the roots of *Belamcanda chinensis* (L.) DC was effective against breast, prostate, and stomach cancer cells [20]. Likewise, BE from the twigs of *Coussarea paniculata* decreased proliferation of human ovarian carcinoma cells [21], whereas BE from *Cyrtomium fortumei* (J.) inhibited growth of human prostate and stomach cancer cell lines [22].

BE has exhibited quite a different range of its antiproliferative activity, depending on cancer cells type, from a weak inhibition of cell proliferation in human erythroleukaemia cell line (K562) to a strong inhibition in human neuroblastoma cells (SK-N-AS), where the effect has been most pronounced (Table 1 and references therein). Additionally, BE has also been found to express significant cytotoxicity against primary cancer cells cultures isolated from tumour samples obtained from ovarian, cervical carcinoma, and glioblastoma patients, where the IC$_{50}$ values have ranged from 2.8 to 3.4 μM [23] (Table 2), being significantly lower, when compared with established cell lines [21, 24].

Other studies have shown clearly pronounced effect of BE towards human neural tumour cell lines with the IC$_{50}$ value 10.3 μM in TE671 (rhabdomyosarcoma/medulloblastoma), neuroblastoma cells—2.5 μM in SK-N-AS [23], 17.1 μM in GOTO, and 16.5 μM in NB-1 cell line [25], and glial tumour—5.9 μM in C6 (rat glioma) [23].

It should be mentioned that BE has also elicited significant antiproliferative potential against human thyroid carcinoma FTC 238 cells and the concentration 6.8 μM has effectively inhibited proliferation of 50% cells after 48 h treatment [23].

BE has been investigated for its anticancer potential in human lung cancer cells Lu1 (with the IC$_{50}$ values >45.2 μM) [19], NCI-H460 (nonsmall cell lung carcinoma, the IC$_{50}$ value 63.5 μM) [26], and A549. Interestingly, A549 cell line has been extensively studied by several authors, and the IC$_{50}$ values have prominently ranged from 3.8 μM [27, 28] through 7.4 μM [23] and 20 μM [29] to 33.4 μM [26]. Another study has shown that the dose of BE required to reach a 10% cell viability inhibition (ID$_{10}$) in A549 cells has been 0.7 μM and the effect obtained after 24 h has been nearly doubled,

TABLE 1: *In vitro* antiproliferative effect of BE on human and animal cancer cell lines by means of IC_{50} values (inhibitory concentration 50%).

Cancer type	Cell line	IC_{50}		References
		μM	μg/mL	
Human myelogenous leukaemia	K562	**14.5**	6.4	[25]
		>225.9	**>100.0**	[26]
		>250.0	>111.0	[35]
Human neuroblastoma	SK-N-AS	**2.5**	1.1	[23]
Human rhabdomyosarcoma/medulloblastoma	TE671	**10.3**	4.6	
Human neuroblastoma	GOTO	**17.1**	7.6	[25]
Human neuroblastoma	NB-1	**16.5**	7.3	
Rat glioma	C6	**5.9**	2.6	[23]
Human thyroid carcinoma	FTC 238	**6.8**	3.0	
Human lung cancer	Lu1	>45.2	**>20.0**	[19]
Human nonsmall cell lung carcinoma	NCI-H460	63.5	**28.1**	[26]
Human lung carcinoma	A549	**20.0**	8.9	[29]
		33.4	**14.8**	[26]
		7.4	3.3	[23]
		3.8	1.7	[27, 28]
		23.3	10.3	
Human breast adenocarcinoma	MCF-7	30.7	**13.6**	[26]
		8.32	3.7	[32]
Human breast carcinoma	T47D	**5.2**	2.3	[23]
		73.2	32.4	[33]
Human cervical carcinoma	HeLa	74.1	**32.8**	24 h [24]
		57.1	**25.3**	48 h [24]
		34.4	**15.2**	72 h [24]
		22.6	**10.0**	[26]
		6.7	2.9	[32]
Human ovarian carcinoma cells	A2780	>45.2	**>20.0**	[21]
Human prostate adenocarcinoma	PC-3	**17.9**	7.9	[27, 28]
		82.9	**36.7**	[26]
Hormone-dependent human prostate cancer	LNCaP	>45.2	**>20.0**	[19]
Human gastric carcinoma	EPG85-257P	**18.7**	8.3	[36]
Human pancreatic carcinoma	EPP85-181P	**21.1**	9.3	
Human colorectal adenocarcinoma	DLD-1	**6.6**	2.9	[27, 28]
Human colorectal adenocarcinoma	HT-29	**4.3**	1.9	[23]
Human colon cancer	Col2	45.2	**>20.0**	[19]
Human colorectal adenocarcinoma	SW707	**51.7**	22.9	[33]
Human hepatoma	HepG2	22.8	**10.1**	[26]
Human hepatocarcinoma	SK-HEP-1	132.1	**58.5**	
Human melanoma	G361	**12.4**	5.5	[25]
Human melanoma	SK-MEL-28	**16.2**	7.2	
Mouse melanoma	B16-F1	**13.8**	6.1	[27]
Mouse melanoma	B16 2F2	**27.4**	12.1	[37]
Human melanoma	MEL-2	>45.2	**>20.0**	[38]
Human melanoma	SK-MEL2	**>250.0**	>111.0	[35]
Human skin epidermoid carcinoma	A431	**6.8**	3.0	[32]
Human promyeloblastic leukaemia	HL60	**14.7**	6.5	[25]
Human leukaemia	U937	**14.4**	6.4	
Human T lymphoblast leukaemia	Jurkat E6.1	**6.7**	3.0	[23]

TABLE 1: Continued.

Cancer type	Cell line	IC$_{50}$		References
		μM	μg/mL	
Mouse leukaemia	P388	**12.4**	5.5	[33]
Human leukaemia	CCRF/CEM	**24.6**	10.9	
Human multiple myeloma	RPMI 8226	**6.4**	2.8	[23]
Human oral epidermoid carcinoma	KB	>45.2	**>20.0**	[19]
Gastric carcinoma, atypical mitoxantrone MDR variant	EPG85-257RNOV	**12.3**	5.4	[36]
Gastric carcinoma, classical daunorubicin MDR variant	EPG85-257RDB	**11.0**	4.9	
Pancreatic carcinoma, atypical mitoxantrone MDR variant	EPP85-181RNOV	**20.6**	9.1	
Pancreatic carcinoma, classical daunorubicin MDR variant	EPP85-181RDB	**26.5**	11.7	
Human myelogenous leukaemia (paclitaxel-resistant)	K562-Tax	**250.0**	111.0	[35]

To facilitate comparison, the doses were recalculated to μM or μg/mL. Original data are presented in bold.

TABLE 2: *In vitro* antiproliferative effect of BE on human tumour primary cultures by means of IC$_{50}$ values (inhibitory concentration 50%).

Tumour type	Primary culture	IC$_{50}$		References
		μM	μg/mL	
Ovarian carcinoma	HPOC	**2.8**	1.2	[23]
Cervical carcinoma	HPCC	**3.4**	1.5	
Glioblastoma multiforme	HPGBM	**3.4**	1.5	

To facilitate comparison, the doses were recalculated to μM or μg/mL. Original data are presented in bold.

when the treatment has been extended to 48 h (0.4 μM) [30]. Moreover, BE has also been found to be slightly more potent antitumour agent than cisplatin (IC$_{50}$ value 25 μM) towards human lung cancer A549 cell line [29], however, was also demonstrated to be inactive towards nonsmall-cell bronchopulmonary carcinoma (NSCLC-N6) [31].

BE has also been evaluated *in vitro* for its anticancer potential towards the most commonly diagnosed cancers in women worldwide [1]. Its inhibitory effect on the growth of human breast, cervical, and ovarian carcinoma cells has been shown. Cell proliferation was 53.2% inhibited by 20 μM of BE in MCF-7 and Bcap-37 cell lines (both breast cancer cell lines) [20]. Other studies have shown that BE at the concentration 10 μM (4.43 μg/mL) and 30 μM (13.28 μg/mL) inhibited 25.81% and 35.54% proliferation of MCF-7 cells, respectively [16], whereas another report has shown the IC$_{50}$ value—8.32 μM [32]. Significantly higher IC$_{50}$ values for MCF-7 cells have been reported in several other studies— 23.3 μM [27, 28] and 30.7 μM [26]. T47D cell line has varied significantly in the sensitivity to the antiproliferative properties of BE with the IC$_{50}$ value from 5.2 μM [23] to 73.2 μM [33]. On the other side, BE has been shown to elicit about three-fold weaker antiproliferative activity (IC$_{50}$ value 17 μM) with respect to cytostatic agent 5-fluorouracil (5-FU, with the IC$_{50}$ value 5.34 μM) against MCF-7 cell line [34]. The proliferation of human cervical cancer cells (HeLa cell line) has been inhibited in dose- and time-dependent manner. The IC$_{50}$ values after 24 h were 74.1 μM [24], after 48 h 22.6 μM [26] and 57.1 μM [24], and 6.67 μM [32] and 34.4 μM [24] after 72 h. The dose required to reach an ID$_{10}$ in HeLa has been 0.47 μM, and the effect obtained after 24 h has been significantly enhanced when the treatment has been extended to 48 h (0.32 μM) [30]. Other authors have reported

BE to inhibit proliferation of HeLa cells at the concentration 10 μM (4.43 μg/mL) and 30 μM (13.28 μg/mL) by 73.02% and 81.39%, respectively [16]. BE at the concentration >45.2 μM has been demonstrated to reach a 50% cell proliferation inhibition in human ovarian carcinoma cells (A2780 cell line) [21].

Furthermore, some studies have also provided evidence that BE elicits antiproliferative activity towards human prostate cancers including androgen-dependent type. However, high discrepancies appear when comparing the IC$_{50}$ values towards the same cell line PC-3, ranging from 17.9 μM [27, 28] through 82.9 μM [26] up to >250 μM [35]. For example, BE inhibited proliferation of PC-3 cells by 18.4% [22] and by 17.3% at concentration 20 μM [20], whereas in LNCaP cells (androgen-dependent human prostate cancer cell line) the IC$_{50}$ was over 45.2 μM [19].

BE has also been shown to display antiproliferative activity towards cancers within human digestive system. BE has inhibited proliferation by 50% in pancreatic carcinoma (EPP85-181) and human gastric (EPG85-257) cell lines at 21.09 μM and 18.74 μM concentration, respectively [36]. The proliferation of another stomach cancer cell line (MGC-803) was inhibited by 43.7% [20] and 45.1% [22] at a concentration of 20 μM. BE has been investigated for its antiproliferative potential towards human colorectal adenocarcinomas, DLD-1, HT-29, Col2, and SW707 cells. Inhibition of cells proliferation in response to BE has been highly dependent on the cell line. The BE IC$_{50}$ values for DLD-1 [27, 28] and HT-29 colon cancer cells [23] have been comparable, 6.6 μM and 4.3 μM, respectively, and considerably much lower than for Col2 cells, with the IC$_{50}$ values of 45.2 μM [19], and for SW707 cells— 51.7 μM [33]. Conversely, BE is ineffective against HT-29 cells, with an IC$_{50}$ value higher than 250 μM [35].

BE has also demonstrated extremely diverse antiproliferative effects on human hepatoma cell lines. The IC_{50} values have ranged from $22.8\,\mu$M in HepG2 cells to $132.1\,\mu$M in SK-HEP-1 cells [26]. The BE dose required to reach an ID_{10} in HepG2 has been $1.02\,\mu$M, and the antiproliferative effect obtained after 24 h has been almost doubled after the treatment time has been extended to 48 h ($0.5\,\mu$M) [30].

Moreover, BE has been tested with promising results for its cytotoxicity and inhibitory activity towards a series of melanoma cell lines. The BE IC_{50} values in human melanoma cells G361 and SK-MEL-28 have been comparable, $12.4\,\mu$M and $16.2\,\mu$M, respectively [25], similar to those for murine melanoma B16-F1 cells—$13.8\,\mu$M [27], but considerably lower than in the case of B16 2F2 [37] and MEL-2 [38] cell lines, suggesting that antiproliferative potential of BE was independent from the cells origin (of human or non-human origin). Similarly, BE (at a concentration $10\,\mu$M) demonstrated a marked decrease in viability of other murine melanoma, B164A5 cell line, resulting in a 52% reduction of viable cells compared to control [39], while it has moderate activity towards epidermoid carcinoma of the mouth KB cells (IC_{50} value $>45.2\,\mu$M) [38] and total inactivity towards melanoma SK-MEL2 cells with an IC_{50} value higher than $250\,\mu$M [35]. Another skin cancer epidermoid carcinoma A431 cell line was much more sensitive to BE treatment; the concentrations $10\,\mu$M ($4.43\,\mu$g/mL) and $30\,\mu$M ($13.28\,\mu$g/mL) have inhibited proliferation by 63.42% and 70.30%, respectively [16], and the IC_{50} value was $6.76\,\mu$M [32].

Cytotoxicity and antiproliferative activity of BE have also been confirmed towards a panel of human and murine haematological malignancies *in vitro*. BE has significantly suppressed cells growth in several models of leukaemia, HL60 and U937 cell lines [25], with the comparable IC_{50} values 14.7 and $14.4\,\mu$M, respectively, but the most pronounced effect has been observed in Jurkat E6.1 cells—$6.7\,\mu$M [23]. Nearly two-fold weaker activity of BE towards human leukaemia CCRF/CEM cells versus mouse leukaemia P388 cell line has been observed (IC50 24.6 versus $12.4\,\mu$M) [33]. Although this results have been contested by other studies that show a total lack of BE activity against CEM cells— IC_{50} value $>250\,\mu$M [35, 40, 41]. Similar discrepancies have been demonstrated towards human chronic myelogenous leukaemia K562 whereas on one hand BE is reported as active, IC_{50} value $14.5\,\mu$M [25], while on the other hand it is completely inactive, IC_{50} values $>200\,\mu$M [26] and $250\,\mu$M [35]. Additional studies have evidenced notable activity of BE in human multiple myeloma RPMI 8226 cell line, where the concentration $6.4\,\mu$M inhibited growth of 50% cells after 48 h treatment [23].

The significant discrepancies between IC_{50} doses of BE towards the same cell lines, A549 [23, 26–29], T47D [23, 33], PC-3 [26–28, 35], CCRF/CEM [33, 35, 40, 41], and K562 [25, 26, 35], evaluated by different authors seem to be the result of various sources of BE and extraction procedures as well as lack of standardised treatment modalities (treatment times, doses, and individual features of each laboratory cell strains).

Conspicuously, BE shows antiproliferative and cytotoxic activity towards cancer cell lines resistant to conventional cytostatic drugs, which suggests a novel mechanism of action. BE has been shown to elicit significantly stronger antiproliferative effect (by means of IC_{50}) values on the daunorubicin- and mitoxantrone-resistant cancer cells, such as the DB-resistant human gastric cancer 257RDB cell line (IC_{50} $10.97\,\mu$M), and NOV-resistant (Novantrone) human gastric cancer 257RNOV cell line (IC_{50} $12.25\,\mu$M), and human pancreatic carcinoma 181RNOV cell lines (IC_{50} $20.62\,\mu$M) than on the drug-sensitive parental 257P and 181P cells [36], whereas BE has been inactive towards K562-Tax (paclitaxel-resistant subline of human chronic myelogenous leukaemia), with the IC50 value $>250\,\mu$M [35]. Nevertheless, BE has been suggested to overcome some forms of drug resistance in cancer cells refractory to conventional chemotherapeutic agents [36].

The purity and purification methods play important roles in the downstream activity of BE and its derivatives. A growing body of evidence suggests that different BE extracts have better therapeutic potential than pure BE. In some cases, isolated BE has been found to elicit a weaker antiproliferative activity against the human gastric cell line (EPG85-257P) (Table 1) as compared with a crude birch bark extract, while in other cases, stronger inhibitory effect towards pancreatic carcinoma cells (EPP85-181P) by isolated BE as compared to the birch bark extract has been observed [36]. The outer bark of the birch trees contains BE as the main component but some other pentacyclic triterpenes as well [42]. Thereby, the synergistic effects of combination of various triterpenes with diverse activities and modes of action could explain to some extent the discrepancies in results obtained *in vitro* between birch bark extract and purified BE. Although this action, or combination of actions, is cell type-dependent, for example, a crude birch bark extract (*B. pendula* Roth, syn. *B. verrucosa*-European White Birch) has been found to elicit more pronounced antiproliferative potential against the daunorubicin- and mitoxantrone-resistant human gastric and pancreatic carcinoma cell lines (IC_{50} values 4.29–$7.08\,\mu$M and 9.07–$23.03\,\mu$M, resp.), compared to the drug-sensitive parental 257P and 181P cell lines [36]. Likewise, the BE-enriched (approximately 97%) birch bark extract (*B. pendula* Roth) has shown strong antiproliferative potential towards human cancer cell lines A431, A2780, HeLa, and MCF7 *in vitro*, with the IC_{50} values from $2.26\,\mu$M up to $11.29\,\mu$M (1 and $5\,\mu$g/mL) [43]. In another study, bark extract from *B. pendula* Roth with content of 57.01% of BE at the concentration of $17.53\,\mu$M ($7.76\,\mu$g/mL) and $52.61\,\mu$M ($23.29\,\mu$g/mL) has inhibited proliferation of A431 (by 70.02% and 78.70%, resp.), MCF-7 (by 45.54% and 55.55%, resp.), and HeLa (by 70.62% and 76.23%, resp.) cells stronger than pure BE [16]. A highly purified triterpene extract (TE) from the *Betulae* cortex with BE as a main component (up to 87.3% w/w of identified triterpenes) demonstrated a dose-dependent cytotoxicity from $0.090\,\mu$M ($0.04\,\mu$g/mL) to $90.35\,\mu$M ($40\,\mu$g/mL) in human nonmalignant, immortalized keratinocytes (HaCaT) and skin cancer A431 (squamous cell carcinoma) cell lines, similar to its main constituents, BE and betulinic acid (BA). TE has been shown to form an oleogel, which facilitates an application on the skin for dermatological indications [44].

An essential advantage of the use of BE as bioactive agent is its relatively low toxicity towards noncancerous cells [45]. BE has shown relatively modest cytotoxicity against human skin fibroblasts (HSF)—doses below 10 μM have no apparent toxicity [23]—and mouse fibroblasts (Balb3T3)—IC_{50} value 106.8 μM (47.3 μg/mL) [33]. Also, BE has expressed low activity towards immortalized human epithelial cells (hTERT-RPE1cell line) and human umbilical vein endothelial cells (HUVEC) with the IC_{50} values >45 μM (20 μg/mL) [19]. BE isolated from the tropical plant *Cyrtomium fortumei* (J.) or BE from the roots of *Belamcanda chinensis* (L.) DC inhibited the growth of NIH3T3 mouse fibroblasts only by 29.8% and 33.5%, respectively, at a concentration 20 μM [20, 22].

On the other hand, BE has shown significant antiproliferative effect against human normal skin fibroblasts (WS1), with the IC_{50} value 3.6 μM [27, 28] and normal lung fibroblasts WI38 (IC_{50} 15.2 μM) [25]. Although there are only few reports concerning BE influence on normal cells, noncancerous cells of various origins have been confirmed to be more resistant to BE treatment than tumour cells pointing to some cell-type selectivity. These encouraging results of *in vitro* studies make BE a promising therapeutic candidate.

BE has been shown to markedly impede the migration of several cancer cell types, including lung (lung carcinoma A549 cells) and central nervous system tumours (cell lines C6—glioma and TE671—rhabdomyosarcoma/medulloblastoma) [23].

In vivo antiangiogenic effects have also been reported for BE. Using the chorioallantoic membrane (CAM) model in chicken embryos, to study blood vessel formation, the antiangiogenic activity of BE has been proved by inhibition of the formation of new capillaries, presumably throughout targeting the endothelial cells [43]. This activity can be further enhanced by using BE in nanoemulsion formulation to increase penetrability to extraembryonic tissues [46]. Similarly, the decrease in melanoma tumour size in C57BL/6J mice model (at post-B164A5 tumour cells inoculation) after BE treatment has been attributed to its antiangiogenic activity. Indeed, immunocytochemical analyses showed a reduced VEGF expression in mice treated with BE-γ-cyclodextrin derivative (GCDG) complex in comparison with the control group [39]. The molecular basis of BE antimigration and antiangiogenic activities remains to be determined.

3. Potential Mechanisms of BE-Mediated Anticancer Activity

A rapidly rising number of studies have shown that the induction of apoptotic cell death is an essential mechanism of anticancer agents activity [47–49], including BE. It has been demonstrated that disruption of the apoptosis machinery is a typical feature of tumour cells [50–52]. Apoptosis is a type of programmed cell death, characterized by a series of complex, specific biochemical and cytomorphological events. Two main pathways of apoptosis have been identified, the extrinsic (death receptor-related) and the intrinsic (mitochondrion-dependent). The extrinsic pathway is initiated by external signals, for instance, the binding of molecules (ligands), such as Fas, TNF, or TRAIL, to their respective death receptors, localized in the cell surface. The intrinsic apoptosis pathway is activated by different stimuli, such as DNA damages, oxidative stress, radiation, and growth factors withdrawal [53].

An ability to trigger apoptosis in tumour cells has been proved as one of mechanisms underlying BE cytotoxicity and its antiproliferative potential. BE treatment has resulted in cytomorphological alterations characteristic for cells undergoing apoptosis, like cell rounding, chromatin condensation, nuclear fragmentation, membrane blebbing, and formation of apoptotic bodies [26]. Likewise, inhibition of HeLa cells proliferation has been accompanied by morphological changes, characteristic of apoptosis: cells have become smaller and the morphology has showed karyopycnosis, when exposed to BE for 24 h, and the effect was a dose-dependent [24]. BE treatment of murine melanoma cells B164A5 has demonstrated almost equal amounts of apoptotic and dead (necrotic) cells [39]. BE has been shown to induce apoptotic cell death in human lung adenocarcinoma cells *in vitro* (A549 cell line). The amount of apoptotic cells has significantly increased by 27.64% in comparison with control, untreated cells [29]. BE has been shown to increase substantially the number of cytosolic oligonucleosomal fragments in A549 cell line [23]. More detailed studies have shown that BE induces apoptosis of human cancer cells through the mitochondrial (intrinsic) pathway in A549, Jurkat [54] and HeLa cancer cell lines [26, 54]. BE proapoptotic activity in HeLa cells has involved the sequential activation of caspases 9, 3, and 7 and the cleavage of poly (ADP-ribose) polymerase (PARP) [24]. The cleavage of caspase-3 substrate PARP to the 85 kDa form of the protein has been observed, which points at a caspase-activated apoptotic cell death. The activity of caspase-8 remained unchanged, suggesting a lack of extrinsic pathway activation, while caspase-9 has been shown to be initially activated, followed by cytochrome c/Smac proteins release from the mitochondrial intermembrane space, mitochondrial membrane potential depolarization, and rapid translocation to the mitochondrion of Bax and Bak proteins (proapoptotic members of the Bcl-2 family) [26]. In another study, BE had no influence on the total expression of Bax and Bcl-2, on mRNA as well as on protein level, and the total expression of Bak protein in HT-29 cancer cells [23]. However, a few reports have demonstrated that BE treatment induced the expression of other cellular proteins indirectly involved in apoptosis. By means of pharmacoproteomic approach, BE has been shown to upregulate aconitate hydratase and malate dehydrogenase in cancer cells, enzymes involved in ATP generation, supporting the involvement of mitochondrial pathway as the main mechanisms of BE-induced apoptotic cell death [29]. BE-mediated downregulation of isoform 1 of 3-hydroxyacyl-CoA dehydrogenase type 2, also known as enoyl-CoA hydratase, an enzyme related to lipid metabolism, should be further investigated to elucidate its involvement in BE-induced apoptosis. BE treatment resulted also in decrease of poly (rC)-binding protein 1 expression. The poly (rC)-binding protein 1 was reported to protect cells from different apoptosis inducers and modulate heat shock protein 90-α 2

(HSP90-α 2) expression, which is involved in the regulation of mitochondrial membrane permeabilization and cytochrome c release. This might be a mechanism by which BE sensitises cancer cells to undergo apoptosis. Moreover, a highly purified TE from *Betulae* cortex, containing BE as a main component, displays a dose-dependent proapoptotic effects on HaCaT and A431 cells, similar to its main constituents, BE and BA [44].

Apoptosis induction is often a consequence of cell cycle disturbances. The cell cycle progression is controlled by cyclins, which are a regulatory proteins family of cell cycle-dependent kinases (CDKs) [55]. Regulation of the cell cycle has become a challenge and a promising target for cancer therapy [56]. Thus, numerous anticancer agents have been reported to arrest cell cycle at the G_0/G_1, S, or G_2/M phases and consequently trigger apoptosis of cancer cells [57–60].

Surprisingly, limited attention has been given to the regulation of cell cycle by BE in cancer cells. BE at a concentration 10 μM has been shown to induce an arrest of murine melanoma B164A5 cells in S phase, with a concomitant decrease in the number of cells in the G_0/G_1 phases [39]. BE treatment of HepG2 cells (hepatoma) induced a late stage G_0/G_1 phase cell cycle arrest, and at the early stage S phase, and a subsequent decrease in the amount of cells in the G_2/M phases at a relatively low concentration (11.29 μM/5 μg/mL). Another study, using hepatoma Hep3B cells, has shown that BE treatment resulted in a cell cycle arrest at the G_2/M phase, showing different effects of BE in regulation of the cell cycle, depending on hepatoma cells type. Furthermore, BE has been reported to slightly reduce DNA replication, without influencing the expression level of cell cycle regulatory genes, p21 and p53 in hepatoma cells [61]. p21 and p53 expression level were also not affected after BE treatment in other tumor cell lines originating from central nervous system (medulloblastoma/rhabdomyosarcoma, neuroblastoma, and glioma) and various peripheral cancers including lung, colon, thyroid, breast, leukaemia, multiple myeloma, and several tumour primary cultures [23].

Cell division perturbations after BE treatment could be linked to direct interactions with DNA topoisomerases (Topo), but not with DNA, at concentrations comparable with those of the well-known inhibitor etoposide. BE, among other lupane- and oleanane-type triterpenoids from the bark of *Phyllanthus flexuosus*, has been reported to selectively inhibit the activity of human Topo II in a dose-dependent manner. Topo are known to play an essential role in DNA metabolism, affecting replication, transcription, recombination, and mitotic chromosome segregation [62]. Thereby, Topo might be a target for the antitumour activity of BE. Topo I inhibitors are known to induce apoptosis in cancer cells [63, 64]. Whereas BE affects Topo II activity, it has no influence on the activity of human Topo I [25].

Another enzyme involved in cell division and affected by BE treatment (IC$_{50}$ 20 μM) is cAK (cyclic AMP-dependent protein kinase) which is activated by a plethora of extra- and intracellular signals. A central network player, cAK, is involved in the regulation of a variety of cellular processes including metabolism, cell division, specific gene expression,

and development [65]. The inhibition of cAK by BE is specific as no changes in the activity of ERK1/2 and AKT kinases were observed [23]; the two latter kinases are frequently pathologically hyper-activated in several human cancers [66, 67].

BE has been searched for its effect on human melanocortin (MC) receptor signalling pathway. Human MC receptors-expressing COS-7 cells bind BE with different specificities depending on the MC subtype. The affinity of BE to the MCRs is MC1>MC3>MC5>MC4. Furthermore, BE antagonizes α-melanocyte-stimulating hormone- (α-MSH-) induced accumulation of cAMP to some extent in the mouse melanoma cell line B16-F1, which naturally expresses MC1 receptor without stimulating MC receptor-associated generation of cAMP [68]. MC1 receptor subtype is expressed almost in each cutaneous cell type, in immune and in melanoma cells [69, 70]. It is also worth mentioning that the MC1 receptor has been suggested to be a crucial modulator of epidermal melanocyte proliferation and differentiation [71, 72] and has been suggested as an important target of the antimelanoma activity of BE and its structurally similar substances, such as BA [68].

4. Inhibition of Carcinogenesis and Antimutagenic Activity *In Vivo*

BE has been confirmed as a potent antimutagenic agent of skin carcinogenesis. The topical formulation with BE nanoemulsion has been tested on C57BL/6J type mouse skin, chemically damaged by DMBA (7,12-dimethylbenz[α]anthracene) as a tumour initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as tumour promoter. Potentially, any damage of the skin surface might lead to significant pathologies, such as skin neoplasms. Observations of cutaneous damages have revealed the activity of BE in reducing skin lesions and irritation by considerably decreasing erythema [73]. Topical application of BE has exhibited distant effects and influenced the respiratory function of isolated liver mitochondria in a two-stage model of skin carcinoma induced in mice. The improvement of liver mitochondrial respiration and increased basal (LEAK state) and active (OXPHOS state) respiration has been observed. Moreover, BE may also influence the penetration of carcinogens and reduce damage in main organs, such as liver, since application of carcinogens on the skin surface, because of their slow penetration, leads to toxic effects especially on liver. BE has also been shown to inhibit apparition and promotion of skin tumours [46]. Similarly, birch bark dry extract (BDE, with BE as a main component—at least 70%) has been applied on mice with chemically-induced mutagenesis. The administration of 150 and 1500 mg/kg BDE to mice resulted in no mutagenic and comutagenic effects. The number of cells with chromosomal aberrations was comparable between control and BDE-treated animals. Furthermore, BDE in doses of 50, 150, and 450 mg/kg notably reduced the cytogenetic effect of mutagens, dioxidine (1,4-di-N-oxide of 2,3-bis-(hydroxymethyl) quinoxaline, DN) and cyclophosphamide (N$'$-bis-(b-chloroethyl)-N$'$-O-trimethyl

ester of phosphoric acid diamine, CP). A single treatment with BDE in doses of 50 and 150 mg/kg results in approximately the same antimutagenic effect and decreased the damaging activity of DN and CP by 53–60% and 60%, respectively. BDE inhibits free radical oxidation and thus the prooxidant mutagenic activity of DN. The protective activity of BDE has been potentially mediated by various mechanisms, for instance, *via* inhibition of cytochromes P450, playing a crucial role in the metabolism of CP, or by stimulation of production of interferons, which may improve DNA repair [74].

5. Potential Application in Therapy

No typical clinical trials have been published using BE for the treatment of human cancer so far [12]. Nevertheless, a nonrandomized pilot study, using a birch bark extract to treat actinic keratoses (AK) [75, 76], suggests a preventive and therapeutic potency of BE in skin pathologies supporting by encouraging *in vivo* studies [73]. AK is considered to represent an early and noninvasive squamous cell carcinoma *in situ*, due to histological similarity [77], and as commonly diagnosed skin damage induced by ultraviolet light should be treated to avoid the development of nonmelanoma skin cancers [78]. A birch bark ointment (containing around 87% of the triterpenes with predominant content of BE, 80%), used as monotherapy for the treatment of AK, resulted in a remission of more than 75% of the lesions in 79% of the patients after treatment as a product that has been approved for use as a cosmetic in Germany [75]. Furthermore, recent tests with water-free BE-based oleogel containing a higher extract concentration have confirmed the effectiveness of the BE-based strategy in the therapy of AK. The treatment resulted in complete clearing of the lesions in 64% and partial remission (more than 75% of lesions) in 86% of the patients, after a three-month treatment period, comparably to standard therapy (cryotherapy) [76]. Additionally, a synergistic effect by the combination of BE and cryotherapy has been reported with no observable undesirable effects [75]. Besides, BE-based oleogel decreased the degree of epidermal dysplasia and number of dyskeratoses in treated patients during a prospective, randomized, and comparative clinical phase 2a study. Excellent skin tolerance for oleogel prepared from a standardized triterpene dry birch bark extract was also noticed [76]. For that reason, the treatment with birch bark ointment or BE-based oleogel is regarded as a new topical alternative for current AK therapy and a promising chemopreventive agent, especially that the risk of AK progression to invasive type of squamous cell carcinoma has been estimated between 1% and 16% [79].

In animal models and pilot studies with BE, BE-based oleogel, or triterpene birch bark extract, no severe adverse effects have been observed. BE, likewise other pentacyclic triterpenes, has also shown no toxicity. Daily administration of BE (doses at 540 mg/kg of body weight i.p. in rats and 300 mg/kg s.c. in dogs) resulted in very low toxicity, if any [42]. Thereby, it seems that triterpene birch bark extract and its representative compound, BE, are safe to use *in vivo*.

6. Concluding Remarks

An increasing number of studies support the antineoplastic activity of BE. A limitation for TE's biological and pharmacological effectiveness is their poor solubility. The solution could be a complexation with hydrophilic carriers. Indeed, BE hydrosolubility can be significantly improved by highly hydrophilic semisynthetic β-cyclodextrin [80], and γ-cyclodextrin derivatives [39] as carriers, which has enhanced antiproliferative potential of BE towards cancer cell lines [80], and by incorporation in nanoemulsion [46], which may increase its bioavailability and consequently improve its activity *in vitro* and *in vivo*. Chemically synthesized cyclodextrin derivatives offer the prospect of preparation highly stable complexes with both BE and other terpenes, such as BA [81], and possibly might be submitted for clinical trials soon. Likewise, application of cholesterol containing BE-liposomes may be considered as a promising method to facilitate the use of BE in the context of anticancer therapy [54].

Due to the multitarget activity of BE on cancer cells, it may be used in combination with commonly used chemotherapeutic drugs, as their synergistic effect can help to eliminate cancer cells, including drug-resistant cells [36]. Another novel approach for the application of BE in cancer therapy may be its chemical modification with various ligands which allows obtaining an enhanced cytotoxicity towards tumour cells, better solubility, and bioavailability than the parental compound [33]. Therefore, BE has been attempted to be used as a precursor in the synthesis of novel BE derivatives with improved anticancer and pharmacokinetic properties.

Many of the molecular mechanisms of action of TE are still elusive which limits our understanding of this potentially beneficial group of natural compounds.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Extracts from *Vatica diospyroides* Type SS Fruit Show Low Dose Activity against MDA-MB-468 Breast Cancer Cell-Line via Apoptotic Action

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Very strong antiproliferative action of *V. diospyroides* type SS fruit extracts (IC_{50} range of 1.60-17.45 μg/mL) in MDA-MB-468 cell-line was observed in an MTT assay. After dosing of an extract concentration at half IC_{50} to cell line for 24 to 72 hours, treated cells were subjected to Annexin V-FITC/PI binding assay, followed by FACS and western blot analyses. Significant apoptotic death was observed with all extract treatments and both exposure times. Dosing with acetone extract of pericarp and cotyledon induced the highest apoptotic populations (33 and 32%, resp.), with the lowest populations of viable cells (65 and 67%, resp.). During 24 to 72 hours of dosing with methanolic extract of pericarp, the populations of viable and early apoptotic cells decreased significantly from 72.40 to 71.32% and from 12.00 to 6.36%, respectively, while the late apoptotic and nonviable cell populations continuously increased from 15.30 to 19.18% and from 0.30 to 3.14%, respectively. The expression of Bax increased within 12–48 hours of dosing, confirming apoptosis induced by time-dependent responses. The mutant p53 of MDA-MB-468 cells was expressed. Our results indicate that apoptosis and time-dependent therapeutic actions contribute to the cytotoxic effects of *V. diospyroides* type SS fruit on MDA-MB-468 cell.

1. Introduction

At present, breast cancer is commonly considered the globally leading cancer type that contributes to mortality of women. The types of breast cancer are categorized based on estrogen level and on responsiveness of the cancer cells. The estrogen-dependent types (ER-rich), such as the MCF-7 cell-line, are responsive to chemotherapy, while the estrogen-independent types (ER-poor), such as the MDA-MB-231 cell-line, are aggressive unresponsive drug-resistant cancers [1, 2]. The therapeutic intervention against a breast cancer usually comprises both chemoradiation therapy and surgical operations, but the survival rates and times remain very low [3] and depend on the type and the stage of cancer. Moreover, patients with the ER-poor type of breast cancer may suffer from

negative toxic effects caused by high drug doses. The dose level of a drug can induce various modes of breast cancer cell death, with apoptosis being dominant at a low dose level in both ER-rich and ER-poor types and necrosis being dominant in the ER-rich type at a high dose level [4, 5]. Only apoptosis is effective and it is critical for the success of a cancer treatment. Therefore, much of current research seeks to discover natural drugs that would be effective and nontoxic at a low dose level [2], for inducing apoptosis of breast cancer both *in vitro* and *in vivo*. Such programs mostly focus on medicinal plants as sources of the active ingredients.

Vatica diospyroides Symington, a plant belonging to the family Dipterocarpaceae, is an important source of breast cancer chemopreventives, such as phytochemical constituents like saponins and terpenoids components [6], and

resveratrol tetramer [7, 8]. The resveratrol derivatives like dibalanocarpol and vaticaffinol purified from plants in this family have been conducted as several biological properties against HIV and funguses [9, 10]. Interestingly, the resveratrol tetramer like vaticanol and vaticaphenol series that play an important role as the major antineoplastic constituent has been purified from both stem and bark of many Dipterocarpaceae plants. Based on *in vitro* breast cancer experiments using *V. diospyroides* fruit, these are a better source of therapeutic agents with antiproliferative effects than the other plant parts such as root [6, 11]. In our previous work, *V. diospyroides* type SS fruit had antiproliferative action and induced apoptosis and/or necrosis, depending on the extract concentration used, in MCF-7 and MDA-MB-231 breast cancer cell-lines [5]. Aside from dependence on the dose level, the inhibition via apoptotic pathways of ER-poor MDA-MB-468 cell-line, an *in vitro* model of malignant and invasive breast cancer, also depends on the duration of incubation [2]. Therefore, in this study, dose levels below the IC_{50} of a *V. diospyroides* type SS fruit extract could also lead to apoptosis in MDA-MB-468 cells, given time for such therapeutic action. We demonstrate the *in vitro* efficacy of *V. diospyroides* type SS fruit extract as a breast cancer chemopreventive, with antiproliferative and apoptotic effects via p53 and Bax protein related mechanisms on an ER-poor breast cancer model.

2. Materials and Methods

2.1. Plant Materials and Extract Preparations. The collection of fruit samples from ten-year-old *V. diospyroides* type SS (Collector number T. Srisawat 002) and the preparation of acetone and methanolic extracts from the fruit followed methods described previously [6]. Extracts of fruit pericarp and cotyledon were kept separate and stored in darkness at 4°C prior to tests against breast cancer (MDA-MB-468 cell, ATCC HTB-132) and normal (Vero cell, ATCC CCL-81) cell-lines in a cytotoxicity assay.

2.2. Cytotoxicity Assay. Two assays were performed, namely, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for breast cancer cell-lines and a green fluorescence protein (GFP) assay for the normal Vero cells. For the MTT assay, the MDA-MB-468 cells were seeded in 96-well plates at a density of 2×10^4 cells, in $100 \mu L$ of medium per well. Each extract was diluted to the desired concentration, added into the cell culture, and then incubated for 72 hours. Adjusting to the desired 5–80 $\mu g/mL$ concentrations of extracts, cell viability determination and fitting of response curves, and the harvest of treated cells followed previously described methods [5]. The half-inhibitory IC_{50} concentration and the labeling of cytotoxic activity were determined according to Alitheen et al. [12] and Wibowo et al. [13] criteria, with very strong inhibiting activity meaning $IC_{50} < 5 \mu g/mL$. For the normal Vero cells, the GFP assay followed the methods of Hunt et al. [14], and less than 50% cell growth indicated cytotoxicity against normal cells.

The mode of MDA-MB-468 cell death and the time of therapeutic action were determined by an Annexin V-FITC/PI binding assay and flow cytometry. To avoid necrotic cell death from acute toxicity with high extract dose levels, half IC_{50} extract concentrations were used.

2.3. Annexin V-FITC/PI Binding Assay. To detect apoptotic cells, the cellular location of phosphatidylserine (PS) and the cell membrane integrity/permeability of treated cells were assessed by staining and by applying Annexin V-FITC/PI binding kit, following the manufacturer's protocol (BD Pharmingen, USA). Briefly, after suspending the cells in a 1x binding buffer (0.1 M Hepes, 0.1 M NaOH pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL, five μL each of Annexin V-FITC and propidium iodide (PI) was subsequently added. The suspensions were vortexed gently and $400 \mu L$ of 1x binding buffer was added before analysis with fluorescence activated cell sorter (FACS).

2.4. Flow Cytometry. For FACS analysis, a FACSCalibur flow cytometer (Becton Dickinson Biosciences {BDB}, San Jose, CA), equipped with a 488 nm argon ion laser, was used. A total of 5,000 events were acquired with CellQuest software (BDB). The populations of viable, early apoptotic, late apoptotic, and dead cells in each experiment were analyzed and dot plot diagrams were generated with WinMDI version 2.9 software. The mode of cell death was determined from the transitions of cells in such plots, following criteria explained earlier [5].

2.5. Western Blotting. The MDA-MB-468 cells were treated with the extract at half IC_{50} (0.5 IC_{50}) concentration for up to 48 hours, then released by trypsinizing the cells, and collected by centrifugation. The cells were washed once in cold PBS, prior to lyse in RIPA buffer (Peirce Biotechnology, IL, USA). Protein concentration was measured using Bradford protein assay (Biorad, CA, USA). Fifty micrograms of each protein sample was separated in 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked in 5% low-fat dry milk in TBS-T (5% low-fat dry milk, 0.1% Tween-20 in Tris-buffered saline) for 1 h at room temperature. After that, each membrane was incubated overnight with primary antibodies against p53 (1 : 1000) and Bax (1 : 1000) with β-actin (1 : 1000) used as an internal control. All the antibodies were purchased from Cell Signaling, MA, USA. The membranes were washed 3 times (5 min/wash) with 1% low-fat dry milk in TBS-T and incubated with ECL anti-rabbit IgG horseradish peroxidase (GE health care) diluted 1 : 5000 in 1% low-fat dry milk in TBS-T, for 1 h at room temperature. After washing with TBS-T 3 times, the signals were detected using a chemiluminescent detection system (Pierce, IL, USA) and then exposed to film [5].

2.6. Statistical Analysis. Three independent cytotoxicity experiments were performed, and the results are expressed as mean ± SD. Statistical analysis used a one-way ANOVA with SPSS software (version 11.0).

TABLE 1: Half-inhibitory dose concentrations (IC_{50}) of both acetone and methanolic extracts from *V. diospyroides* type SS fruit against MDA-MB-468 cell-lines.

Fruit part	Solvent	MDA-MB-468 cell	
		IC_{50}, mean ± SD (μg/mL)	Activity
Cotyledon	Acetone	1.60 ± 0.00[a]	Very strong
	Methanol	3.50 ± 0.60[a]	Very strong
Pericarp	Acetone	17.45 ± 2.10[c]	Moderate
	Methanol	10.64 ± 3.22[b]	Moderate

Statistically significant differences at $P < 0.05$ were determined by one-way ANOVA using SPSS version 11.0 program. Different superscripts (a, b, or c) indicate significant differences according to Tukey's multiple comparison test. Each result is based on three replicates.

3. Results

3.1. Cytotoxicity on MDA-MB-468 and Normal Vero Cell-Lines. The inhibitory effects, of cotyledon and pericarp extracts of *V. diospyroides* type SS fruit, on MDA-MB-468 and normal Vero cell-lines were evaluated by MTT and GFP assays, respectively. An overview of the results shows moderate to very strong antiproliferative ($IC_{50} \leq 20\,\mu$g/mL) effects on MDA-MB-468 when this cell-line was cultured in the presence of any extract. Table 1 lists the half-inhibitory IC_{50} concentrations of the extracts for 72-hour treatment. The acetone and methanolic extracts of cotyledon were the best chemopreventives against MDA-MB-468 cells based on their 1.60 and 3.50 μg/mL IC_{50} values. Meanwhile, very strong inhibition by the acetone and methanolic extracts of pericarp was observed, with 17.45 and 10.64 μg/mL IC_{50} values. The cytotoxicity against normal cells is shown in Table 2. All extracts were noncytotoxic with statistical significance, and the cell growth ranged between 88 and 100%. The extracts were inactive on normal cells and IC_{50} levels could not be determined. However, the acetone extract of cotyledon and the methanolic extract of pericarp at 50 μg/mL concentrations would be toxic to normal cells, with 43.90 and 48.75 μg/mL IC_{50} values. Although the 50% of inhibitions by *V. diospyroides* fruit extracts indicate successful antiproliferative activity against MDA-MB-468 breast cancer; this might be due to apoptosis and/or necrosis depending on the dose level [5]. Therefore, half IC_{50} dose levels of each extract were tested for their modes of cancer cell death and for times of therapeutic action.

3.2. Mode of Cancer Cell Death and Time of Therapeutic Action. Time experiments on the therapeutic action, assessing the population of MDA-MB-468 cells following an apoptotic pathway, were carried out by treating the cells at half IC_{50} concentration for 24–72 hours with each extract. After staining the treated cells with Annexin V-FITC/PI, the mode of cell death was determined from the transition paths of the cells in the dot plot diagrams from FACS. The diagrams of FACS results are shown in Figure 1. The plots are divided into four quadrants, so that viable cells fall into the lower left quadrant (Annexin V-negative, PI-negative), early apoptosis is at lower right (Annexin V-positive, PI-negative),

late apoptosis is at upper right (Annexin V-positive, PI-positive), and the nonviable cells are at upper left (Annexin V-negative, PI-positive). The apoptotic and necrotic pathways are distinguished as follows: apoptosis is identified by a continuous trace of treated cell from viable to early-late apoptosis and further to nonviable cells, while a direct trace from viable to nonviable quadrant is labeled as necrosis [5]. As shown in Figures 1(a)–1(l), in most extract treatments the lower left quadrant had half or more of the total cells, considered viable. Interestingly, by the above criteria the apoptotic pathway but not the necrotic pathway was clearly indicated by the traces in these plots. At 48 hours of treatment, the lowest viable counts and the highest apoptotic counts (early + late apoptosis) were found in the treatment with acetone extract of pericarp (65.92 + 33.22%; see Figure 1(b)). However, at 72 hours of treatment, the acetone extract of cotyledon was the most effective with 67.56 + 32.08% of the cells apoptotic (Figure 1(i)). This indicates that the therapeutic action of acetone extracts of cotyledon and pericarp peaked in their effects at different treatment times. In the treatment with methanolic extracts of pericarp, the populations of viable and early apoptotic cells declined continuously during 24 to 72 hours of treatment, from 72.40 to 71.32% and from 12.00 to 6.36%, respectively (see Figures 1(d)–1(f)). Simultaneously, in the same treatment, the populations of late apoptotic and nonviable cells increased from 15.30 to 19.18% and from 0.30 to 3.14%, respectively. On the other hand, over 24–72 hours of treatment, the cells treated with methanolic extract of cotyledon underwent apoptosis with only moderate numbers of nonviable cells and apoptotic cells (early + late apoptosis) (Figures 1(j)–1(l)).

Interestingly, in western blotting analysis, the expression of tumor suppressor protein (p53) and proapoptotic protein (Bax) confirmed that the fruit extracts of *V. diospyroides* could induce apoptosis pathway of MDA-MB-468 cells in time-dependent responses (12–48 hours). Although, there was detectable p53 protein expressed in all treated cells and times, continuous changes in the expression levels of Bax were detected in all treated cells, in a consistently time-dependent manner, indicating activation downstream of p53 (Figures 2(a)–2(d)). There were upregulations of Bax at 12–48 h for methanolic extract of pericarp, at 12–24 h for acetone extract of pericarp and methanolic extract of cotyledon, and at 12 h for acetone extract of cotyledon. The expression of Bax protein will be motivated leading finally to apoptosis. Simultaneously, apoptosis in all treated cells was also induced by the Bax protein in a consistently time-dependent response (12–48 hours).

4. Discussion

A previous study has shown that the crude extracts of *V. diospyroides* type LS fruit have strongly *in vitro* activity against the ER-rich MCF-7 and the ER-poor MDA-MB-468 breast cancer cell-lines [6]. In the present work instead of the type LS we used extracts of *V. diospyroides* type SS fruit and observed remarkable effects against the viability of MDA-MB-468 human breast cancer cell-line associated with the
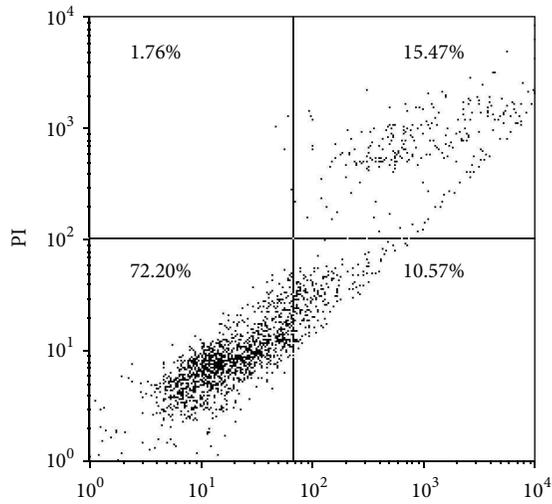
(a) Cells treated with acetone extract of pericarp for 24 hours
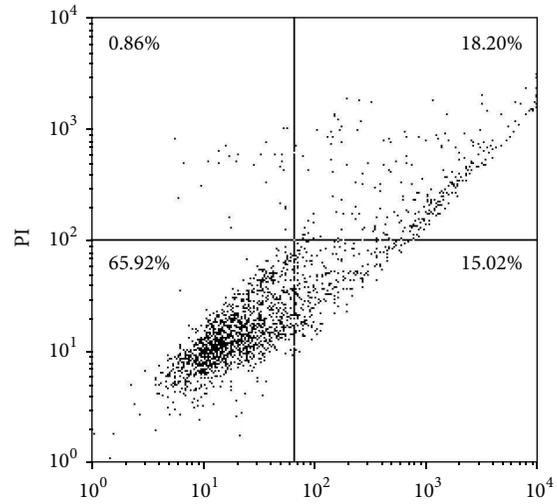
(b) Cells treated with acetone extract of pericarp for 48 hours
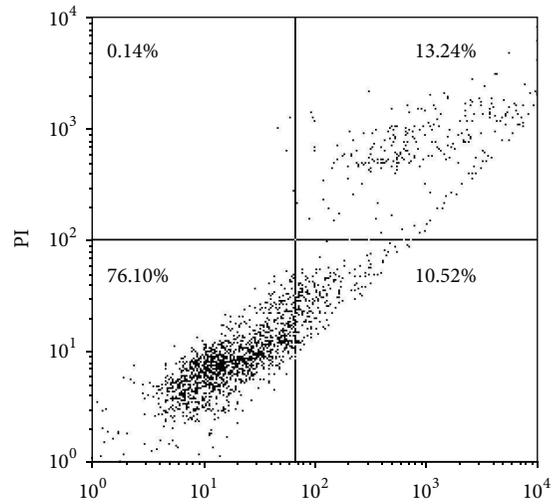
(c) Cells treated with acetone extract of pericarp for 72 hours
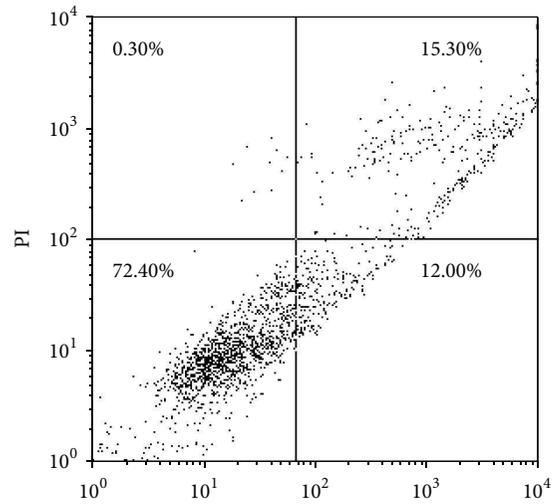
(d) Cells treated with methanolic extract of pericarp for 24 hours
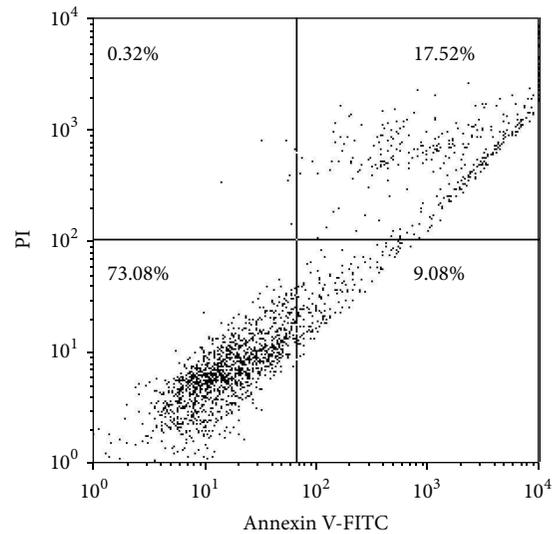
(e) Cells treated with methanolic extract of pericarp for 48 hours
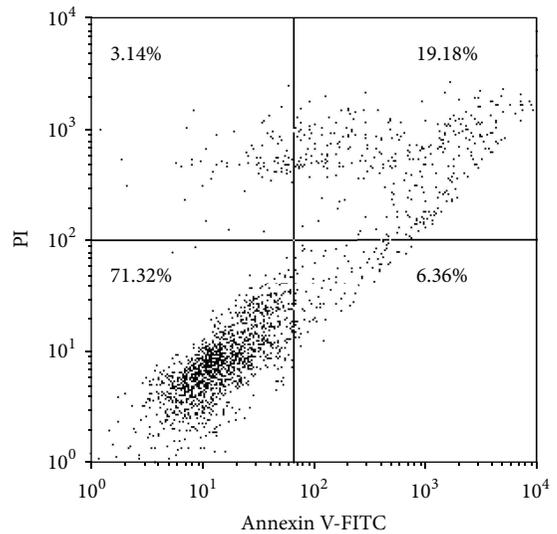
(f) Cells treated with methanolic extract of pericarp for 72 hours

FIGURE 1: Continued.

(g) Cells treated with acetone extract of cotyledon for 24 hours

(h) Cells treated with acetone extract of cotyledon for 48 hours

(i) Cells treated with acetone extract of cotyledon for 72 hours

(j) Cells treated with methanolic extract of cotyledon for 24 hours

(k) Cells treated with methanolic extract of cotyledon for 48 hours

(l) Cells treated with methanolic extract of cotyledon for 72 hours

FIGURE 1: (a–l) Dot plots from FACS analysis of MDA-MB-468 cells treated with acetone extract of pericarp (a–c), methanolic extract of pericarp (d–f), acetone extract of cotyledon (g–i), and methanolic extract of cotyledon (j–l). All these extracts from *V. diospyroides* type SS fruit were used at half IC_{50} dose level and compared across a range of treatment times (24–72 h).

Pericarp-methanol	Pericarp-acetone	Cotyledon-methanol	Cotyledon-acetone	
0 12 24 48	0 12 24 48	0 12 24 48	0 12 24 48	p53
				Bax
				Actin
MDA-MB-468	MDA-MB-468	MDA-MB-468	MDA-MB-468	
(a)	(b)	(c)	(d)	
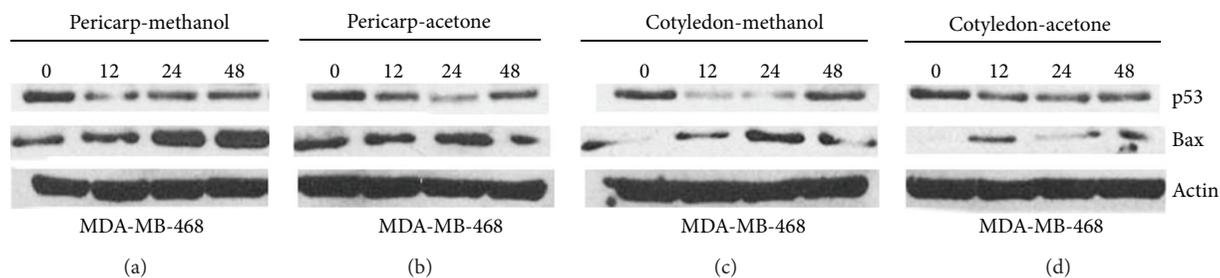
Figure 2: Effect of *V. diospyroides* type SS fruit on expression of the tumor suppressor protein p53 and proapoptotic protein, namely, the Bax. (a-b) MDA-MB-468 cells were treated with the extract of fruit pericarp, whereas (c-d) the cells were treated with the extract of fruit cotyledon, all at concentration of half IC_{50}. The cellular proteins were separated on SDS-polyacrylamide gels and subsequently transferred to membranes. The protein levels were determined by western blots with appropriate antibodies directed against each protein.

Table 2: The acetone and methanol extracts of *V. diospyroides* type SS fruit at their IC_{50} concentrations (see Table 1) were inactive on normal Vero cells. The cell growth was assessed by a green fluorescent protein (GFP) assay.

Extract-fruit part	Extract concentrations (μg/mL)	% Cell growth*	Activity	IC_{50} (μg/mL)
Acetone-cotyledon	50.00	26.53 ± 11.34^b	Cytotoxic	43.90 ± 4.67
	16.67	102.74 ± 2.56^a	Noncytotoxic	N/A
	5.56	104.17 ± 14.26^a	Noncytotoxic	N/A
	1.85	108.71 ± 10.00^a	Noncytotoxic	N/A
	0.62	107.94 ± 10.79^a	Noncytotoxic	N/A
	0.21	110.78 ± 12.40^a	Noncytotoxic	N/A
Acetone-pericarp	50.00	103.49 ± 32.03^a	Noncytotoxic	N/A
	16.67	114.56 ± 7.96^a	Noncytotoxic	N/A
	5.56	110.46 ± 11.19^a	Noncytotoxic	N/A
	1.85	108.13 ± 10.80^a	Noncytotoxic	N/A
	0.62	110.54 ± 5.94^a	Noncytotoxic	N/A
	0.21	111.26 ± 6.65^a	Noncytotoxic	N/A
Methanol-cotyledon	50.00	88.01 ± 15.42^a	Noncytotoxic	N/A
	16.67	115.46 ± 11.87^a	Noncytotoxic	N/A
	5.56	112.69 ± 8.84^a	Noncytotoxic	N/A
	1.85	116.70 ± 11.63^a	Noncytotoxic	N/A
	0.62	116.26 ± 11.07^a	Noncytotoxic	N/A
	0.21	117.57 ± 7.79^a	Noncytotoxic	N/A
Methanol-pericarp	50.00	25.34 ± 15.50^b	Cytotoxic	48.75 ± 0.79
	16.67	112.68 ± 6.99^a	Noncytotoxic	N/A
	5.56	104.09 ± 6.55^a	Noncytotoxic	N/A
	1.85	104.45 ± 11.47^a	Noncytotoxic	N/A
	0.62	104.64 ± 6.67^a	Noncytotoxic	N/A
	0.21	104.89 ± 9.67^a	Noncytotoxic	N/A

N/A means not applicable IC_{50} calculation.
-*Cell growth reduced to less than 50% would indicate cytotoxicity against the Vero cells; otherwise an extract was considered inactive and the IC_{50} level could not be determined (N/A). Three independent replicates were performed.
-Different superscripts within a column indicate statistically significant differences in the Vero cell growth (mean ± SD) according to Tukey's multiple comparison test.

induction of apoptosis. A higher cytotoxic activity against cancer cells, indicated by a lower IC_{50}, is anticipated with active apoptosis. Our results suggest that all extracts from type SS fruit have cancer chemopreventive potential *in vitro,* inhibiting cell proliferation with moderate to very strong activity ($IC_{50} < 20\,\mu$g/mL) [12, 13]. Furthermore, testing against the normal Vero cell-line did not show cytotoxicity; instead we observed cell growth >50% indicating that at IC_{50} the extracts might not inhibit normal cells. The various fruit parts and various organic solvents for extraction were explored. Acetone and methanolic extracts of cotyledon were the best in terms of cytotoxicity with 1.6 and 3.5 μg/mL IC_{50}

values. Both the more polar methanol and acetone have been widely used in the extraction of chemopreventive agents from various plant species.

In agreement with earlier results on the type LS fruit, acetone and methanol have been generally described as most suitable solvents for extraction that recover terpenoids, anthraquinones, and saponins in the crudes [6]. These phytochemicals have been investigated and shown as chemopreventives for various cancer therapies [15–17]. The crude extract of *V. diospyroides* type SS fruit might combine several active ingredients with antiproliferation effects on MDA-MB-468 cancer cells. On the other hand, the resveratrol tetramers vaticanol and vaticaphenol A have been first purified from the stem of *V. diospyroides* and have therapeutic properties that are documented against various cancers [7]. Subsequently resveratrol as a nontoxic agent has shown antineoplastic activity and very high efficacy in various cancer therapies [18–20]. Therefore, the therapeutic effects of *V. diospyroides* type SS fruit against MDA-MB-468 cells might be caused not only by the phytochemicals but also by the resveratrol compounds.

Most anticancer agents with minimal or absent side effects on normal cells inhibit cancer cell proliferation by cytotoxicity and activation of apoptosis [21, 22]. Our fruit extracts from type SS *Vatica* inhibited the proliferation of both ER-rich MCF-7 and ER-poor MDA-MB-231 breast cancer cell-lines, along pathways that might depend on the dose level [5]. An extract dose level at half IC_{50} was effective and only activated apoptosis of MCF-7. In contrast, death by necrosis was rapidly induced by acute toxicity at IC_{50} or higher dose levels. Apoptosis is precisely indicated by the loss of plasma membrane phospholipid asymmetry, with enzymatic cleavage of the DNA into oligonucleosomal fragments, and the subsequent segmentation of a cell into apoptotic bodies [23]. This is an important defensive mechanism against cancer progression with successes in cancer treatment [2]. Therefore, to avoid acute toxicity and side effects typical with a high dose level, low dose efficacy is critical to cancer chemopreventive agents. In the present work, half IC_{50} doses of the fruit extracts of *V. diospyroides* type SS were highly effective *in vitro* and induced apoptosis of breast cancer cell-lines.

Several techniques, including immunohistochemistry, western blots, and FACS, are known to elucidate the progression and mechanisms and discriminate between various types of cell death. By FACS analysis, various events, such as the blocking of cell cycle, activation of apoptotic genes or proteins, and loss of plasma membrane phospholipid asymmetry, can be detected and quantified to determine the apoptosis pathway [23]. Using live cancer cells without fixation, plasma membrane changes associated with apoptosis have been detected with Annexin V-FITC/PI bindings that represent apoptotic exploration and by monitoring the location of phosphatidylserine (PS) and integrity/permeability of cell membranes after treatment [5]. We investigated the apoptosis of breast cancer cells treated with extracts of type SS fruit, by Annexin V-FITC/PI binding assay using FACS method. After the FACS analysis, the traces of MDA-MB-468 cells treated at half IC_{50} concentration are shown in dot plots by symbol "7," and criteria earlier described by us were applied to

label the type of cell death [5]. The fruit extracts significantly induced apoptosis without necrosis in the MDA-MB-468 cells. Interestingly, with 48–72 hours of treatment, the acetone extracts of cotyledon and pericarp gave the highest fractions of apoptotic cells and the lowest fractions of viable cells. On the other hand, with a methanolic extract of pericarp, a continuous inhibition response to treatment time was observed. The viable cells and early apoptotic cells decreased consistently from 24 to 48 to 72 hours of treatment, while the counts of late apoptotic and nonviable cells increased. This indicates long-term therapeutic effects, not present with the acetone extract. Also, for the efficacy of therapeutic effects, at least 48 hours of treatment with the methanolic extract of cotyledon are required. These results confirm that the various extracts had different basic pharmacokinetic actions, in terms of both cytotoxic dose levels and dosing time effects.

The detailed mechanisms of apoptosis cannot be fully determined with an Annexin V-FITC/PI binding assay. Those mechanisms that involve the tumor suppressor protein (p53) and its products are still under study in several laboratories [2]. The p53 protein promotes apoptosis by proapoptotic and antiapoptotic pathways involving the Bcl2-family, whereas lack of p53 protein increases the risk of tumor emergence. The target of p53 in the Bcl-2 family is the Bax protein, a proapoptotic protein that promotes the release of cytochrome *c* from mitochondria inducing apoptosis [23]. In the present study, although mutant p53 is commonly known, the expressions of p53 and Bax protein are closely related to the dynamics of cells in apoptotic pathway detected by FACS method. Twelve to twenty-four hours of dosing with acetone extract of cotyledon and pericarp and methanolic extract of cotyledon gave the best results in terms of high percentage of apoptotic cells (up to 33%) and low percentage of living cells (down to 65%). On the other hand, treating with methanolic extract of pericarp in long period (more than 48 hours) could induce continuously apoptosis in time-dependent responses resulting in the highest percentage of dead cells (3.14%).

Therefore, p53 and Bax protein are now critical to the successful induction of apoptosis, and other key factors such as Bcl-2 protein, caspase-3, and PARP will be detailed in our future research on the MDA-MB-468 cells treated with *V. diospyroides* type SS fruit extracts.

5. Conclusions

The cotyledon and pericarp extracts of *V. diospyroides* type SS fruit significantly inhibited the ER-poor MDA-MB-468 breast cancer cell-line *in vitro*, even at half IC_{50}. The mechanism of this therapeutic action was apoptosis induced during 48–72 hours of treatment, with appropriate dose depending on the type of extract used. The results suggest these extracts might support future clinical therapies, especially against both ER-rich and ER-poor breast cancers.

Conflict of Interests

The authors have declared that there is no conflict of interests regarding this paper.

Acknowledgments

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Review Article

Emerging Anticancer Potentials of Goniothalamin and Its Molecular Mechanisms

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The treatment of most cancers is still inadequate, despite tremendous steady progress in drug discovery and effective prevention. Nature is an attractive source of new therapeutics. Several medicinal plants and their biomarkers have been widely used for the treatment of cancer with less known scientific basis of their functioning. Although a wide array of plant derived active metabolites play a role in the prevention and treatment of cancer, more extensive scientific evaluation of their mechanisms is still required. Styryl-lactones are a group of secondary metabolites ubiquitous in the genus *Goniothalamus* that have demonstrated to possess antiproliferative activity against cancer cells. A large body of evidence suggests that this activity is associated with the induction of apoptosis in target cells. In an effort to promote further research on the genus *Goniothalamus*, this review offers a broad analysis of the current knowledge on *Goniothalamin* (GTN) or 5, 6, dihydro-6-styryl-2-pyronone ($C_{13}H_{12}O_2$), a natural occurring styryl-lactone. Therefore, it includes (i) the source of GTN and other metabolites; (ii) isolation, purification, and (iii) the molecular mechanisms of actions of GTN, especially the anticancer properties, and summarizes the role of GTN which is crucial for drug design, development, and application in future for well-being of humans.

1. Background

Cancer continues to be one of the major causes of death worldwide, despite technological advancements in various fields during the last two decades [1, 2]. Current estimates from the American Cancer Society and from the International Union against Cancer indicate that 12 million cases of cancer were diagnosed last year, accounting for 8.2 million deaths in 2012 worldwide; these numbers are expected to double by 2030, of which 62% arise in developing countries (27 million cases with 17 million deaths) [1–4]. As many as 95% of all cancers are caused by life style (lack of physical activity, tobacco, and alcohol use) and may take as long as 20–30 years to develop [5]. Due to its complex nature, treatment such as surgery, chemotherapy, photodynamic therapy (PDT), and radiation varies according to each type, location, and stage [6].

Medicinal plants are widely used by majority of populations as primary healthcare to cure various diseases and illnesses and have high an economic impact on the world economy [7, 8]. The increasing interest and scope of the drug of natural origin provides opportunities for its exploration, investigation, and utilization for biological activity [9–11] and particularly considered as cancer preventive or anticarcinogenic agents if they show good availability, low toxicity, suitability for oral application, and a vast variety of mechanisms of their action to prevent or at least delay and inhibit multiple types of cancer [12]. Various bioactive compounds from plant extracts have been experimentally tested to expand the clinical knowledge for its biological effects. As such, natural products have provided a continuous source of novel chemical structures in the development of new drugs and approximately 119 pure compounds isolated from plants are being used as medicine throughout the world.

2. Plants as Source of Anticancer Agents

Plants have a long history of use in the treatment of cancer. More than 3000 plant species have been reported to be

involved in the development of anticancer drugs [13] and 60% of current anticancer agents have come from natural sources [14, 15] which include vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivative (etoposide, teniposide), camptothecin and its derivatives (topotecan, irinotecan), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin), and others. Anticancer drugs target several cellular components and activate responses that go from cell repair to cell death [16, 17].

3. *Goniothalamus* spp.

Goniothalamus is one of the largest genera of palaeotropical Annonaceae, with over 160 species distributed throughout tropical southeast Asia; the centre of diversity lies in Indochina and Western Malaysia [18]. Only 22 (13.7%) out of 160 species of *Goniothalamus* have so far been recognized and investigated out of which only five are medicinal, which are used to treat asthma, rheumatism, fever, malaria, cholera, stomachache, postpartum protective remedy, abortifacient, and insect repellent [19]. Various compounds have been isolated from *Goniothalamus* species, especially the low molecular weight phenolic styryl-pyrone derivatives as lactonic pharmacophore, quinoline, and isoquinoline alkaloid derivatives and phenanthrene lactones, terpenes, acetogenins, and flavonoids [20–25]. Few styryl-lactones extracted from *Goniothalamus* are (i) goniothalamin, (ii) altholactone, and (iii) cardiopetalolactone [26].

4. Bioactive Components of *Goniothalamus* spp.

Acetogenins and styryl-lactones from *Goniothalamus* species have shown to be cytotoxic to different human tumor cell lines [27–29]. Other reported biological properties of some compounds are antifungal, antiplasmodial, antimycobacterial, insecticidal, antimalarial, anti-inflammatory, immunosuppressive, and inhibitor of platelet-activating factor (PAF) receptor binding activities [30, 31]. Currently, 100 styryl-lactones are available approximately which are either discovered from natural products or made as synthetic analogs. These compounds have been demonstrated to be cytotoxic with preference to kill cancer cells [28, 32–34].

It was reported [26] that GTN as the active constituent of the bark of *G. andersonii, G. macrophyllus* Miq., and *G. malayanus* and altholactone was characterized from *G. arvensis* Scheff. and from the *G. borneensis* Mat-Salleh [35, 36]. Cardiopetalolactone was characterized from the stem bark of *G. cardiopetalus* Hook.f. & Thoms. with altholactone, (iv) goniopypyrone, goniothalamin, (v) goniodiol, (vi) goniofufurone, and (vii) goniofupyrone [37, 38]. Goniofufurone, goniopypyrone, goniothalamin, goniodiol, (viii) goniotriol, and (ix) 8-acetylgoniotriol were isolated from the roots of *G. griffithii* [21–23]. An isomer of altholactone and (x) (+)-isoaltholactone was isolated from stem bark of *G. malayanus*, and from the leaves of *G. montanus* J. Sincl. and the roots of *G. tapis* Miq. [39] whereas goniolactones were identified
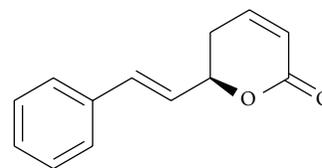

FIGURE 1: Chemical structure of goniothalamin.

from the roots of *G. cheliensis* [40]. Digoniodiol, deoxygoniopypyrone A, goniofupyrone, goniothalamin, deoxygoniopypyrone A, gonodiol-8-monoacetate, and gonotriol (xi) were characterized from the aerial parts of *G. amuyon*, collected in the southern part of Taiwan near the coastal regions [25, 41–45]. The petroleum ether extract of the stem bark of *G. sesquipedalis* collected in Bangladesh yielded 5-isogoniothalamin oxide [44] and 5-acetyl goniothalamin (xii) was characterized from *G. uvaroides* King collected in Bangladesh [34] and Chen et al. [46] isolated howiinol A from *G. howii* Merr. (xiii). The mode of cytotoxic action of styryl-lactone is described subsequently.

5. Isolation and Purification of Goniothalamin

Styryl-lactone GTN (Figure 1) was first isolated in 1972 [26, 47] since then it was subjected to extraction, isolation, and characterization. In most cases, the extracts were prepared by hot and cold extraction methods, that is, Soxhlet and percolation techniques, respectively. The crude methanol extracts were obtained by removing the solvent under reduced pressure and the yields were calculated based on dry weight. Bioactive compounds were isolated using various chromatographic techniques (VLC, column chromatography, Prep-TLC, etc.). The structures of bioactive compounds were also elucidated using spectroscopic techniques (1D, 2D NMR spectroscopy, FTIR, UV, mass spectrometry, etc.). Chromatographic fingerprint (HPLC) and spectrophotometric fingerprinting (ATR-FTIR) analyses with reference markers were also carried out on the plant extract. Briefly, the herbs were ground to powder, extracted in MeOH by ultrasonication for 30 min, and filtered. The chromatographic system consists of a HPLC equipped with a secondary pump, a diode-array detector, an autosampler, and a column compartment, a C18 column packed with 5 μm diameter particles. A suitable solvent system was used for extraction process, for example, trifluoroacetic acid and acetonitrile was used with a linear gradient elution. Analytical technique using HPLC-DAD was developed and used to quantify the bioactive components of each extract as marker compounds. Preparation of the herb and the HPLC setup varied as per individual laboratory set up [48, 49].

6. Synthesis of Goniothalamin

Due to its diverse pharmacological properties, GTN gained huge interest from researchers because several successful approaches have been adopted for its synthesis [50–54]. The absolute configuration in the pyran-2-one
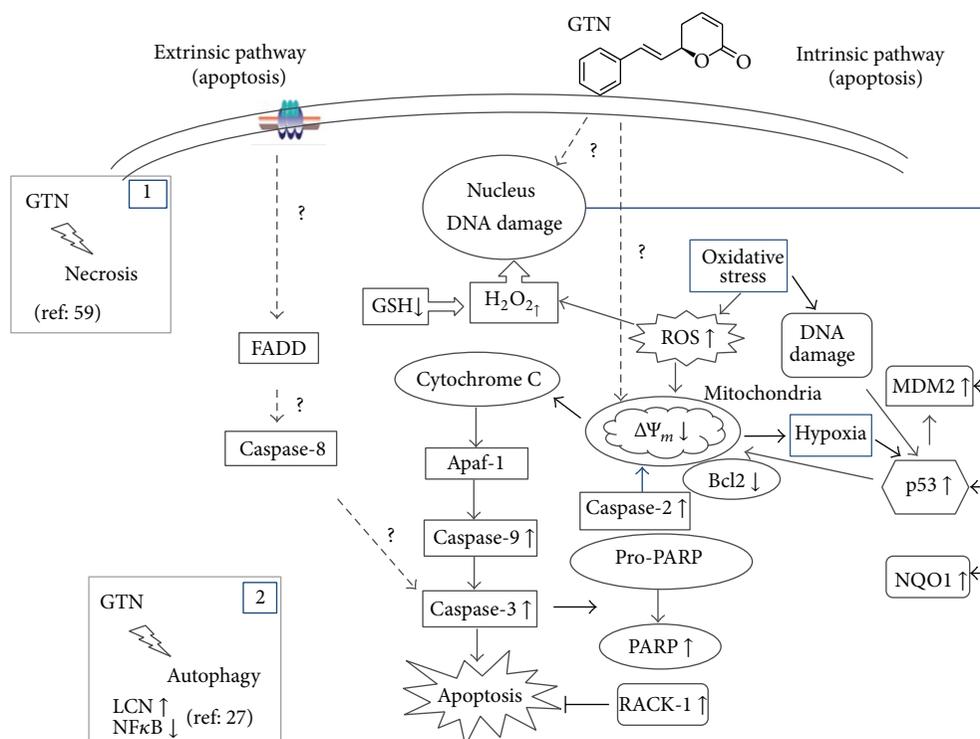
FIGURE 2: Schematic representation of mechanism of action of goniothalamin (GTN) in cancer cells. GTN mostly induces apoptosis either by DNA damage from oxidative stress where GTN decreases GSH level and increases ROS production or direct effect on DNA. Alternatively, GTN may directly affect mitochondria leading to ROS production. The GTN induced cellular stress response leads to the upregulation of p53 as an initial signal for apoptosis. Once activated, the p53 protein can directly or via processing caspase-2 trigger the release of cytochrome c without loss of membrane potential. This is followed by caspase-9 and caspase-3 subsequently. GTN may also act directly on mitochondria or induce the upregulation of Fas/FasL but that needs to be further investigated.

moiety has generally been secured from chiral starting material, asymmetric allylboration of aldehydes with β-allyldiisopinocampehylborane [50, 55, 56], or through asymmetric reduction using enzymes or microorganisms [51, 53, 54, 57–61]. De Fátima and Pilli [51] reported the syntheses of GTN via catalytic asymmetric allylation of α-benzyloxyacetaldehyde, followed by ring-closing metathesis and Wittig olefination, and via catalytic asymmetric allylation of trans-cinnamaldehyde, followed by ring-closing metathesis [62]. Gruttadauria et al. [54] along with coworkers reported that the high-yielding three-step synthesis of GTN involves an enzymatic kinetic resolution in the presence of vinyl acrylate followed by ring-closing metathesis [54]. GTN has been synthesized by lipase catalyzed resolution of (1E)-1-phenylhexa-1, 5-dien-3-ol using vinyl acrylate as acyl donor followed by ring-closing metathesis of the formed (1R)-1-[(E)-2-phenylvinyl] but-3-enyl acrylate. The unreacted alcohol from the resolution, (1E, 3S)-1-phenylhexa-1, 5-dien-3-ol, was esterified nonenzymatically and used for synthesis of GTN [53]. Das et al. [63] reported that the stereo selective total synthesis of GTN is achieved via a common intermediate. The synthesis employed the reduction of a propargyl ketone and olefin cross-metathesis as the key steps [63]. Fournier et al. showed that the diastereoselective [2+2]-cycloaddition of β-silyloxy aldehydes with trimethylsilylketene followed by

HF-induced translactonization is a useful short method for the efficient synthesis of α, β-unsaturated-δ-lactones [64].

7. Mechanism of Action

7.1. Cytotoxic Activity against Cancer Cells.
GTN, a simple styryl-lactone has significant potential in the development of a cancer drug as it has been reported to possess a wide range of biological activities (Figure 2) including anticancer [34], anti-inflammatory [65], immunosuppressive, and apoptotic effects [21, 24, 28, 66–68]. GTN had been able to induce cytotoxicity in a variety of cancer cell lines including vascular smooth muscle cells (VSMCs), Chinese hamster ovary cells, renal cells [69–71], hepatoblastoma [72, 73], gastric, kidney cells, breast carcinomas, leukemia, Jurkat cells [67, 69, 74–84], hepatocellular carcinoma [85], lung cancer cells [86], oral cancer cells [87, 88], and HeLa cells [89, 90] but sparing the normal cells including blood cells [83].

Besides the above, GTN has been proved to be only cytotoxic to ovarian cancer cell line (Caov-3) without causing cell death in normal kidney cell (MDBK) when compared to tamoxifen or taxol treated cells [32]. In addition, GTN showed lower toxicity to normal liver Chang cell line as compared to doxorubicin (known chemotherapeutic drug) [72, 73]. On the other hand a study by [75] reported the

antiproliferative activity of GTN in some solid tumor experimental model with no evidence of toxic effects in the animals after single and repeated doses.

7.2. Induction of Apoptosis.
GTN initially induces DNA damage which subsequently leads to cytotoxicity primarily via apoptosis in VSMCs [78]. This finding indicates that apoptosis that had occurred on this method was previously described by Cohen [91] and Ren et al. [92] and others on HeLa cells [92, 93]. The above findings were confronted by Alabsi et al. [90] that GTN stimulate DNA fragmentation, a characteristic feature of apoptosis in HeLa cell line at 24, 48, and 72 h after treatment. DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 base pairs subunit. DNA ladder formation is observed only when the extent of oligonucleosomal cleavage is prominent. Alabsi et al. [90] suggested that internucleosomal cleavage of DNA is likely to be in the later phase of apoptotic process [91, 94, 95]. Some evidence has indicated that GTN exposure can alter the membrane properties [67].

Apoptosis can be either caspase-dependent or caspase-independent [96, 97]. However, the mechanism of caspase-independent apoptosis was still poorly understood until recently. Caspase plays important roles in execution of apoptosis through either extrinsic or intrinsic pathways [33]. The ability of GTN to induce apoptosis via caspase-3 activation against hepatoblastoma (HepG2) cells, whereas in human Jurkat T-cells both caspases 3 and 7 activation is involved, which is totally absent in normal Chang liver cells [24] and caspases 3 and 7 in human Jurkat T-cells [81]. In this study, HepG2 and Chang cells were treated with GTN for 72 h and analysed by TUNEL and Annexin-V/PI staining. Furthermore, the postmitochondrial caspase-3 was quantified using ELISA and alteration of cellular membrane integrity and cleavage of DNA were also observed. On the other hand, postmitochondrial caspase-3 activity was significantly elevated in HepG2 cells treated with GTN after 72 h. These findings suggest that GTN induced apoptosis on HepG2 liver cancer cells via induction of caspase-3 with less sensitivity on the cell line of Chang cells. Besides the above, it was also shown that the executioner caspase-3/7/9 activity, not initiator caspase-8, was increased in low level, less than onefold at 6 hours and 24 hours of treatment with GTN as compared to untreated cells [90]. Previous study also reported that the sequential activation of caspase-9 but not caspase-8 leading to the downstream caspase-3 cleavage was observed in GTN-treated coronary artery smooth muscle cells (CASMCs) [79].

It has also been reported that GTN induced apoptosis in HL-60 and Jurkat cells via mitochondrial pathway [67, 82]. Thus, these findings suggested the insignificant role of caspase-8 as an initiator caspase. Caspase-8 is not essential in GTN induced apoptosis in HeLa cells. In order to rule out the possibility of caspase-8 involvement in GTN induced apoptosis, a detailed appropriate study is still required. de Fátima et al. [70] reported that R-GTN and S-GTN markedly downregulated Bcl2, an antiapoptotic protein, and also induced PARP cleavage by causing apoptosis in renal cancer

cells. In this study, authors have also reported interestingly that S-GTN enhanced the expression of LC3; a typical marker of autophagy and NFkappaB was downregulated in S-GTN-treated cells. Overall, these results indicate that the antiproliferative activity of the two enantiomers of GTN on renal cancer cells involved distinct signaling pathways, apoptosis, and autophagy as dominant responses towards R-GTN and S-GTN, respectively. Also, it was reported that GTN treatment induces cell cycle arrest at G2/M level [33] and concentration dependent necrotic type of cell death [74]. However, most of the studies have reported that GTN induced cell death predominantly occurred through apoptosis mode only.

It has been reported that cytotoxic stress either from DNA damage or mitochondrial impairment leads to apoptosis via the intrinsic pathway [78, 98]. The intrinsic pathway involves the release of proapoptotic proteins including cytochrome c from the inner membrane of mitochondria to the cytosol leading to activation of caspase-9 [99]. Most of the styryl-lactones including GTN and altholactone induce oxidative stress in MDA-MD-231 breast cancer cells, and Jurkat and HL-60 leukemic cells leading to apoptosis [40, 92, 100]. Although previous work has demonstrated that GTN induces DNA damage in CASMCs, which subsequently leads to apoptosis induction [101] and this study hypothesizes that GTN-induced oxidative stress and DNA damage resulted in p53 upregulation which was stabilized by NQO1 leading to caspase-2-dependent mitochondrial-mediated apoptotic pathway. However, the mechanisms of oxidative stress induced by styryl-lactones have not been unraveled. Numerous studies have demonstrated that the oncoprotein Bcl-2 can inhibit apoptosis by inhibiting the release of cytochrome c and can also modulate oxidant induced apoptosis [102]. Since the discovery of the caspase-9 apoptosome complex [103], more recent studies have shown that the initiator caspase-2 also forms a complex with RAIDD, a death receptor molecule, and the p53 inducible death domain PIDD forming a PIDDosome complex [104]. Importantly, caspase-2 has been demonstrated in a variety of cell lines to be activated upstream of mitochondria in genotoxin-induced apoptosis. Cleavage of the proapoptotic Bcl-2 family member Bid by caspase-2 has been shown to be required for cytochrome c release suggesting a potentially crucial role for caspase-2.

Although a large body of evidence suggests that various plant metabolites exerted their potentials against many cancer types through their unique mechanism of action for example, vincristine inhibits microtubule assembly, inducing tubulin self-association into coiled spiral aggregates [105]. Etoposide, a topoisomerase II inhibitor [106, 107] causes the stabilization of the clevable DNA- topoisomerase II covalent complexes, preventing subsequent DNA religation and stimulate enzyme-linked DNA breaks [108]. The taxanes paclitaxel and docetaxel has shown antitumor activity against breast, ovarian, and other tumor types in the clinic trial. Paclitaxel stabilizes microtubules and leads to mitotic arrest [109]. In addition, the camptothecin derivatives irinotecan and topotecan have shown significant antitumor activity against colorectal and ovarian cancer, respectively [100, 110], by inhibiting topoisomerase I [111]. Despite the above development, the unequal distribution of cancer burden

TABLE 1: Mechanism of action of Goniothalamin (GTN) in various cancer cells and their molecular effects.

S. no	Cell line (in vitro)	Animals (in vivo)	Mode of cell death	Molecular targets/effects	References
1	786-0 (renal cells)	—	Cytotoxicity/apoptosis	NOS↑/BCL2↓	[27, 70]
2	786-0 (renal cells)	—	Cytotoxicity/autophagy	LC3↑/NFκB↓	[27]
3	Jurkat T-cells	—	Cytotoxicity/apoptosis	Caspases 3 and 7↑, oxidative stress, DNA damage RACK1↑	[81, 82] [80] [70]
4	HepG2 (hepatoblastoma) Chang (normal cells)	—	Cytotoxicity/apoptosis No toxicity	Caspase-3↑ Sparing normal cells	[72, 73] [72]
5	HCC (hepatocellular carcinoma)	—	Cytotoxicity/apoptosis	ROS↑	[85]
6	Caov-3 (ovarian) Caov-3 (ovarian) MDBK (normal kidney cells)	—	Cytotoxicity/apoptosis Antiproliferative No toxicity	Caspase-3↑ bcl-2↓ and bax↑ Sparing normal cells	[32] [77] [80]
7	MCF-7, T47D, MDA-MB-231 (breast cancer)	—	Cytotoxicity/apoptosis	Cell cycle arrest/modulating redox status	[33, 89]
8	MCF-7 (breast cancer)	—	Cytotoxicity/necrosis	Membrane integrity loss	[74]
9	COR-L23 (large cell lung carcinoma)	—	Cytotoxicity	Good cytotoxic compound to cancer cells	[68]
10	NCI-H460 (human nonsmall cell lung cancer cells)	—	Cytotoxicity/apoptosis	DNA damage	[86]
11	Ca9-22 (oral cancer)	—	Cytotoxicity/apoptosis	DNA damage, ROS↑, ΔΨ ↓	[88]
12	U251 (glioma)	—	Antiproliferative	Good cytotoxic compound to cancer cells	[65]
13	OVCAR-03 (ovarian)	—	Antiproliferative	Good cytotoxic compound to cancer cells	[65]
14	PC-3 (prostate)	—	Antiproliferative	Good cytotoxic compound to cancer cells	[65]
15	W7.2 T-cells	—	Cytotoxicity/apoptosis	DNA damage, RACK1↑	[70]
16	NCI-460 (lung, nonsmall cells)	—	Antiproliferative	Good cytotoxic compound to cancer cells	[65]
17	NSCLC lung cancer	—	Cytotoxicity/apoptosis	DNA damage, MMP-2 and MMP-9↓	[87]
18	UACC-62 (melanoma)	—	Antiproliferative	Good cytotoxic compound to cancer cells	[65]
19	HL-60 (leukemia)	—	Genotoxicity/apoptosis	Ψ ↓, caspase-9↑	[67, 80] [84, 101]
20	U937 (lymphoma)	—	Cytotoxicity/apoptosis	ΔΨ ↓, caspase-9↑	[84]
21	CASMC (coronary artery smooth muscle cells)	—	Cytotoxicity/apoptosis	Caspase-2↑, p53↑	[78, 79]
22	HeLa (cervical)	—	Cytotoxicity apoptosis	Good cytotoxic compound to cancer cells DNA damage, caspase-9↑	[80–82] [90]
23	HGC-27 (gastric)	—	Cytotoxicity	Good cytotoxic compound to cancer cells	[74, 80–82]
24	768-0 (kidney)	—	Cytotoxicity	Good cytotoxic compound to cancer cells	[80–82]
25	HT-29 (colon) LS174T (colon)	— —	Cytotoxicity/apoptosis	Cell cycle arrest at S-phase	[89] [68]
26	3T3 (normal fibroblast) ST3 fibroblast	— —	No toxicity Cytotoxicity	Sparing normal cells Kills MMP1 expressing cells	[89] [68]
27	PANC-1 (pancreatic cancer)	—	Cytotoxicity/necrosis	Loss of cell membrane integrity	[74]
28	CHO (Chinese hamster ovary)	—	Genotoxicity	Causing damage to DNA	[69]
29	K562 (chronic myelogenous leukemia)	—	Cytotoxic and anti-inflammatory	NF-κB↓	[83]

S. no	Cell line (in vitro)	Animals (in vivo)	Mode of cell death	Molecular targets/effects	References
30	Platelets (rabbits)	—	Inhibitory	Platelet activating factor binding	[31]
31	Ehrlich tumor cells	Balb/C mice	Cytotoxicity	Tumor regression	[65]
32	Blood and serum parameters	Long Evans rats	Cytotoxicity	Biochemical/hematology and histopathology evaluation	[47]

between the developing and developed world is still largely looking for a better and safer anticancer compound for human use. Based on the data obtained from both *in vitro* cell culture and few *in vivo* animal models, GTN has demonstrated its potential against cancers and proven its insignificant effects on normal cells (Table 1). Taken together, undoubtedly GTN is emerging as promising agent in anticancer drug development with potential applications in cancer chemotherapy.

8. Conclusion

In conclusion, styryl-lactones are a group of secondary metabolites ubiquitous in the genus *Goniothalamus* that has demonstrated to possess interesting biological properties. These findings revealed that *Goniothalamus* plants do possess anticancer activity in a selective manner towards several tumor cell lines and initiate them to undergo different mode of cell death mainly apoptosis. Although the anticancer activity of the potential biomarker of this herbal plant, GTN on multiple cancer cells was through its regulation on cancer cell cycle and apoptosis induction mediated via oxidative stress and caspases activation and the antimetastatic and antiangiogenesis effects observed in GTN treated cells and animal, indicate its potential in inhibiting the development of secondary tumour. Further investigations into the mechanism of anticarcinogenic, antimetastatic, antiangiogenesis, and apoptotic regulation properties of GTN against various in vivo cancer models are still required. This may create an opportunity for the compound not only to be designed and developed as anticancer agent, but also to be used as an adjuvant or immunomodulators for combination chemotherapy against cancer. However, the preliminary *in vitro* data is insufficient and less convincing due to its limitation as most of the experiments are done in an *ex vivo* environment outside an animal or human body. Thus, more *in vivo* studies using various experimental cancer animal models are needed to determine the pharmacological and toxicological data as well as antitumour effect of GTN. Due to its diverse pharmacological properties, this compound gained huge interest among researchers that lead to the cost effective approaches for its synthesis; hence, this activity will further strengthen the efforts to identify more pathways and therapeutic action of this compound before it enters into the next phase of development. Overall, this compound provides information on the safe use and effectiveness that is crucial for drug design, development, and application in future for well-being of human.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Anticancer Activity of Marine Sponge *Hyrtios* sp. Extract in Human Colorectal Carcinoma RKO Cells with Different p53 Status

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Drug development using marine bioresources is limited even though the ocean occupies about 70% of the earth and contains a large number of biological materials. From the screening test of the marine sponge extracts, we found *Hyrtios* sp. sponge collected from Chuuk island, Micronesia. In this study, the *Hyrtios* sp. extract was examined for anticancer activity against human colorectal carcinoma RKO cells that are wildtype for p53 and RKO-E6 that are p53 defective. The *Hyrtios* sp. extract dose-dependently inhibited viability in both cell lines. Multinucleation as an indication of mitotic catastrophe was also observed. Cytotoxicity tests gave significantly different results for RKO and RKO-E6 cells after 48 h exposure to *Hyrtios* sp. extract. In RKO cells treated with *Hyrtios* sp. extract, cell death occurred by induction of p53 and p21 proteins. In p53-defective RKO-E6 cells, *Hyrtios* sp. extract decreased expression of JNK protein and increased p21 protein. These results indicate that *Hyrtios* sp. extract induced apoptosis *via* different pathways depending on p53 status and could be a good natural product for developing new anticancer drugs.

1. Introduction

The ocean occupies about 70% of the earth and contains a huge number of marine organisms. Collection and identification of marine organisms were difficult for researchers and drug developers, but marine resources are still attractive for the application of pharmaceutical fields. Among marine resources, marine sponges are known to have about 15,000 species worldwide [1]. Marine sponges take in nutritious through body pores and produce secondary metabolites with bioactivity. In our ongoing research, we investigated the bioactivity of marine sponges before classifying and isolating their active compounds. Crude extracts were made from marine sponges collected from the Chuuk islands in Micronesia and investigated for anticancer effect. Screening tests identified some specimens with anticancer effects. One of the specimens was identified as *Hyrtios* sp. (Figure 1). Recently, *Hyrtios* sp. was reported to have cytotoxic [2, 3] and antioxidant activities [4]. Several *Hyrtios* metabolites

[5, 6] and active compounds [2, 4] have been reported, but the anticancer effects of *Hyrtios* sp. have not been reported. Herein, *Hyrtios* sp. extract was investigated for anticancer activity in human colorectal carcinoma RKO cells with different p53 status.

2. Materials and Methods

2.1. Specimen Preparation. Marin sponge specimens were collected by hand with scuba equipment at Chuuk state, Federated States of Micronesia, in October, 2010. Freshly collected specimens were washed by sterilized artificial sea water three times, immediately frozen, and stored at −20°C until use. Lyophilized specimens were extracted with methanol (3 × 3 L) as previous study [7]. All of the samples were provided from KIOST (Korea Institute of Ocean Science & Technology). The extracts of specimens (10 mg) were dissolved in sterile distilled water (the final concentration,

FIGURE 1: Morphology of *Hyrtios* sp. specimen before methanol extraction.

50 mg/mL). Aliquots of samples were stored at −20°C until use.

2.2. Cells and Treatment. Human colorectal carcinoma RKO (CRL-2577) and RKO-E6 (CRL-2578) cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, GenDEPOT) supplemented with 10% fetal bovine serum (GenDEPOT) and 1% penicillin/streptomycin (GenDEPOT) in a humidified 5% CO_2 incubator. Cells used for the assays were in exponential growth phase. The samples were treated to the cell culture for 24 h or 48 h.

2.3. Cell Cytotoxicity. Cell cytotoxicity was examined by Cell Counting Kit-8 (CCK-8, DOJINDO, Japan). Briefly, cells were seeded in 96-well plates at a density of 3×10^3 cells/well. After incubation for 24 h, cells were treated with sponge samples for 24 h or 48 h. CCK-8 reagent (10 μL) was added to each well and incubated for 3 h at 37°C. Absorbance at 450 nm was determined through microplate reader (Infinite M200 PRO, TECAN, Austria).

2.4. Cellular Morphology. Cells were seeded in 6-well plate at 4–6 × 10^4 cells/well and treated with *Hyrtios* sp. extract (80 μg/mL) for 48 h. Cells were observed under light microscopy (×40 and ×400) (Nikon eclipse TS100, Japan).

2.5. Western Blot Analysis. Cells were seeded in 6-well plate at a density of 4~6 × 10^4 cells/well. Samples were treated to each well and incubated for 24 h or 48 h. Cells were harvested and lysed in RIPA buffer (GenDEPOT) with protease inhibitors (Xpert protease inhibitor cocktail solution, GenDEPOT) and phosphatase inhibitors (Xpert phosphatase inhibitor cocktail solution, GenDEPOT). Cell lysates were boiled in 5× sample buffer and separated by 10% SDS-PAGE. Proteins were transferred onto PVDF membranes (Millipore) using a semidry electroblotter (Peqlab, Germany). Membranes were blocked with 5% skim milk in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated sequentially with primary antibodies at 4°C, overnight. After washing membranes with TBST, the membranes reincubated with secondary antibody at room temperature for 3 hr. Immunoreactive protein was visualized using ECL reagents and developed with X-ray film. Antibodies and ratios at

which they were used were p53, p21 (1 : 2000, Millipore), c-Jun N-terminal kinase (JNK, 1 : 500, Santa Cruz Biotechnology), β-actin (1 : 5000, Sigma-aldrich), and anti-mouse IgG (H+L) horseradish peroxidase conjugate and anti-rabbit IgG (H+L) horseradish peroxidase conjugate (1 : 3000, Bio-Rad).

2.6. Apoptosis. Cells were seeded in 6-well plate and treated as described for western blots. After incubation, cells were stained with Annexin V-FITC (Nexcelom Bioscience LLC) and propidium iodide (PI) solution. Apoptosis was detected using a Cellometer (Nexcelom Bioscience LLC).

3. Results and Discussion

3.1. Cytotoxicity of Hyrtios sp. Extract in RKO and RKO-E6. To evaluate cytotoxicity to cells with different p53 status, serially diluted samples of *Hyrtios* sp. extracts were treated to RKO and RKO-E6 cells for 24 h and 48 h. As shown in Figure 2(a), cytotoxicity slightly increased in both RKO and RKO-E6 cells. *Hyrtios* sp. extract (100 μg/mL) inhibited 52.7 ± 0.9% viability of RKO cells and 66 ± 0.9% viability of RKO-E6 cells. *Hyrtios* sp. extract (100 μg/mL) for 48 h inhibited 26.5 ± 0.7% and 44.5 ± 1% of cell viability in RKO and RKO-E6 cells, respectively (Figure 2(b)). *Hyrtios* sp. extract increased cytotoxicity time-dependently for RKO cells and RKO-E6 cells. The result showed that RKO cells were more sensitive than RKO-E6 cells to *Hyrtios* sp. extracts and indicated that the anticancer effects of *Hyrtios* sp. were different for RKO and RKO-E6 cells depending on their p53 status.

3.2. Hyrtios sp. Extract-Induced Mitotic Catastrophe. To investigate cell death induced by *Hyrtios* sp. extracts, cellular morphology was observed. RKO cells had fewer cells than RKO-E6 cells after treatment with *Hyrtios* sp. extracts (Figure 3(a)) consistent with cytotoxicity results. In particular, RKO cells treated with *Hyrtios* sp. extracts exhibited multinucleation and increased cell size, indications of mitotic catastrophe (Figure 3(b), *upper right arrow*). *Hyrtios* sp. might trigger the cell death induced by DNA damage.

3.3. Changes in p53, p21, and JNK Levels after Treatment with Hyrtios sp. Extract. To examine differences in cellular mechanisms between RKO and RKO-E6 cells, protein levels were determined by western blots. Expression of p53 protein was detected in RKO cells but not RKO-E6 cells (Figure 4). RKO cells treated with *Hyrtios* sp. extracts showed an increase of p53 protein levels and also increased expression of p21 protein which is encoded by a p53 target gene. In addition, p21 protein also increased in RKO-E6 cells treated with *Hyrtios* sp. extracts. JNK protein expression was slightly reduced in RKO and RKO-E6 cells (Figure 4). These results indicated that RKO cells exposed to *Hyrtios* sp. extracts induced cell death by increase of p53 protein, which activated the expression of p21 protein for 24 h (not shown) and
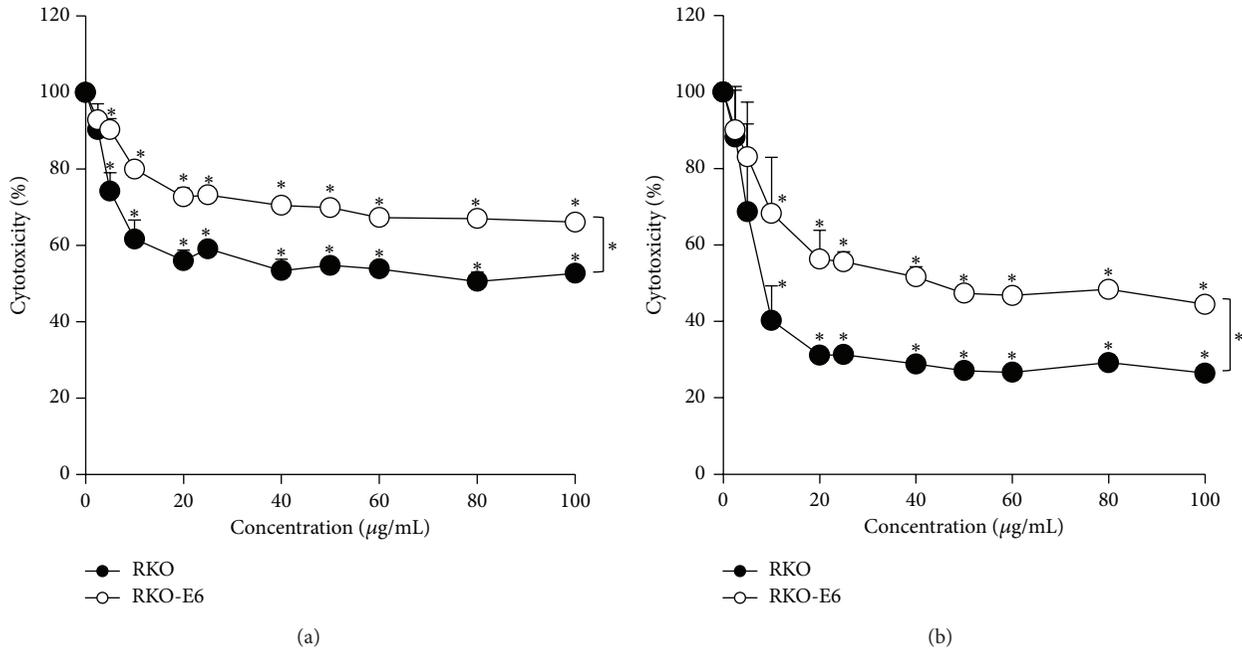
FIGURE 2: Inhibition of cell viability by *Hyrtios* sp. in RKO and RKO-E6 cells. Cells were treated with *Hyrtios* sp. extract and incubated for 24 h (a) and 48 h (b). Cytotoxicity was determined by CCK-8 assay. Data represent mean ± standard deviations ($n = 7$), *t*-test (*$P < 0.001$).
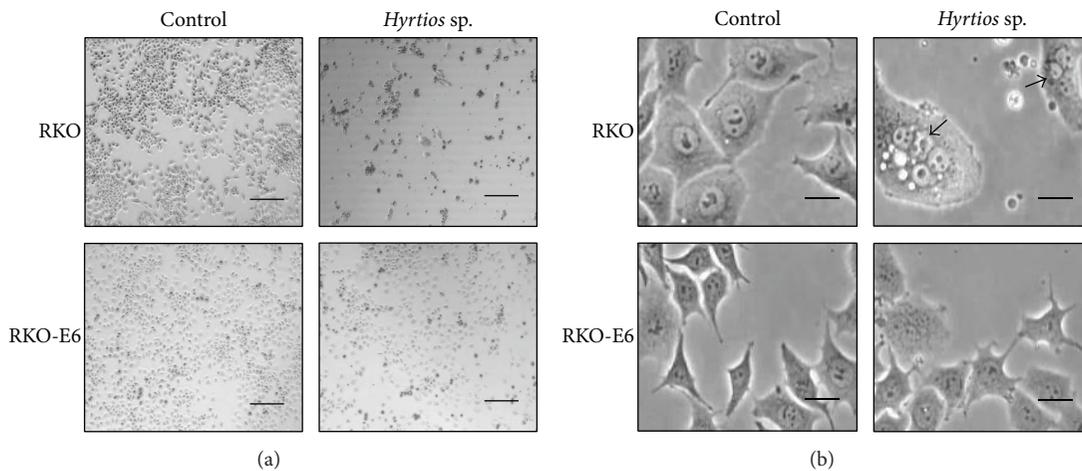

FIGURE 3: Change in cellular morphology after treatment with *Hyrtios* sp. extracts. Cells were treated with *Hyrtios* sp. extract (80 μg/mL) and incubated for 48 h. (a) Scale bar = 500 μm. (b) Scale bar = 50 μm.

48 h, consistent with previous studies [8–10]. Surprisingly, RKO-E6 cells, which lack functional p53, showed slightly induced p53 protein expression after treatment with *Hyrtios* sp. extracts. However, RKO-E6 cells treated with *Hyrtios* sp. extracts inhibited cell growth through induction of p21 protein *via* a p53-independent pathway. These results suggested that suppression of JNK protein might be involved in p21 protein expression as previous study [11]. The JNK pathway is involved in phosphorylation of c-Jun [12] and p53 [13] and regulation of cell growth [14]. As reported by Potapova

[15], inhibition of JNK reduced the growth of p53-deficient cells and induced p21 protein expression. Our results were consistent with this study [15].

3.4. Induction of Apoptosis by Hyrtios sp. Extract. To verify cellular apoptosis, we used Annexin V and PI staining to investigate RKO and RKO-E6 cells treated with *Hyrtios* sp. extracts. As shown in **Figure 5**, apoptotic cells were detected similarly in RKO and RKO-E6 cells without treatment of
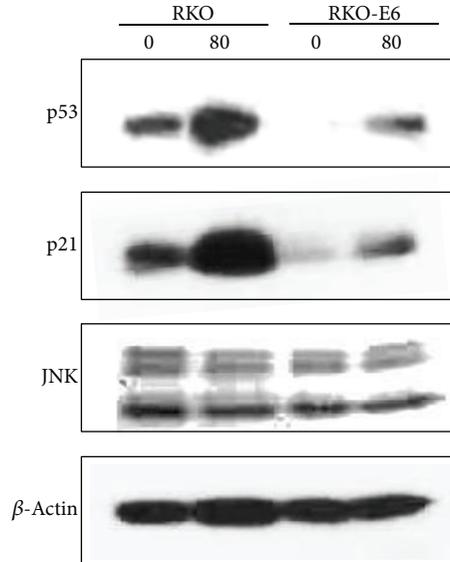
FIGURE 4: Expression of p53, p21, and JNK. Cells were treated with *Hyrtios* sp. extract (80 μg/mL) and incubated for 48 h. Protein expression was determined by western blots according to "Section 2".
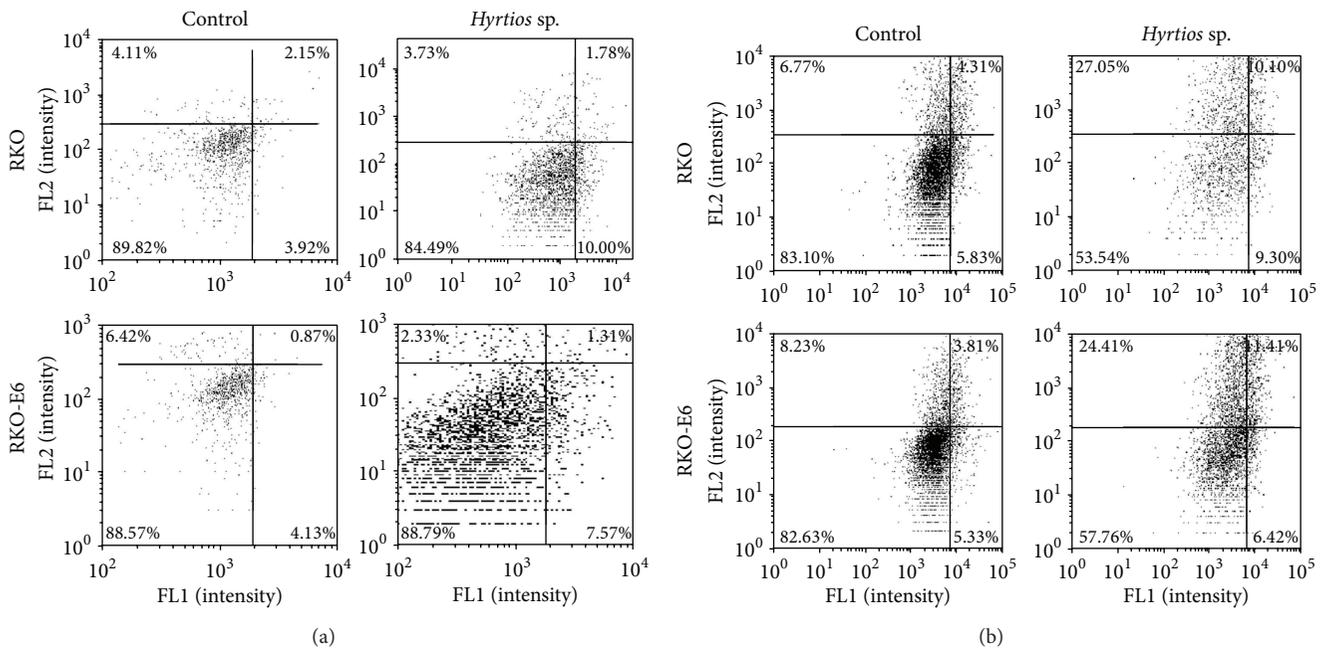
FIGURE 5: *Hyrtios* sp. extracts induced apoptosis. Cells were treated with *Hyrtios* sp. extract (80 μg/mL) and incubated for 24 h (a) or 48 h (b). Apoptotic cells were detected by Annexin V and PI staining.

Hyrtios sp. extracts. *Hyrtios* sp. extracts time-dependently decreased live cells and increased apoptotic cell death. RKO cells treated with *Hyrtios* sp. extract showed more apoptotic cells than RKO-E6 cells treated with *Hyrtios* sp. Extract. Therefore, *Hyrtios* sp. extracts induced more apoptosis in RKO cells than in RKO-E6 cells.

4. Conclusions

In this study, we investigated anticancer effects of *Hyrtios* sp. extract on p53 wild-type RKO cells and p53-deficient RKO-E6 cells. Our results indicated that *Hyrtios* sp. extract suppressed cell growth in both cell lines, but RKO cells,

which have functional p53, were more sensitive to the cytotoxic effects of *Hyrtios* sp. extract than RKO-E6 cells. The anticancer effect of *Hyrtios* sp. extract was induced *via* activation of the p53 pathway in RKO cells and suppression of the JNK pathway in RKO-E6 cells. *Hyrtios* sp. is a natural marine sponge that could be a good candidate for cancer treatments. In a further study, the bioactive components of *Hyrtios* sp. will be investigated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Epigallocatechingallate Inhibits Migration of Human Uveal Melanoma Cells via Downregulation of Matrix Metalloproteinase-2 Activity and ERK1/2 Pathway

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The effects of epigallocatechingallate (EGCG) on the migration and expression of MMP-2 of uveal melanoma cells have not been reported. We studied this effect and relevant signaling pathways in a human uveal melanoma cell line (M17). MTT study found that EGCG did not affect the cell viability of M17 cells up to 100 μM. Wound-healing assay showed that EGCG significantly reduced the migration of melanoma cells in a dose-dependent manner from 20 to 100 μM. Gelatin zymography showed that secreted MMP-2 activity was dose-dependently inhibited by EGCG, whereas the MMP-2 expression at protein and mRNA levels was not affected as determined by western blot and RT-PCR analysis. EGCG significantly increased the expressions of MMP-2 endogenous inhibitors (TIMP-2 and RECK) in M17 cells. Western blot analysis of MAPK signal pathways showed that EGCG significantly decreased phosphorylated ERK1/2 levels, but not p38 and JNK levels, in melanoma cells. ERK1/2 inhibitors also reduced the migration and activity of MMP-2 in M17 cells. The present study suggested EGCG at nontoxic levels could inhibit migration of melanoma cells via downregulation of activities of secreted MMP-2 through the inhibition of the ERK1/2 phosphorylation. Therefore, EGCG may be a promising agent to be explored for the prevention of metastasis of uveal melanoma.

1. Introduction

Uveal melanoma is the most common primary malignant intraocular tumor in adults and eventually metastasizes to the liver in up to 50% of patients within 15 years after the initial diagnosis [1–3]. Various procedures have been reported for the treatment of metastatic uveal melanoma, but the response rate is very poor. Most uveal melanoma patients with liver metastasis die within 6 months and the median survival time after diagnosis of metastasis is only 3.6 months [2, 3]. Because of the poor prognosis of metastatic melanoma, new therapies

for inhibiting cell migration, invasion, and metastasis are urgently required.

Tea is the most popular and polyphenol-rich beverage worldwide. Epigallocatechingallate (EGCG), the major polyphenol in green tea, has been considered to be the major active compound with chemopreventive properties [4]. Numerous previous studies showed that dietary phytochemicals or EGCG have many beneficial effects such as suppressing inflammatory processes [5], increasing antioxidant activity [6–10], inducing tumor cells apoptosis [11], and protecting cells from tumor development. Its cost effectiveness

and natural abundance make it an attractive substance to investigate.

The tumorigenesis process and progression to metastasis is generally recognized as a multistep process in which the cellular and molecular mechanism changes [12]. Five major classes of proteases (serine, aspartic, cysteine, threonine, and metalloproteinases) are involved in cancer cell metastasis [12]. Matrix metalloproteinases (MMPs) are one of the major proteases in the degradation of extracellular matrix (ECM), especially MMP-2 and MMP-9. This process may affect the adhesive capacity between cancer cells, promote the migration of cancer cells, and lead to the metastasis [13]. Numerous studies have showed that the relative expression levels of MMPs seem to increase with tumorigenesis and have connected elevated MMP-2 and MMP-9 levels with an increased metastasis and invasion [14, 15]. High levels of MMP-2 expression have been demonstrated in many different cancers, including the liver [16], lung [17], colon [18], breast [19], prostate [20], skin [21], and ovary [22]. The activated MMP-2 expression was also correlated with cancer cell invasion and metastasis in various cancers [23–25]. In uveal melanoma, Cottam et al. [26] observed that most of cell lines secreted MMP-2 in vitro and the expression of MMP-2 was associated with a poor prognosis [27].

Multifunctional effects of EGCG in downregulation of MMP-2 by interfering with the activation, secretion, and regulation of the molecule have been demonstrated in many cancer cell types such as breast [28], oral [29], lung [30], and others. However, the effect of EGCG on the cell migration and MMP-2 secretion in human uveal melanoma cells has not been reported. The purpose of this study was to investigate the effect and mechanism of EGCG on cell migration in human uveal melanoma cells.

2. Materials and Methods

2.1. Cell Line and EGCG Treatment. M17 cell line, a human uveal melanoma cell line, was isolated from a primary choroidal melanoma patient and was established as an immortal cell line by us (DNH) in the Tissue Culture Center of the New York Eye and Ear Infirmary as previously reported [31]. M17 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL streptomycin sulfate. Cells were incubated at 37°C in a CO_2 regulated incubator in a humidified 95% air/5% CO_2 atmosphere. Epigallocatechingallate (EGCG) and U0126 (ERK 1/2 inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For EGCG treatments, stock solutions of EGCG were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 50 mM, and cells were treated with EGCG at a final concentration between 0 and 100 μM for 24 hours.

2.2. Cell Viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to detect the cytotoxicity of EGCG. Cells were seeded in 24-well plate and treated with different concentration of EGCG at the range from 0 to 100 μM for 24 hours. After 24 hours, the media

were removed and the cells were washed with PBS, followed by the addition of MTT (0.5 mg/mL) to the culture medium for 4 hours at 37°C. Subsequently, the absorbance values were measured by a microplate photometer at 570 nm.

2.3. Wound-Healing Assay. An in vitro wound-healing assay was used to observe the migration of M17 cells after EGCG treatment. M17 cells were seeded into 6-well plate for 24 hours until they visibly reached confluence. Before EGCG treatment, a pipette tip was used to create a straight scratch on the plate to simulate a wound. The width of the remaining gap was captured using phase-contrast microscopy (×100) at 0, 24, 48, and 72 hours.

2.4. Western Blot Analysis. Cells were seeded into 6 cm dish. EGCG at different concentrations (0, 20, 40, 60, 80, and 100 μM) was added. After 24 hours, total cell lysates were collected using lysis buffer containing a protease inhibitors cocktail and then lysed by sonication using an ultrasonic processor. Afterward, cells extracts were microcentrifuged at 13000 rpm for 20 min at 4°C and the supernatants were collected. The protein concentrations of total cell lysates were determined by Bradford assay. The cell extracts were separated by 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Subsequently, the membrane was incubated with 5% nonfat milk in TBST buffer for 1 h blocking and then the primary antibodies of MMP-2, TIMP-2, RECK, phosphor-ERK1/2, phosphor-JNK, phosphor-p38, total JNK, total p38, and total ERK1/2 were added. After incubating for overnight at 4°C, secondary antibodies with horseradish peroxidase were used for the indirect detection of specific primary antibody for 1 hour at room temperature. Finally, the protein expression was detected by chemiluminescence with an ECL detection kit. The relative photographic density was quantified by Multi Gauge V2.2 software.

2.5. Gelatin Zymography. The activities of MMP-2 in a condition medium and cell lysate were measured by gelatin zymography protease assays. Cells were plated at a density of 5×10^4 cells/well in 24-well plates for 24 hours and then treated with indicated concentrations (0, 20, 40, 60, 80, and 100 μM) of EGCG for another 24 hours. Otherwise, cells were pretreated with an indicated concentration of specific inhibitors, 10 μM U0126 (ERK 1/2 inhibitor), for 60 min followed by incubation with or without 60 μM EGCG for an additional 24 hours. For MMP-2 activity detection, conditioned medium and cell lysate from treated cells were prepared without boiling or reduction and subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 30 min to remove SDS and incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM CaCl2, 0.02% NaN3) at 37°C for 16 hours. Finally, the gel was stained with Coomassie brilliant blue R-250 [29].

2.6. RNA Isolation, Semiquantity PCR, and Quantitative Real-Time PCR. Total RNA was isolated from 1×10^6 M17 cells treated with and without EGCG using Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's

(a)

(b)
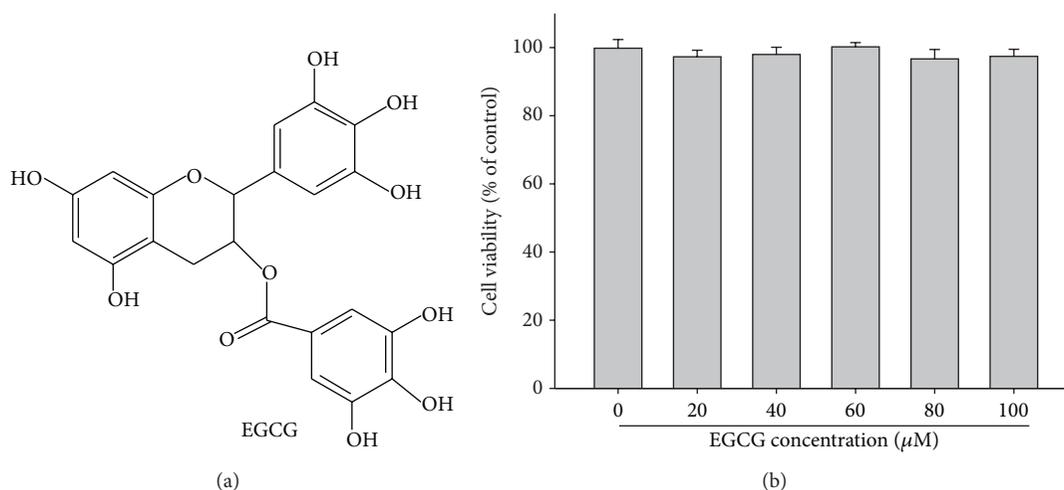
FIGURE 1: Effect of EGCG on cell viability. (a) Structure of EGCG. (b) Cell viability analysis of M17 cells cultured in presence of EGCG for 24 hours by MTT assay. M17 cells were treated with different concentrations of EGCG (0–100 μM) for 24 hours. Data represent mean of 3 determinations per condition repeated 3 times. Results are shown as mean ± SD.

instructions. Total RNA (2 μg) was reverse transcribed into cDNA by SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA). The PCR was performed in a reaction mixture containing 2 μL cDNA, 0.2 mM dNTP mixture, 2 μM of each of the primers, 1 U Taq DNA polymerase, and 1-fold concentration of Thermal Pol Buffer (New England BioLabs, MA, USA) by denaturation at 95°C for 5 min, followed by amplification of indicated cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. The specific primer sequences for these genes are as follows: MMP-2: 5′-GGCCCTGTCACTCCTGAGAT-3′ (forward), 5′-GGCATCCAGGTTATCGGGG A-3′ (reverse) and TIMP-2: 5′-GGCGTTTTGCAATGCAGATGTAG-3′ (forward), 5′-CACAGGAGCCGTCACTTCTCTTG-3′ (reverse). Quantitative real-time PCR analysis was carried out using TaqMan one-step PCR Master Mix (Applied Biosystems). 100 ng of total cDNA was added per 25 μL reactions with MMP-2 or GAPDH primers and TaqMan probes. The MMP-2 and GAPDH primers and probes were designed using commercial software (ABI PRISM Sequence Detection System; Applied Biosystems). Quantitative real-time PCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected.

2.7. Statistical Analysis. For all of the measurements, analysis of variance followed by Scheffe posteriori comparison was used to assess the differences between control and cells treated with various concentration of EGCG. A difference at $P < 0.05$ was considered to be statistically significant and the experiments were repeated three times.

3. Results

3.1. Effect of EGCG on Cell Viability. The chemical structure of EGCG is shown in Figure 1(a). Cytotoxic effects of EGCG

on M17 cell was determined by MTT assay. As shown in Figure 1(b), after 24-hour treatment with various concentrations (0–100 μM) of EGCG, the cell viability was not significantly affected. Therefore, a concentration range of 20–100 μM of EGCG was chosen for subsequent experiments.

3.2. Effect of EGCG on Cell Migration. Wound-healing assay was used to investigate the migration of M17 cells cultured with or without EGCG. The results showed that EGCG significantly reduced the migration of M17 cells in a dose-dependent manner during 72 hours (Figure 2).

3.3. Effect of EGCG on the Expression and Secretion of MMP-2. Gelatin zymography was used to analyze the activity of secreted and cytosolic MMP-2 in M17 cells treated with and without EGCG. Figure 3(a) shows that only secreted MMP-2 activity was significantly inhibited by EGCG in a dose-dependent manner. The protein and mRNA level of MMP-2 expression was indicated by western blot, semiquantity, and quantitative real-time PCR assay, respectively (Figures 3(b), 3(c), and 3(d)). The results showed that EGCG did not affect both protein and gene expressions of MMP-2. This suggested that EGCG inhibited activities of secreted MMP-2 but did not affect the expression of MMP-2.

3.4. Effect of EGCG on Endogenous Inhibitor Expression of MMP-2. From Figure 3, it was deduced that EGCG reduced the activities of secreted MMP-2. The physiological activities of MMP-2 are related to their specific endogenous inhibitors, tissue inhibitor of metalloproteinase- (TIMP-) 2, and reversion-inducing-cysteine-rich protein with kazal motifs (RECK). We analyzed the effect of EGCG on TIMP-2 and RECK expressions by western blot analysis. The results showed that EGCG significantly increased the expressions of TIMP-2 and RECK in M17 cells (Figure 4(b)). We further observed that EGCG substantially increased the TIMP-2 and RECK levels of M17 cells in a dose-dependent manner, with
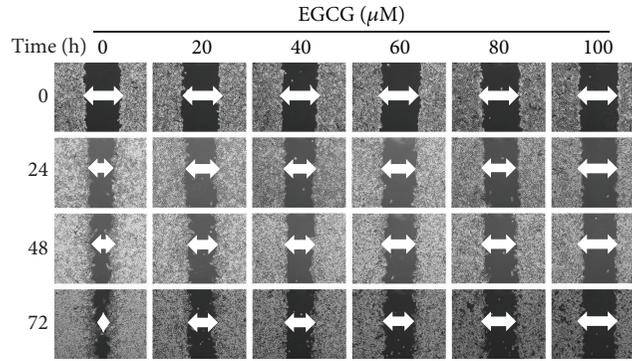
FIGURE 2: Effect of EGCG on cell migration in M17 cells. M17 cells were wounded and then treated with vehicle (DMSO) or EGCG (0, 20, 40, 60, 80, and 100 μM) for 0 h, 24, 48, and 72 hours in 10% FBS-containing medium. At 0, 24, 48, and 72 hours, phase-contrast pictures of the wounds at three different locations were taken.

(a)

(b)

(c)

(d)
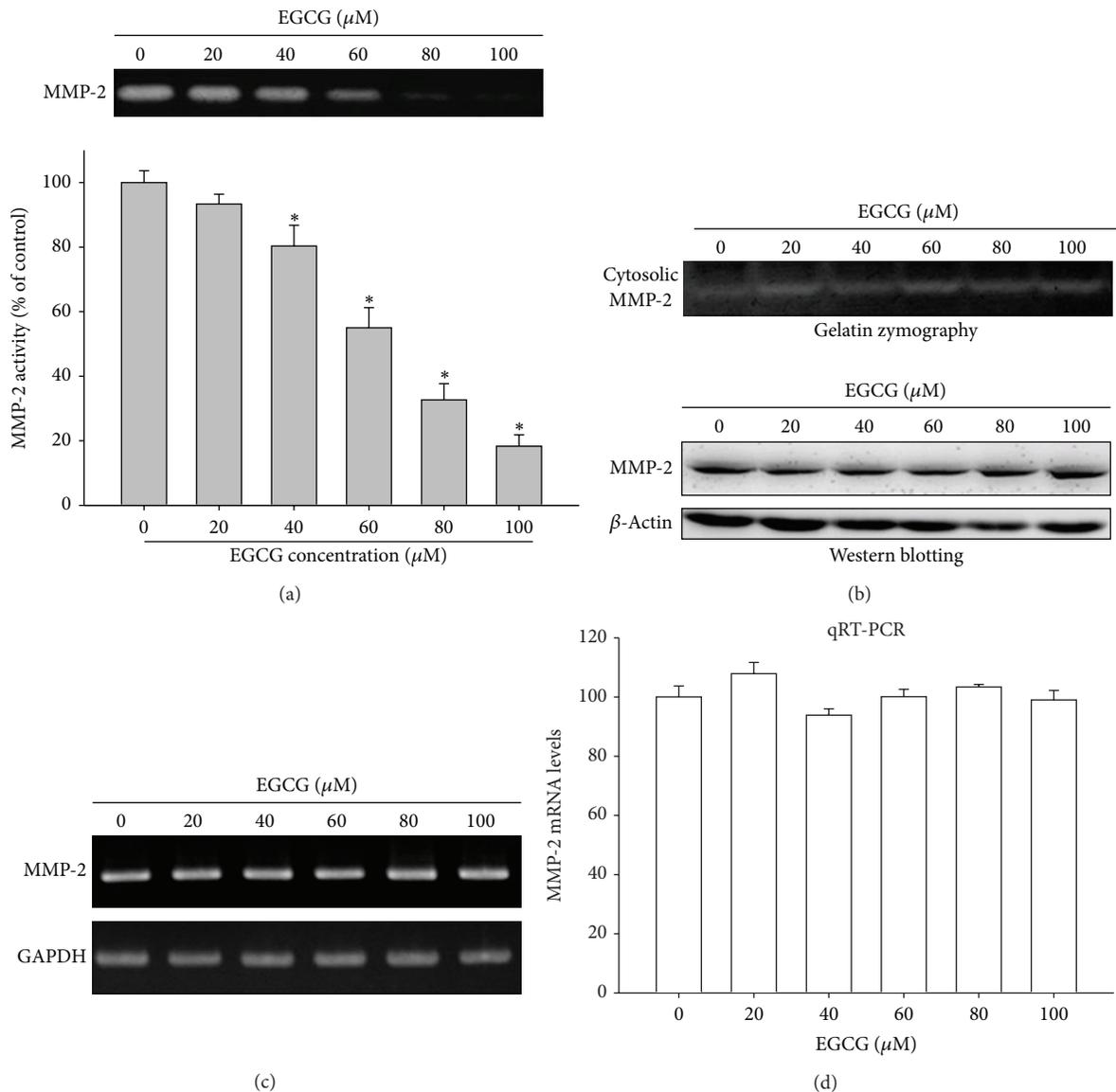
FIGURE 3: Effect of EGCG on the secretion, protein, and mRNA expression of MMP-2. M17 cells were treated with various concentrations (0, 20, 40, 60, 80, and 100 μM) of EGCG for 24 hours. (a) The conditioned media were collected and the activity of MMP-2 was detected. (b) A gelatin zymography and western blot were performed for cell lysates. (c) Semiquantitative RT-PCR was performed to compare MMP-2 mRNA levels. (d) The mRNA levels of MMP-2 were quantified using a real-time PCR assay. The values represented the means ± SD of at least three independent experiments. $^{*}P < 0.05$ as compared with the control.

(a)

(b)
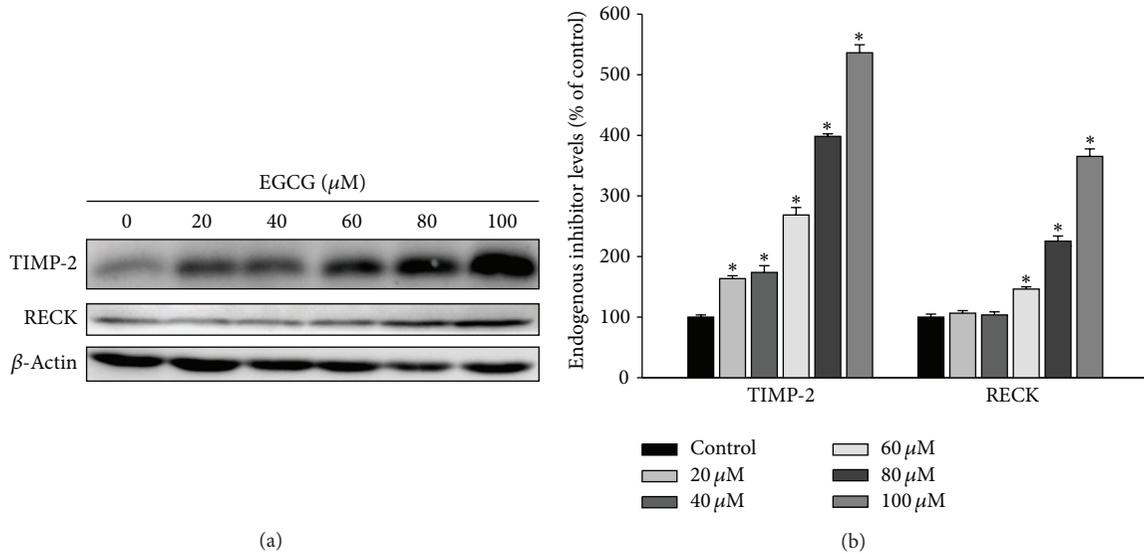
FIGURE 4: Effects of EGCG on the protein level of the endogenous inhibitor TIMP-2 and RECK. (a) M17 cells were treated with EGCG (0–100 μM) for 24 hours and then subjected to western blotting to analyze the protein levels of TIMP-2 and RECK, respectively. (b) Quantitative results of TIMP-2 and RECK protein levels which were adjusted with β-actin protein level. The values represented the means $\pm$ SD of at least three independent experiments. $^*P < 0.05$ as compared with the vehicle group.

5.36-fold and 3.65-fold increase after treating with 100 μM of EGCG, respectively (Figure 4(b)).

3.5. Effect of EGCG on the Phosphorylation of ERK1/2 Pathway. As we have shown that the treatment of EGCG to M17 cells inhibited the cell migration and activities of secreted MMP-2, the underlying mechanisms were further investigated. As shown in Figure 5, the treatment of EGCG could significantly reduce the activation of phospho-ERK1/2 in a dose-dependent manner (Figure 5(a)). However, the phosphorylation of the JNK1/2 and p38 pathways remained unaffected (Figures 5(b) and 5(c)).

3.6. Inhibitory Effect of ERK1/2 Inhibitor on MMP-2 Activities and Cell Migration. To further study whether the inhibition of MMP-2 activities by EGCG was mainly through an inhibition of ERK1/2 signaling pathway, we investigated the effects of specific inhibitor of ERK l/2 pathway (U0126) on M17 cells. Results showed that U0126 treatment led to the inhibition of MMP-2 activity, similar to the EGCG treatment (Figure 6(a)). In addition, a combined treatment of ERK1/2 inhibitor with EGCG could further decrease the MMP-2 activity (Figure 6(a)) and increase the expression of TIMP-2 and RECK protein but not affect the MMP-2 expression (Figure 6(b)). Furthermore, a similar result for an inhibition on the cell migration of M17 cells by a sole treatment of ERK1/2 inhibitor and combination treatment with ERK1/2 inhibitor and EGCG was also observed (Figure 6(c)). This suggested that the inhibition of ERK l/2 signaling pathways could result in the inhibition of activities of secreted MMP-2 as well as the cell migration of melanoma cells.

4. Discussion

M17 uveal melanoma cell line was isolated and cultured from a primary choroidal melanoma patient, has been cultured in vitro for more than 20 years, and has been divided more than 200 times, indicating that this is an immortal cell line. Cells grew actively, with a doubling time of 24–48 hours, and are tumorigenic in immune nude mice. This cell line has been used widely in several melanoma research centers for the study of melanogenesis, role of microRNA in the pathogenesis of uveal melanoma, and various pharmacological and toxicological studies [32–39]. Therefore, M17 cell line was selected for use in the present study. The effects of EGCG on the growth, migration, and invasion of cutaneous melanoma cells have been reported [40–42]. However, the effect of EGCG on the migration and expression of MMPs in uveal melanoma cells has not been reported.

Cutaneous melanomas are biologically different from uveal melanomas in many respects. Most cutaneous melanomas occur in area exposed to sun radiation, whereas most of the uveal melanomas occur in the posterior segment of the eye and are not exposed to sun radiation. UV radiation increases the incidence of cutaneous melanoma but not uveal melanoma [43, 44]. Gene mutations in cutaneous melanoma (BRAF, N-Ras, etc.) were entirely different from those in uveal melanoma (GNAQ, GNA11, etc.) [45]. Furthermore, the karyotypes of cutaneous melanomas are also different from those of uveal melanomas [46]. Therefore, cutaneous melanoma and uveal melanoma should be considered as two different and independent disease entities. Independent studies are required for each type of melanoma to develop relevant novel treatments.

(a)

(b)

(c)
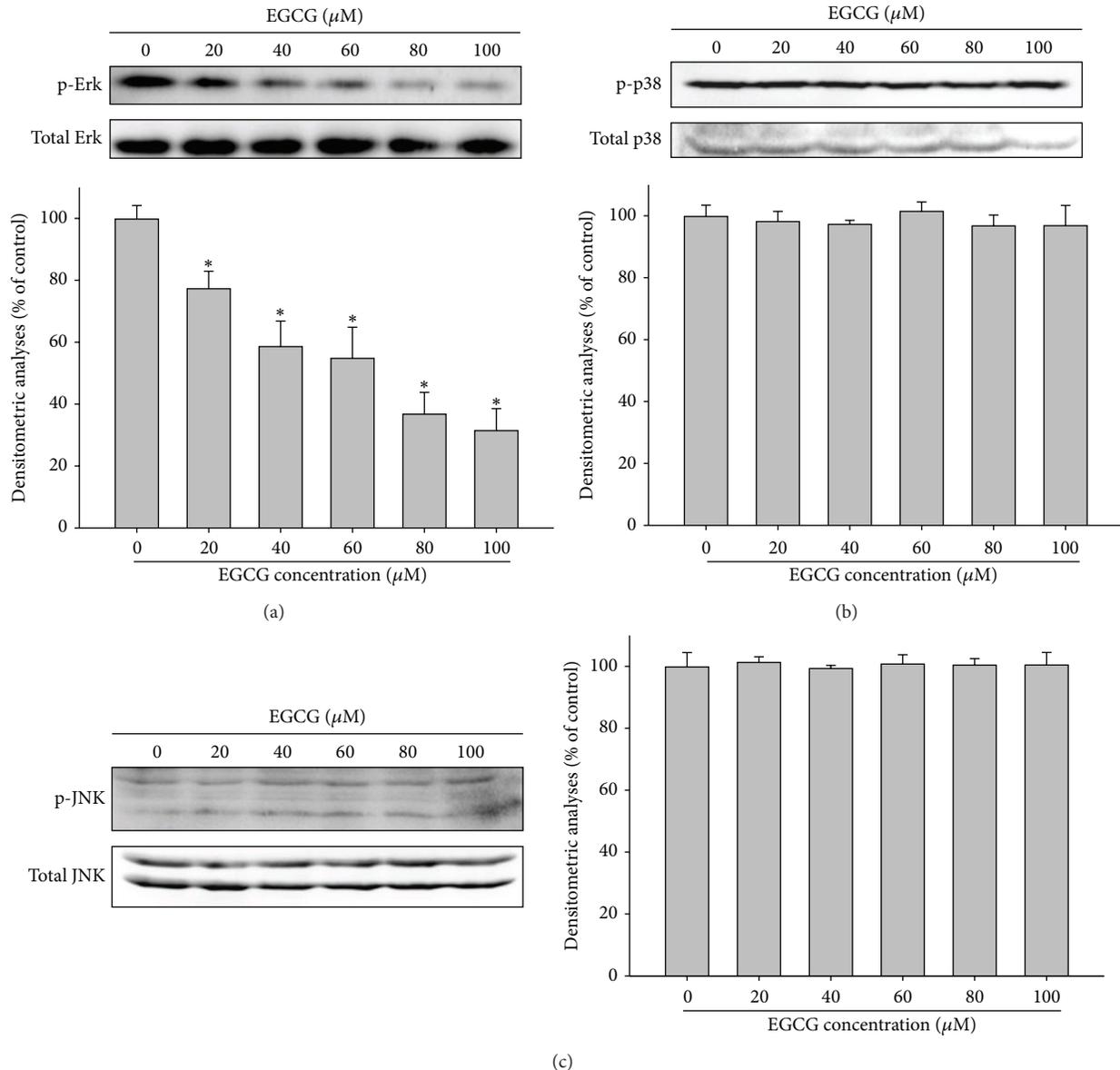
FIGURE 5: Effects of EGCG on the MAPK signaling pathways. M17 cells were treated with various doses of EGCG (0, 20, 40, 60, 80, and 100 μM) for 24 hours and whole cell lysates prepared from these cells were used for western blot analysis with (a) anti-ERK, (b) anti-p38, and (c) anti-JNK (total and phosphorylated) antibodies as described in Materials and Methods section. The values represented the means $\pm$ SD of at least three independent experiments. $^{*}P < 0.05$ as compared with the control.

The present study revealed that EGCG inhibited the migration and decreased the MMP-2 activity of uveal melanoma cells. To our knowledge, this study provides the first demonstration that EGCG is capable of inhibiting invasive behaviors and MMP-2 activities in uveal melanoma cells.

Previous studies have well established the role of the mitogen-activated protein kinase (MAPK) pathway in regulating MMP-2 expression [47]. Lin et al. indicated that kaempferol reduces MMP-2 expression by downregulating ERK1/2 signaling pathways in oral cancer cells [47]. Our previous study also showed that silibinin inhibits the invasion of oral cancer cells by suppressing the activation of ERK1/2

and MMP-2 expression [48]. Furthermore, in the study by Sen et al., EGCG downregulated MMP-2 in human breast cancer cell line via Erk 1/2 signal pathway [28]. In another study, JNK 1/2 pathway modulated MMP-2 production by EGCG treatment in lung cancer cells [30]. However, the present study showed that EGCG only inhibited ERK phosphorylation and no significant effects were detected on the JNK and p38 signaling pathways. The involvement of the MAPK pathway in the modulation of MMP-2 activities was demonstrated by treating uveal melanoma cells by ERK inhibitor, which showed that ERK inhibitor could lead to an inhibition of MMP-2 secretion and cell invasion of uveal melanoma cells.

(a)

(b)

(c)
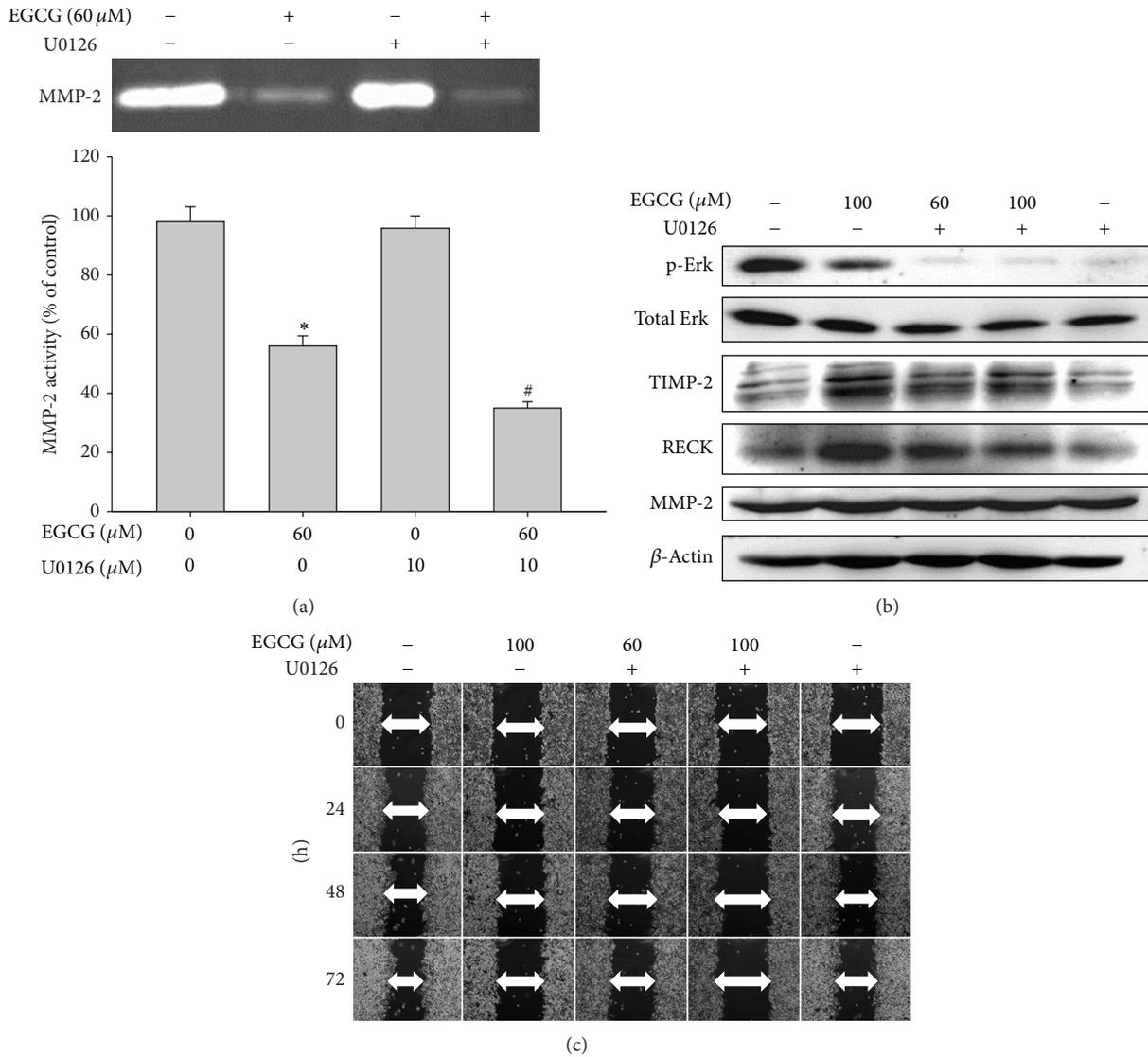
FIGURE 6: Effect of U0126 and EGCG on the expression of MMP-2, TIMP-2, and ERK1/2 pathway and cell migration. M17 cells were pretreated with U0126 (10 μM) for 1 hour and then incubated in the presence or absence of EGCG (60 or 100 μM) for 24 hours. (a) The culture media were used as subjects for analysis of MMP-2 activity. (b) The cell lysates were used as subjects for analysis of MMP-2, TIMP-2, and RECK protein levels. (c) Cells were used for wound-healing assay as described in Materials and Methods section. The values represented the means ± SD of at least three independent experiments. $^{*}P < 0.05$ as compared with the control. $^{#}P < 0.05$ as compared with the EGCG-treated only.

In conclusion, our study results suggested that one of the antimetastatic effects of EGCG on uveal melanoma cells was the downregulation of activities of secreted MMP-2 through the inhibition of ERK1/2 phosphorylation. Overall, these data suggest that EGCG may be a promising agent to be explored for the prevention of metastasis of uveal melanoma.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chi-Wu Chang and Yi-Hsien Hsieh contributed equally to this work.

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Research Article

Andrographolide Induces Apoptosis of C6 Glioma Cells via the ERK-p53-Caspase 7-PARP Pathway

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Background. Glioma is the most malignant tumor of the central nervous system. Efforts on the development of new chemotherapy are mandatory. Andrographolide (AND), a diterpenoid lactone isolated from the *Andrographis paniculata*, has been shown to have antitumor activities in several types of cancer cells. Whether AND can exert its antitumor activity in glioblastoma cells remains unknown. This study examined the anticancer effects of AND, both *in vitro* and *in vivo*. *Methods.* Cell apoptosis was assayed by flow cytometry and nuclear staining. The signaling pathway for AND was determined by western blotting. The effects of AND on tumor growth was evaluated in a mouse model. *Results and Conclusion. In vitro*, with application of specific inhibitors and siRNA, AND-induced apoptosis was proven through ROS-ERK-P53-caspase 7-PARP signaling pathway. *In vivo*, AND significantly retarded tumor growth and caused regression of well-formed tumors *in vivo*. Furthermore, AND did not induce apoptosis or activate ERK and p53 in primary cultured astrocyte cells, and it may serve as a potential therapeutic candidate for the treatment of glioma.

1. Introduction

Glioma is the most common malignant tumor of the central nervous system [1]. These tumors, including astrocytoma, oligodendrogliomas, ependymomas, and other rare types of glial tumors, arise from glial cells. Due to their infiltrative nature and frequent involvement of eloquent regions in brain and spinal cord, surgical removal is usually not possible. These patients often need to control their diseases through adjuvant therapies such as radiotherapy and chemotherapy. Other therapeutic agents against specific targets, including antivascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab) and epidermal growth factor receptor (EGFR) inhibitors, are also being used for disease control in glioma [2, 3]. However, failure of treatment inevitably

occurs. Among all kinds of glioma, glioblastoma, which is associated with extremely poor prognosis, is the most frequent and malignant type of glioma. The 2-year survival rate is 7.5%, and 5-year survival rate reduced to only 5% [4, 5]. Most patients die of glioblastoma within 2 years. Therefore, scientists and clinicians worldwide are still searching for better therapies for malignant gliomas.

Andrographolide (AND) is a diterpenoid lactone molecule that possesses various biological activities, including anti-inflammatory [6], immunomodulatory [7], hepatoprotective [8], antiviral [9], and antitumoral effects [10]. It is extracted from the stem and leaves of the medicinal plant, *Andrographis paniculata*. AND treatment blocked the *in vitro* proliferation of a variety of tumor cell lines, such as neuroblastoma, melanoma, hepatoma, prostate cancer, and gastric

cancer [11–14]. This compound exerts anticancer activity on tumor cells by several mechanisms, such as cell-cycle arrest [13], growth factor signaling modulation, cellular migration [15], and angiogenesis. For example, AND inhibited the growth of colorectal carcinoma LoVo cells by inducing expression of p53, p21, and p16, resulting in repression of Cyclin D/Cdk4 and/or Cyclin E/Cdk2 activities, as well as Rb phosphorylation, thus leading to G1-S phase arrest [16]. AND also inhibits human hepatoma Hep3B cell growth through JNK activation [17]. In epidermoid carcinoma cells, AND decreased cell proliferation through enhanced degradation of EGFRs on the cell surface [18]. It also inhibited migration of colorectal carcinoma LoVo cells and non small cell lung cancer A549 cells by suppression of PI3K/Akt signaling pathway, which decreased the mRNA and protein levels of matrix metalloproteinase-7 (MMP-7) [19, 20]. Furthermore, AND reduced VEGF level in both B16F-10 melanoma cells and A549 lung cancer cells [21, 22], which blocked angiogenesis around tumors. In addition, AND induces cell death in various tumor cell types. In HL-60 leukemic cells, AND treatment resulted in disappearance of mitochondrial cytochrome C, increased expression of Bax, and decreased expression level of Bcl-2 proteins [23]. In B16F-10 melanoma cells, AND modulated p53-induced-caspase-3 expression [24]. A recent study demonstrated that AND inhibited cell proliferation via inactivation of PI3K/AKT signaling in human glioblastoma cells [25]. Beside, AND also sensitizes cancer cells to TRAIL-induced apoptosis via p53 [26]. Whether AND induces programmed cell death (apoptosis) in glioma cells and the mechanisms underlying AND-induced cell death remain to be determined.

In this report, we aimed to study the antitumor effects of AND on C6 glioma cells, which is an experimental model of glioblastoma [27], and the underlying mechanisms.

2. Materials and Methods

2.1. Cell Culture. C6 glioma cells, a rat cell line of astrocytic origin, were purchased from the American Type Culture Collection (Rockville, MD, USA). The primary rat astrocyte cell line was a generous gift from Dr. Jiahn-Chun Wu (National Yang-Ming University, Taiwan) [28]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (both from Gibco BRL, Grand Island, NY), 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA), and 100 IU/mL penicillin and streptomycin (pH 7.2) (Gibco BRL, Grand Island, NY). Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C.

2.2. Drugs. AND, propidium iodide (PI), and 4,6-diamidino-2-phenylindole dilactate (DAPI) were purchased from Sigma. 3AB, Z-VAD, and DEVD were purchased from Biomol (Enzo Life Sciences Inc., NY, USA). PD98059 was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

2.3. Cell Survival Assay. Cells were plated at 8×10^3 cells per well of a 24-well plate and incubated for 24 h for cell adhesion. Different concentrations of AND or 0.2% dimethyl sulfoxide

(DMSO, Sigma) were added to the culture medium for 12 or 24 h as indicated. After washing twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 Mm KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), 0.5 mL of DMEM medium containing 0.5 mg/mL of 2.3.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to each well and incubation was continued for another 2 h. The reaction solution was then removed, and the cells were lysed with 0.5 mL of DMSO and the absorbance at 590 nm was determined using a spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

2.4. Apoptosis Detection Assays. For detection of apoptosis, two methods were used in the study. First, cells were treated with AND for 0–24 h and then trypsinized. After washing with cold PBS, the cells were stained with Apoptosis Detection kit (Strong Biotech Corporation, AVK050, Taipei, Taiwan), containing identified annexin V-FITC and PI in 100 μL of binding buffer, for 15 min and analyzed by flow cytometry. FL1 and FL2 represented the intensity of FITC and PI, respectively. DAPI stain was also used to detect the apoptotic process in cells. Cells were seeded on the cover slides. After various treatments, cells were washed with ice cold PBS and stained for 15 min with 1 μg/mL DAPI in 0.9% NaCl. Cover slides were mounted on the slides using fluorescence mounting medium (70% glycerol and 2% propyl gallate in PBS). Cell images were captured using a fluorescence microscope and a digital camera.

2.5. Small Interfering RNA (siRNA) Transfection. A siRNA for p53, which targeted the RNA coding sequence, was designed by Dharmacon (ON-TARGET plus SMARTpool, Dharmacon Corporation, Lafayette, CO, USA). Negative control and GAPDH siRNAs were purchased from Ambion (Silencer Select Predesigned siRNA, Ambion, Austin, TX, USA). The siRNAs were transfected through electroporation, as specified in the instruction manual (Amaxa, Germany). After transfection, cells were cultured for 48 h to detect target expression. Briefly, 10^6 cells were trypsinized and resuspended in 100 μL of Nucleofector solution (Amaxa), and 100 nM of siRNA duplexes was electroporated.

2.6. Western Blotting. After the various treatments, cells were washed once with ice cold PBS, homogenized in lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, 25 mM HEPES, 0.15% triton X-100, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) and sonicated twice for 10 s each time. The concentrations of proteins were determined using a Bio-Rad Protein Assay kit (Bio-Rad Life Science, Hercules, CA, USA), and samples of proteins (80 or 120 μg per lane) were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH, USA). Strips from the membrane were then blocked by incubation with 5% nonfat milk in Tris-buffered saline (pH 8.2, containing 0.1% Tween (TBS-Tween)) for 1 h at room temperature and then incubated overnight at 4°C with a 1 : 5000 dilution of monoclonal rabbit antibody

against GAPDH (GeneTex Inc., Irvine, USA), 1 : 500 dilution of phosphor-extracellular-signal-regulated kinases (ERK) or phospho-P38 (Santa Cruz Biotechnology, Inc., California, USA). Other blots were incubated with a 1 : 500 dilution of monoclonal rabbit antibodies against caspase 3, cleaved caspase 3, caspase 7, cleaved caspase 7, cleaved poly (ADP-ribose) polymerase (PARP), p53, phospho-p53 (Ser15), or phospho-c-Jun N-terminal protein kinase (phospho-JNK) (Cell Signaling Technology, Inc., Beverly, MA, USA), all diluted in TBS-Tween. After washing with TBS-Tween, the strips were incubated for 2 h at room temperature with a 1 : 7500 dilution of alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibodies (Promega Corp., Madison, WI, USA), and the bound antibody was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as a chromogen. The density of the bands on the nitrocellulose membrane was quantified by densitometry using Gel Pro 3.1 (Media Cybernetics, Silver Spring, MD, USA), setting the density of the band in the control sample as 100% and expressing the density of the band in the test sample as a percentage of the control band density.

2.7. Animals. Adult ICR male mice (8-week old) were purchased from the National Taiwan University Animal Center and housed in individual cages in a temperature- and humidity-controlled room (12 : 12 h light-dark cycle) with free access to tap water and diet. All of the animal experiments were performed according to National Institutes of Health guidelines and were approved by the Laboratory Animal Committee of the College of Medicine, National Taiwan University.

2.8. In Vivo Experiment. The *in vivo* tumor growth model in the ear was performed according to previous studies [29–32] with some modifications. Two kinds of *in vivo* experiments were performed, coinjection or postimplantation AND injection. First, the ears of 8-week-old male ICR mice were subcutaneously injected in the center with 1×10^7 C6 cells with (right ear) or without (left ear) $20\,\mu$M AND. The ears were photographed under a dissecting microscope at day 5 after injection. The tumor tissues were weighted and photographed, and the results were expressed as a relative percentage of that of the control side (left ear). Second, in the postimplantation AND injection experiment, 1×10^7 C6 cells were injected in the middle of both ears in ICR mice. Pictures of tumors were taken at day 3. $30\,\mu$L of saline (left ear) or $20\,\mu$M AND (right ear) was injected into the tumors twice at day 3 and day 6. The tumor tissues were removed from ears at day 9, weighted, and pictured. The weight of tumor tissues was calculated by microbalance, and take left tissue volume as 100%.

2.9. Statistical Analysis. All experiments were performed at least 3 times, and the results are expressed as the mean ± SEM for the total number of experiments. We assessed statistical differences between means by using one-way ANOVA test and posttested them using Dunnett's test. A P value of less than 0.05 was considered statistically significant (* or #), and a value of less than 0.01 was considered more statistically significant (**). *: compared to CTL group, #: compared to AND group.

3. Results

3.1. AND Induced Cell Death of C6 Glioma Cell by Apoptosis. The chemical structure of AND is shown in Figure 1(a). C6 glioma cells were treated with various concentrations of AND for 24 h, and cell viability was analyzed by MTT assay (Figure 1(b)). The effect of AND glioma cell survival was found to be dose-dependent. Compared to cells treated with DMSO (control group), cells treated with $5\,\mu$M AND showed either no survival benefit or no toxic effect. The cell survival rate of cells treated with 10 to $20\,\mu$M of AND decreased from 70% to 30%, and the IC50 of AND was approximately $15\,\mu$M. Therefore, $15\,\mu$M of AND was used in the subsequent time-dependent experiments. Following treatment with DMSO or $15\,\mu$M of AND for different intervals, C6 glioma cells were stained by annexin V and PI or DAPI for analyzing the cell death pattern. As determined by flow cytometry, the proportion of apoptotic cell with annexin V labeling increased with time. The cell population shift from negative stain (Figure 1(c), left down square) to annexin V-positive (Figure 1(c), right down square), and double positive (Figure 1(c), right up square) sequentially defined that AND induced cell death by most apoptosis (Figure 1(c)). DAPI staining identified apoptotic cells by the presence of apoptotic nuclei (Figure 2, arrows). The results revealed that there were very few apoptotic cells in the DMSO group but significant number of apoptotic cells in the AND groups. The percentage of apoptotic cells was 6.7% ± 1.6% in the DMSO group and 28.9% ± 1.6% in the AND group ($15\,\mu$M, 12 h).

3.2. AND Triggered Caspase 7-PARP Signaling in C6 Glioma Cells. To delineate the signal transduction pathway of apoptosis, DEVD ($5\,\mu$g/mL, caspase 3/7 inhibitor) or 3AB ($5\,\mu$g/mL, PARP inhibitor) was used for 30 min before AND treatment. Pretreatment of C6 cells with DEVD or 3AB inhibited AND-induced apoptosis, and the percentages of apoptotic cells were 7.8% ± 1.3% and 15.8% ± 2.0%, respectively, which were significant compared to AND alone (Figure 2). MTT assay and annexin V binding assay were performed to further investigate whether caspase 7 and PARP were involved in AND-induced cell death. Both inhibitors blocked the cytotoxicity of AND (see Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/312847). These findings indicated that AND-induced cell death was caspase 3/7- and PARP-dependent.

Because the caspase 3/7 inhibitor, DEVD, effectively blocked AND-induced apoptosis, we further analyzed the role of caspase 3/7 in the apoptotic pathway. Several activated caspases are self-cleaved into 2 subunits, permitting identification of the activation of caspase by the presence of cleaved caspase (c-caspase). Following AND treatment, the levels of c-caspase 3 in C6 cells did not change significantly in comparison to DMSO treatment (Figure 2(c)), but c-caspase 7 levels increased significantly, and this increase showed

(a)

(b)

(c)
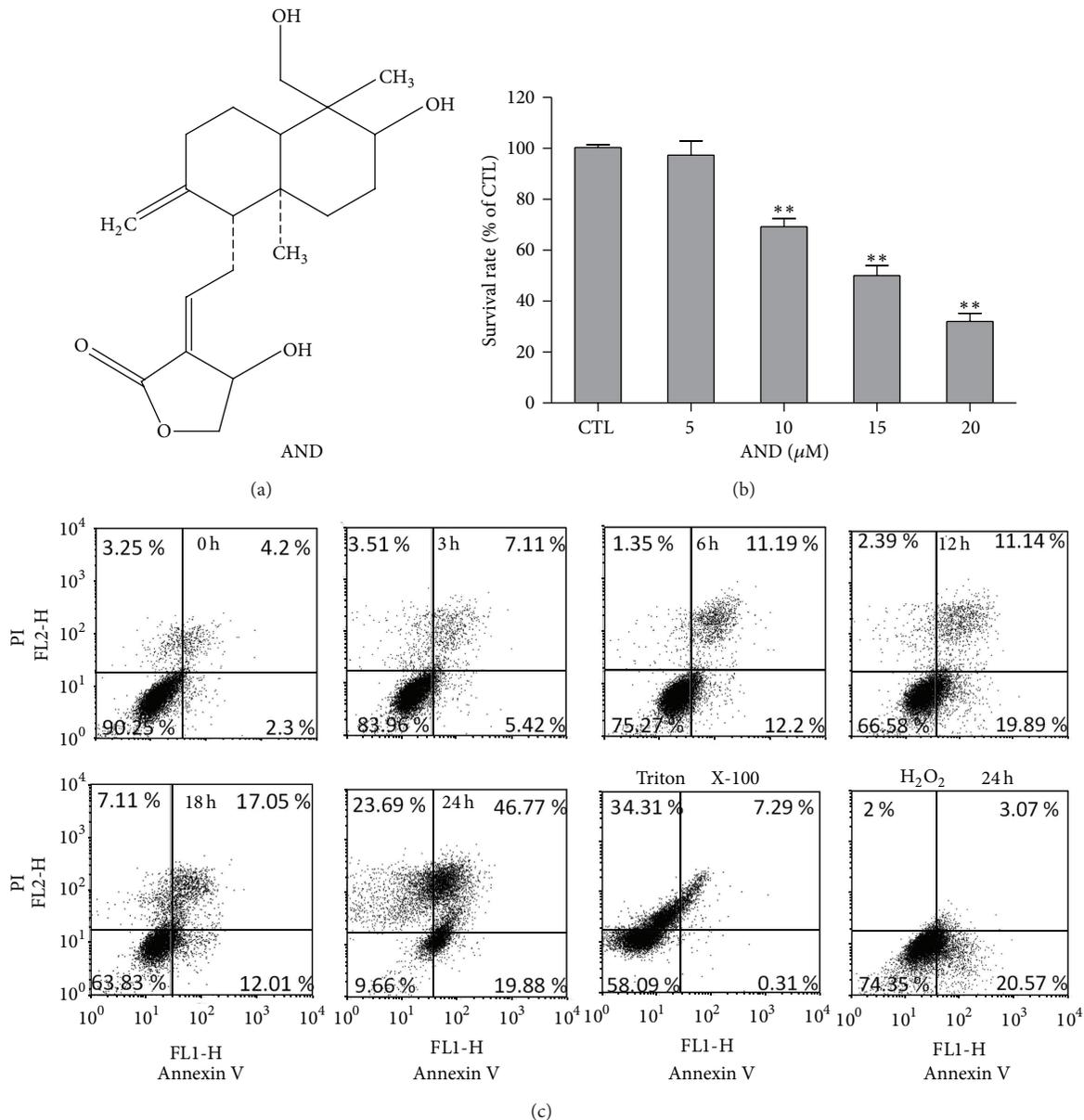
FIGURE 1: The structure of AND and the effect of AND on the survival of C6 glioma cells. (a) The chemical structure of AND. (b) The cells were treated with 0.1% dimethyl sulfoxide (DMSO) (CTL), 5, 10, 15, or 20 μM of AND for 24 h, and cell viability was determined using the MTT assay. $N = 3$. $**P < 0.01$, compared to the control group. (c) Flow cytometric analysis of AND-induced apoptosis. Cells were treated with 15 μM AND for different intervals and stained with annexin V and propidium iodide PI for flow cytometric analysis.

both a dose-dependent (Supplementary Figure 2(a)) and a time-dependent trend (Figure 2(d)). The protein levels of c-caspase 7, following treatment with 20 μM of AND for 12 and 24 h, increased to 1.8- and 2.2-fold, respectively (Figure 2(d)). These results suggest that AND induced caspase 7 activation.

Once activated, caspase 7 cleaves many of the same substrates as caspase 3, including poly (ADP-ribose) polymerase or PARP [33, 34]. Activation of caspase 3 or 7 results in cleavage of the downstream protein PARP, which is an excellent marker for apoptosis [35]. Like caspases, activated PARP is self-cleaved into 2 subunits, permitting the activation of PARP to be identified. With the PARP inhibitor, 3AB, which

effectively blocked AND-induced apoptosis (Figures 2(a) and 2(b)), we further analyzed the role of PARP in the apoptotic pathway. Following AND treatment, the levels of cleaved PARP (c-PARP) in C6 cells increase significantly and showed a dose-dependent (Supplementary Figure 2(b)) as well as a time-dependent trend (Figure 2(e)). Quantitative analysis showed that treatment with AND for 24 h at concentrations of 10 μM, 15 μM, and 20 μM induced c-PARP to 1.5-, 3.5-, and 3.8-fold, respectively (Supplementary Figure 2(b)). Treatment with 15 μM AND for 12 h and 24 h elevated the levels of cleaved PARP to 1.9- and 2.9-fold, respectively (Figure 2(e)). Pretreatment with the caspase 3/7 inhibitor, DEVD, blocked
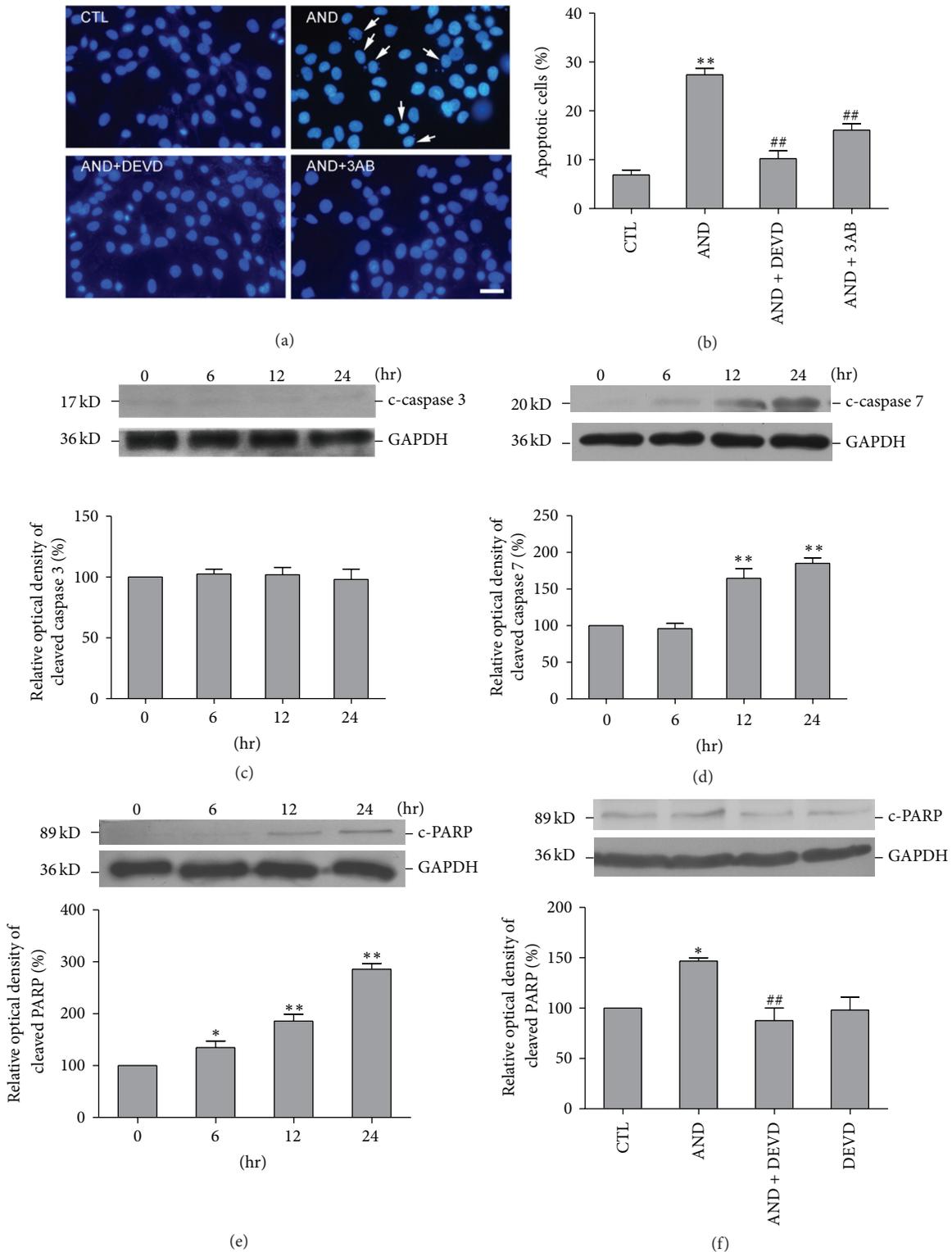
FIGURE 2: The apoptotic effects of AND on C6 glioma cells, and the involved signaling molecules. (a) 4,6-Diamidino-2-phenylindole dilactate (DAPI) staining. The cells were treated with 0.1% DMSO (CTL), 15 μM AND, 15 μM AND plus 50 μM DEVD, and 15 μM AND plus 5 μg/mL 3AB for 12 h and stained with DAPI. Apoptotic nuclei (arrowheads) were identified by nuclear morphology. Bar = 20 μm. (b) Quantitative data from (a). $N = 7$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control group. $^{\#\#}P < 0.01$, as compared to the AND group. ((c)–(e)) The protein expression levels of cleaved caspase (c-caspase) 3 (c), c-caspase 7 (d), and cleaved PARP (c-PARP) (e). Cells were treated with 15 μM AND for 0, 6, 12, or 24 h, and cell lysates were analyzed for target proteins and GADPH (internal standard). $N = 3$. (f) Effects of DEVD. The cells were treated with 0.1% DMSO, 15 μM AND, 15 μM AND plus 50 μM DEVD, or 50 μM DEVD for 24 h, and cell lysates were analyzed for cleaved PARP and GADPH. $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the control group. $^{\#\#}P < 0.01$ as compared to the AND group.

the AND-induced elevation of c-PARP levels (Figure 2(f)). Therefore, AND induced apoptosis via the caspase 7-PARP signaling pathway.

3.3. AND Increased the Expression of p53 and Activated p53.

Procaspase 7 is cleaved to an active form, a heterotetramer of 2 large and 2 small subunits, by many enzymes, including caspases 3 and 9 [33, 36, 37]. In our study, caspases 3 and 9 were apparently not involved in AND-induced apoptosis, because these 2 caspases were not activated by AND treatment (Figure 2(a) and Supplementary Figure 3). The promoter region of caspase 7 is known to contain a binding site for p53 [38]. Further, p53 activation has been shown to lead to downstream activation of caspases 3 and 7, causing apoptosis in human glioblastoma cells [39]. First, we want to examine whether p53 is activated under AND treatment. After 24 h of AND treatment, the protein levels of both phosphorylated p53 and total p53 increased in a dose-dependent (Supplementary Figure 2(c)) and time-dependent (Figure 3(a)) manner. In Supplementary Figure 2(c), the phosphorylated p53 protein levels in C6 cells increased to 2.2-, 2.5-, and 4.1-fold following treatment with 10 μM, 15 μM, and 20 μM AND, respectively, compared to treatment with DMSO, whereas the total p53 protein levels in C6 cells also increased to 2-, 2.1-, and 2.8-fold, respectively (Supplementary Figure 2(c)). As shown in Figure 5, the levels of phosphorylated p53 protein in C6 cells increased to 1.3-, 2.5-, and 3.2-fold following treatment with AND for 6 h, 12 h, and 24 h, respectively, relative to treatment for 0 h, whereas the total p53 protein levels in C6 cells also increased to 1.2-, 1.8-, and 2.8-fold (Figure 3(a)). To serve as a transcription factor, the activation of p53 included both phosphorylation and nuclear translocation. Immunofluorescent staining showed that p-p53 was expressed in the nucleus compared to control with AND treatment (Supplementary Figure 5). These results show that AND induced both the phosphorylation of p53 and p53 activation.

We then examined whether p53 plays a key role in AND-induced apoptosis. We pretreated C6 cells with a p53 inhibitor, pifithrin-α, and evaluated the extent of apoptotic cell death using DAPI stain (Figure 3(b)). The proportions of apoptotic cells were 5.0% ± 0.6% for the DMSO groups, 20.0% ± 2.0% for 15 μM AND, and 7.5% ± 0.6% for 15 μM AND plus pifithrin-α (Figure 3(b)). MTT and annexin V binding assays also showed that the effect of AND could be blocked by pifithrin-α (Supplementary Figure 4). Thus, AND induced apoptosis by p53 activation.

3.4. AND Induced Apoptosis of C6 Glioma Cells via the p53-Caspase 7-PARP Pathway.

Because AND increased cellular p53 levels and the p53 inhibitor pifithrin-α reversed the effects of AND on apoptosis, we investigated the role of p53 in apoptosis. AND treatment led to increased levels of c-PARP, and pifithrin-α blocked this AND-induced PARP activation (Figure 3(c)). Further, AND treatment also led to increased levels of c-caspase 7, and pifithrin-α blocked this AND-induced caspase 7 activation (Figure 3(c)). The above findings suggest that AND can induce increased activation of p53 protein, which in turn activates the downstream caspase 7-PARP cascade.

3.5. Knockdown of p53 by siRNA Blocked AND-Induced Apoptosis.

We further confirmed the role of p53 in AND-induced apoptosis by using RNA interference. A siRNA against p53 was introduced into C6 glioma cells, which decreased the level of total p53 protein to 55% compared to that in cells transfected with a negative siRNA (Figure 4(a)). After 12 h treatment, DAPI stain showed that the proportion of apoptotic cells was 4.8% ± 0.6% for cells treated with DMSO, 18.6% ± 2.9% for cells treated with 15 μM AND, and 8.3% ± 0.6% for cells first transfected with p53 siRNA and then treated with 15 μM AND (Figures 4(b) and 4(c)).

Since p53 siRNA reversed the apoptotic effect of AND, we examined how p53 siRNA affected the activation of PARP and caspase 7 by AND in C6 glioma cells. The levels of cleaved PARP and caspase 7 were elevated to 1.6- and 2.2-fold in negative siRNA groups following AND treatment for 24 h. In p53 siRNA-transfected cells, AND failed to activate caspase 7 and PARP (Figure 4(d)). This further supported the hypothesis that AND caused apoptosis of C6 glioma cells via the p53-caspase 7-PARP pathway.

3.6. Activation of p53 by AND Was Regulated by ERK.

ERK has been implicated in the regulation of p53 in the literature [40]. Following AND treatment, the levels of pERK and pP38 in C6 cells increased significantly in a time-dependent manner (Figure 5(a)), while the phosphorylation of JNK was not affected by the same treatment (Figure 5(a)). The pERK levels were elevated to 2.3-, 5-, and 4.5-fold after AND treatment for 6 h, 12 h, and 24 h, respectively (Figure 5(a)). Pretreatment of C6 cells with the ERK signaling inhibitor, PD98059, for 30 min, blocked the increased expression of p53 protein by AND (Figure 5(b)). Since inhibition of p38 kinase by SB203580 did not abrogate AND-induced p53 phosphorylation, we concluded that p38 kinase was not involved in this event (data not shown). Accordingly, p53 activation by AND was dependent on ERK signaling (Figure 5(b)).

To further confirm the role of ERK in C6 cell apoptosis triggered by AND, glioma cells were treated with an ERK signaling inhibitor, PD98059, for 30 min, followed by 15 μM AND for 12 h. The apoptotic cell ratios were 8.3% ± 0.6% in AND groups pretreated with PD98059 and 18.3% ± 2.3% in AND-only groups (Figures 5(c) and 5(d)). MTT and annexin V binding assay also showed the blocking effect of AND (Supplementary Figure 6). Therefore, AND could induce apoptosis of C6 glioma cells via the ERK-p53-caspase 7-PARP signal transduction pathway.

We used normal astrocytes to compare the cytotoxicity of AND between normal cells and glioma cells. Cell viability was not affected by the presence of AND at various concentrations, ranging from 5 μM to 20 μM, compared to the control group (Figure 6(a)). Following treatment with 15 μM AND for 24 h, the primary cultured astrocytes showed no increase of p53 or pERK protein levels (Figure 6(b)). This indicates that AND induces apoptosis, providing a tumoricidal effect, in C6 glioma cells.

In order to further verify the effect of AND on tumor growth *in vivo*, two types of experiments were designed.

(a)

(c)
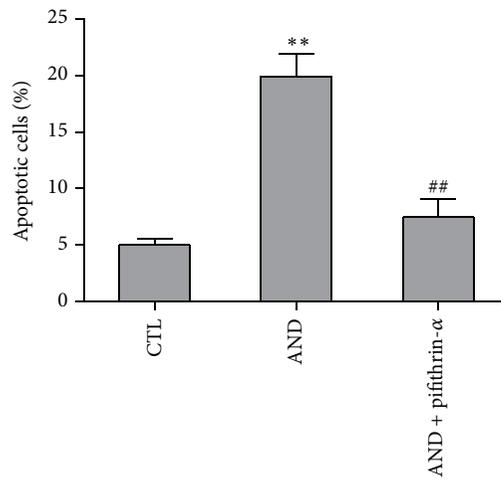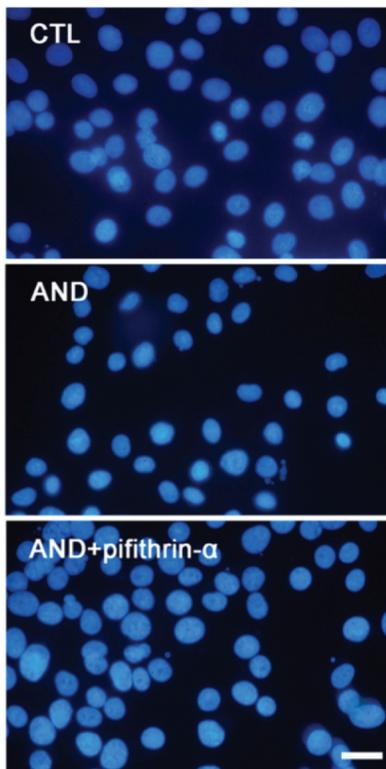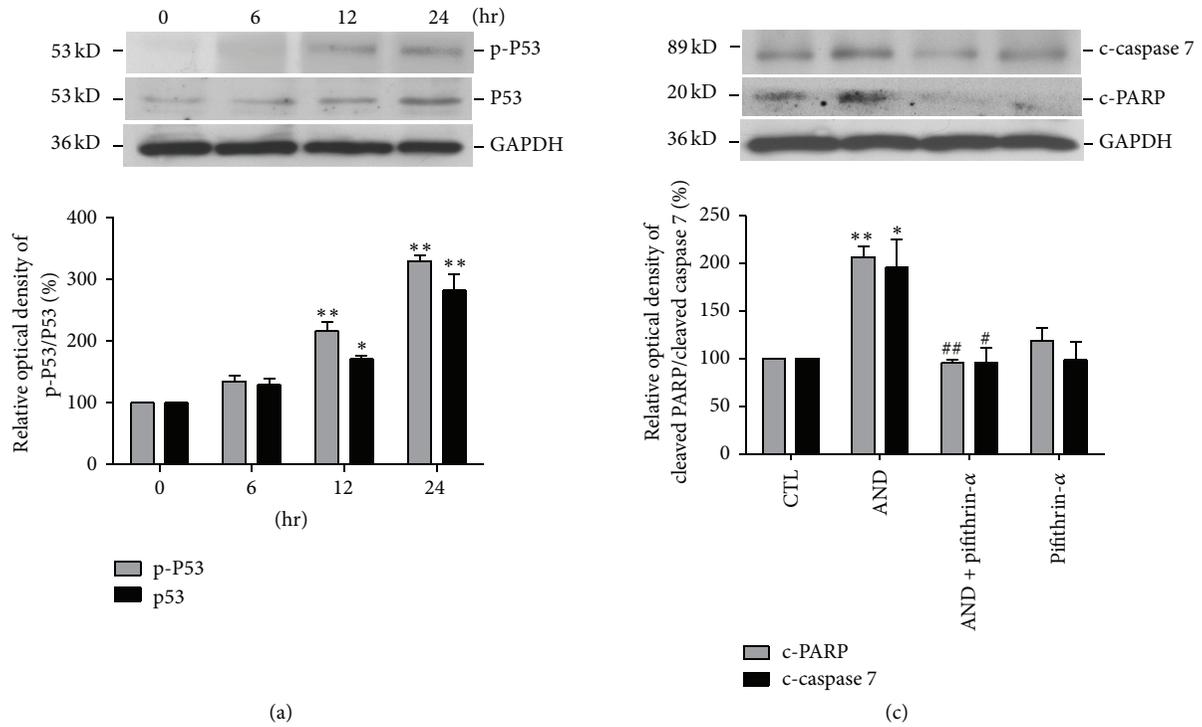
(b)

FIGURE 3: p53 and its downstream molecules were involved in AND-induced apoptosis in C6 glioma cells. (a) The expression of p-p53 and p53. Cells were treated with 15 μM AND for 0, 6, 12, or 24 h, and cell lysates were analyzed for total p53 and p-p53. $N = 3$. (b) DAPI stain. Cells were treated with 0.1% DMSO, 15 μM AND, or 15 μM AND plus 15 μM pifithrin-α for 12 h and stained with DAPI. Bar = 20 μM. Data is quantitated by cell counting. $N = 4$. (c) The protein expression of c-PARP and p-caspase 7. Cells were treated with 0.01% DMSO (CTL), 15 μM AND with or without 15 μM pifithrin, or pifithrin alone for 24 h, and cell lysates were analyzed for cleaved PARP (c-PARP) and c-caspase 7. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the 0 h or CTL group, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, as compared to the AND-group. $N = 4$.
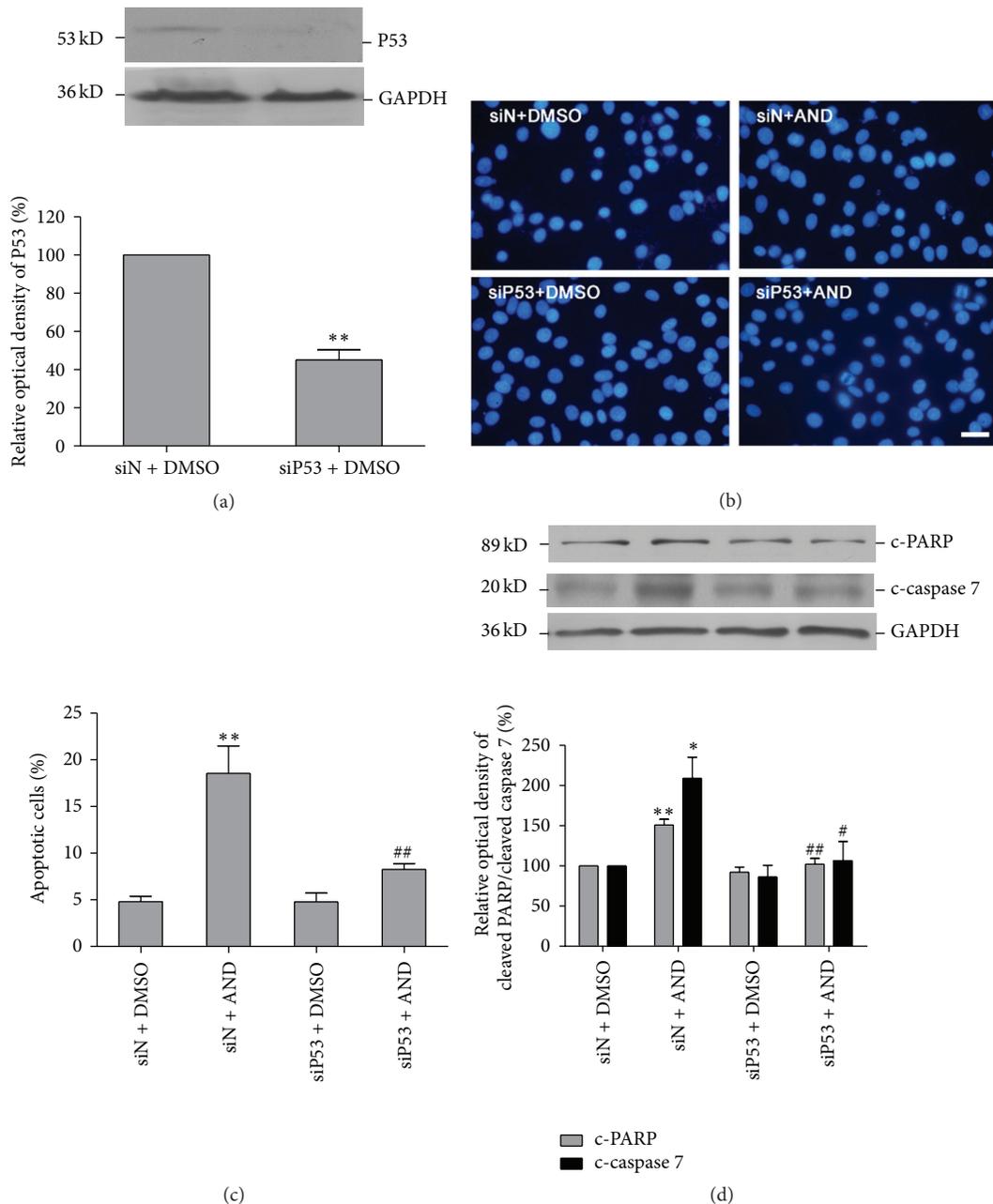
FIGURE 4: Effect of p53 siRNA on AND-induced apoptosis in C6 glioma cells. (a) Knockdown efficiency. Cells were transfected with p53 siRNA for 48 h, and cell lysates were analyzed for total p53 expression. $N = 3$. $^{**}P < 0.01$, as compared to the siRNA-negative (siN) group. ((b)-(c)) Effect of p53 siRNA on AND-induced apoptosis. The cells were transfected with siN and siRNA-p53 (siP53) for 48 h and then treated with 0.01% DMSO or 15 μM AND for 12 h and stained with DAPI (b), and the ratio of apoptotic cells counted (c). $N = 5$. Bar = 20 μm. $^{**}P < 0.01$, compared to the siN + DMSO group. $^{##}P < 0.01$ compared to the siN + AND group. (d) Cells were transfected with siN or siP53 for 48 h and then treated with 0.01% DMSO or 15 μM AND. Cell lysates were analyzed for c-PARP and c-caspase 7. $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the siN + DMSO. $^{#}P < 0.05$, $^{##}P < 0.01$, compared to the siN + AND group.

In the first coinjection of AND way, C6 cells were injected subcutaneously into two ears with (right) or without (left) 20 μM AND for 5 days (Figure 7(a)). AND treatment decreased the tumor weights by 86% (Figures 7(b) and 7(c)). In the second postimplantation AND injection of AND group, C6 cells were injected to both ears of ICR mice and allowed to grow for 3 days. At this stage, tumor masses on both sides appeared to be similar (Figure 7(d)). Then, PBS or 20 μM AND were injected into the tumors of the left and right ear twice (at day 3 and day 6), respectively. AND treatment caused tumor regression as shown by 67% decrease of the tumor weight at day 9 (Figures 7(e), 7(f), and 7(g)).
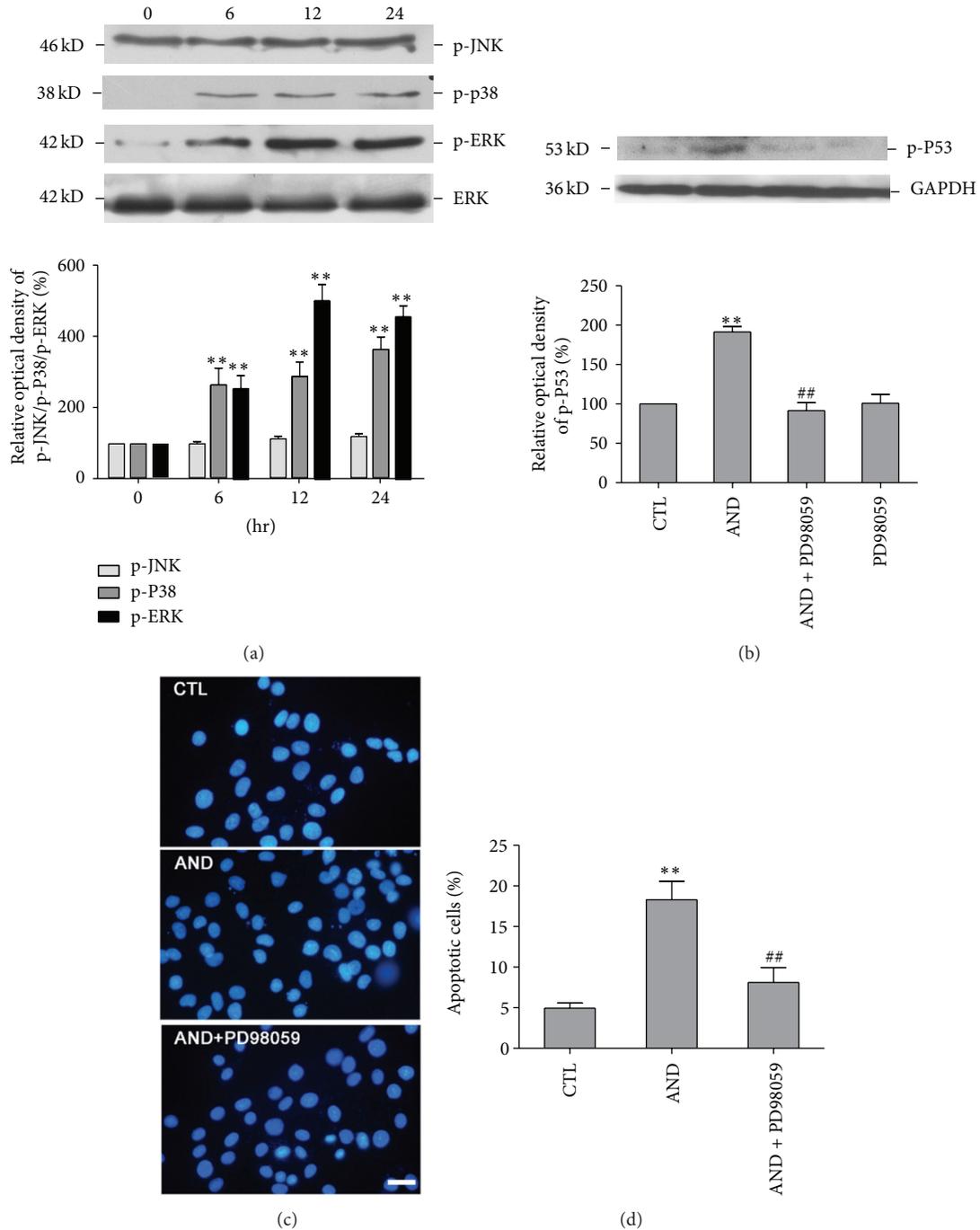
FIGURE 5: The expression of MAPK and the effect of MAPK inhibitors on AND-induced apoptosis in C6 glioma cells. (a) Time course study on MAPK activation. Cells were treated with 15 μM AND for 0, 6, 12, or 24 h, and cell lysates were analyzed for p-JNK, pERK, p-38, or GADPH. The lower panel is the quantization of p-JNK, p-ERK, and p-p38 levels $^*P < 0.05$, $^{**}P < 0.01$, compared with the 0 h control. (b) The effect of ERK inhibitor on p53 phosphorylation. Cells were treated with 0.01% DMSO (CTL) or 15 μM AND with or without 30 μM PD98059 and were blotted for p-p53. $N = 4$. $^{**}P < 0.01$, as compared to the CTL group. $^{\#\#}P < 0.01$, compared to the AND group. (c) The effect of ERK inhibitor on AND-induced cell death. Cells were treated with 0.01% DMSO (CTL) or 15 μM AND with or without 30 μM PD98059 and then were stained with DAPI. (d) Quantization of the apoptotic cell percentage. $N = 4$. $^{**}P < 0.01$, compared to the DMSO group. $^{\#\#}P < 0.01$, as compared to the AND group.

(a)

(b)
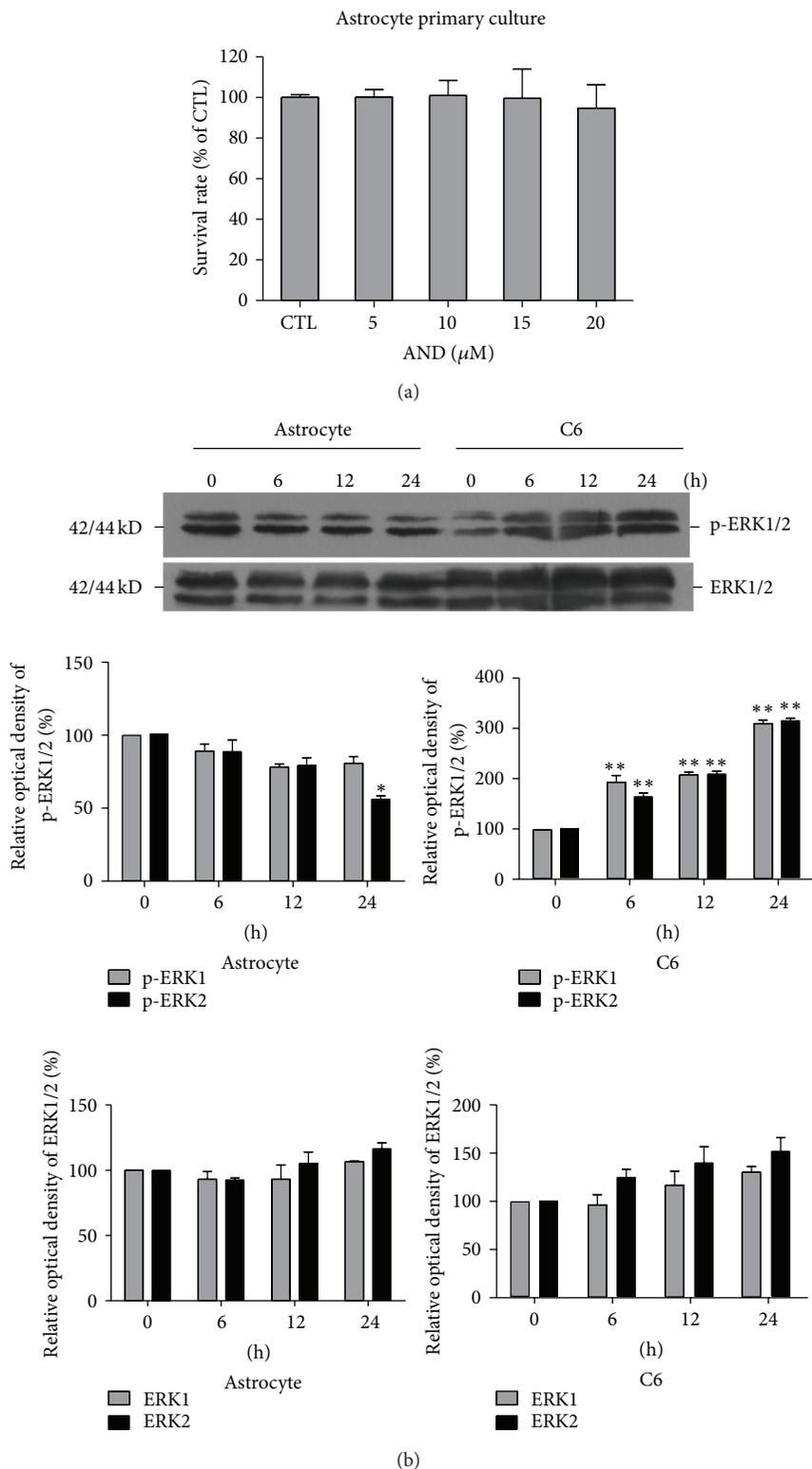
FIGURE 6: Effect of AND on cell viability and the expression of pERK in normal cultured rat astrocytes and C6 glioblastoma cells. (a) Cell survival analysis. Normal astrocytes were treated with 0.1% DMSO (CTL), 5, 10, 15, or 20 μM of AND for 24 h, and the cell viability was determined by MTT assay. $N = 3$. (b) Blot analysis. Astrocytes and C6 cells were treated with 15 μM AND for 0, 6, 12, or 24 h, and cell lysates were analyzed for pERK and ERK (upper panel). The quantization of p-ERK1, p-ERK2, ERK1, and ERK2 was presented in the following plots (lower panel). $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the 0 h group.
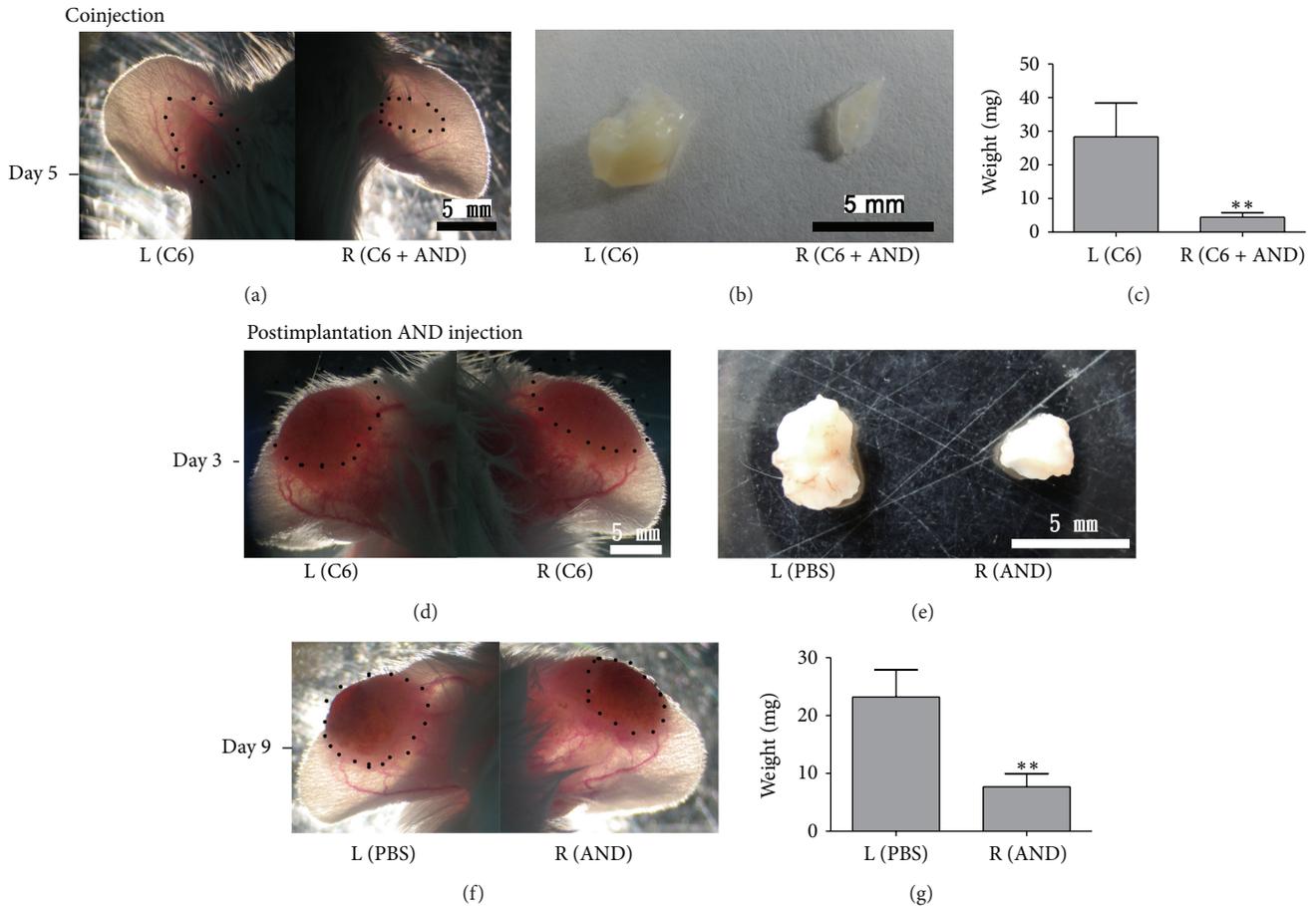
FIGURE 7: AND prevented the growth of C6 glioma *in vivo*. ((a)–(c)) The ears of ICR mice were injected with C6 cells with or without 20 AND μM for 5 days. (a) An example of cell injection alone ear (C6) and AND plus cells injection ear (C6 + AND). (b) Tumors isolated from (a) at day 5. (c) Quantitation of tumor weights. $N = 3$, $**P < 0.01$ compared to the C6 group. ((d)–(g)) The ears of ICR mice were injected with C6 cells for 3 days (d) and then received injection with PBS (PBS) or 20 AND μM (AND) twice at day 3 and day 6, and pictures were taken at day 3 (d) and day 9 (f). (e) Tumors isolated from (f) at day 9. (g) Quantitation of tumor weights. $N = 3$, $**P < 0.01$ compared to the PBS-group.

4. Discussion

The poor prognosis of glioblastoma is due to therapeutic resistance and tumor recurrence after surgical removal. Treatment of high-grade gliomas is still only palliative. Studies have explored many techniques for glioblastoma treatments, including new chemotherapeutic agents such as camptothecin (CPT) [41], etoposide (VP) [42], emodin [43], and As_2O_3 [44]. This study used the C6 glioma cell line to evaluate the cytotoxic effects of AND and its potential therapeutic use. We have shown that AND effectively induced apoptosis in glioma cells via a novel signaling pathway, the ERK-P53-caspase 7-PARP pathway.

AND, the main constituent of *A. paniculata*, exhibits pharmacological effects on various cancers, including cell cycle arrest [45], autophagy [46], and apoptosis [24]. The effects of AND on cancer cells depend on the cell types and the concentrations applied. The concentrations used in previous studies were very wide, ranging from 0.7 to 100 μM, and the concentration of AND we used in this study, 15 μM,

was within this range. AND only caused cell cycle arrest in hepatoma cells and in human glioblastoma [47, 48] but induced cell death in other cancer cells [24]. Interestingly, in this study, 15 μM AND caused apoptosis in C6 glioma cells but had no effects on normal astrocytes (Figure 6), suggesting its potential use as a chemotherapeutic drug that has a selective cytotoxic effect on glioblastoma cancer cells.

In recent years, several studies focused on the apoptotic effect of AND on tumor cells. These studies found that AND-induced apoptosis occurred by the activation of proapoptotic JNK pathway [17, 26] and the suppression of antiapoptotic PI3K/AKT and ERK pathways [15, 19]. In addition, AND also triggered apoptosis through P53-induced caspase 3 activation [24]. In our system, we found that AND-induced apoptosis in C6 cells was mediated through the ERK-p53 pathway, since activation of p53 was decreased by an ERK1/2 inhibitor (Figure 5(b)). Although activation of ERK has been reported to be involved in AND-induced cell death in melanoma [24] and decreased invasion process in colon cancers [49], inhibition of ERK blocked the cytotoxic effect of AND in

C6 cells (Figures 5(c) and 5(d)), suggesting that it is the upstream key regulator in AND-induced C6 cell death. ERK signaling, which was activated in AND-treated C6 cells, is an important signaling pathway involved in cell growth or apoptosis [50, 51]. The major differences in ERK signal activation in cell growth or cell death are the starting time and the duration of phosphorylation of ERK. In response to growth factor (EGF), ERK activation is rapid and transient, occurring within minutes of treatment [52]. We found that when cells were treated with AND, ERK was significantly activated and its phosphorylation remained high up to 24 h (Figure 5(a)). The same pattern of ERK activation has been observed in many anticancer drugs such as doxorubicin, quercetin [50], and paclitaxel [53]. p53, a tumor suppressor, is involved in the apoptotic effects of many drugs on cancer cells [54] and plays a central role in AND-induced apoptosis in C6 cells, as seen from the effect of a specific inhibitor (Figure 3) and siRNA (Figure 4). p53 is characterized as a stress-response protein, which is induced by DNA damage [55], oxidative stress [56], and deregulated oncogene expression [40]. Two major events are noticed in p53 activation. First, the half-life of the p53 protein is increased dramatically, which leads to p53 accumulation in stressed cells. Second, the phosphorylation and conformational change forces p53 to become a transcription factor. It has become clear that the p53 protein interacts functionally with the mitogen-activated protein kinase (MAPK) pathways, including JNK, the p38MAPK, and the ERK pathways. With stress exposure, MAPK phosphorylates and activates p53, leading to p53-mediated cellular responses [57]. Among the MAPK-mediated phosphorylations, ERK-mediated phosphorylation of p53 has been well observed in a number of experimental systems, including in ovarian cells induced by cisplatin [58] and in epidermal cell treated with resveratrol [59]. Our data correlates with these previous findings.

In many cancer cells, PARP is reported to be cleaved by activation of both caspases 3 and 7 during cell death induced by chemotherapeutic drugs, including camptothecin [60] and sorafenib [61]. It was also shown that caspase 7, which shares the same substrate preference as caspase 3, can cleave PARP more efficiently [62]. In our study, we were unable to detect caspase 3 by western blot when we induced cell death by AND in C6 cells (Figure 2(c)), and inhibition of caspase 7 prevented PARP cleavage (Figure 2(f)). These data suggested that PARP was cleaved by caspase 7 in our system. The same signaling was responsible for the apoptosis induced by β-lapachone in human prostate cancer cells [63] and by etoposide (VP16) phosphate in human leukemia cells [35].

Whether p53-induced activation of caspase 7 was due to a direct or indirect effect was a question that remained unanswered in this study. p53 is implicated in the induction of 2 distinct apoptotic signaling pathways—the intrinsic and extrinsic pathways. The extrinsic pathway involves death receptors, which lead to a caspase activation cascade, including caspase 8 and caspase 3. The intrinsic pathway is triggered by DNA damage and is associated with the release of cytochrome c from the intermembrane space of mitochondria into the cytoplasm. Cytochrome c forms a complex, termed the apoptosome, with apoptotic protease-activating factor 1 (APAF-1) and procaspase 9, and caspase 9 is activated to promote the activation of caspase 3, caspase 6, and caspase 7 [64, 65]. Both these pathways can trigger the activation of caspase 7 and PARP and lead cells to apoptosis. This study found that caspases 3 was not activated by AND (Figure 2(c)), and inhibition of caspase 9 by LEHD did not prevent AND-induced cell apoptosis of C6 cells (Supplementary Figure 3). Thus, caspase 9 was not involved in AND-induced caspase 7 activation. We believe that some regulatory signaling molecule(s), which may be caspase 8 in the intrinsic pathway, act between p53 and caspase 7. Despite being an intracellular signaling molecule, ERK also responds to stress, including oxidative stress [66] and ER stress [67]. Recent studies have suggested that both AND [68] and an AND derivatives (AL-1) [69] exert cytotoxic effects on cells through a ROS-dependent mechanism. We also demonstrated that ROS is involved in AND-induced apoptosis in C6 cells by ROS chelators, NAC, and DTT (Supplementary Figure 7) with MTT and annexin V binding assay. Thus, further studies should explore whether ROS activates ERK signaling, as well as the underlying mechanisms.

5. Conclusion

In conclusion, AND exerts its cytotoxicity on C6 glioma cells through the ERK-p53-caspase 7-PARP apoptotic pathway. AND treatment inhibited the tumor growth in coinjection experiment and caused the regression of the tumors in postinjection experiment. This regression of well-formed tumors was mediated by AND-induced cell death. The successful application of AND on animal models strengthens its clinical use in cancer therapy. Because of the selective toxicity to only glioma cells, and not to normal astrocytes, AND has great potential to be an anticancer drug.

Conflict of Interests

There is no conflict of interests for all authors.

Acknowledgment

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Research Article

Mesenchymal Stem Cell-Induced Doxorubicin Resistance in Triple Negative Breast Cancer

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Triple negative breast cancer (TNBC) is an aggressive histological subtype with limited treatment options and a worse clinical outcome compared with other breast cancer subtypes. Doxorubicin is considered to be one of the most effective agents in the treatment of TNBC. Unfortunately, resistance to this agent is common. In some drug-resistant cells, drug efflux is mediated by adenosine triphosphate-dependent membrane transporter termed adenosine triphosphate-binding cassette (ABC) transporter, which can drive the substrates across membranes against concentration gradient. In the tumor microenvironment, upon interaction with mesenchymal stem cells (MSCs), tumor cells exhibit altered biological functions of certain gene clusters, hence increasing stemness of tumor cells, migration ability, angiogenesis, and drug resistance. In our present study, we investigated the mechanism of TNBC drug resistance induced by adipose-derived MSCs. Upon exposure of TNBC to MSC-secreted conditioned medium (CM), noticeable drug resistance against doxorubicin with markedly increased BCRP protein expression was observed. Intracellular doxorubicin accumulation of TNBC was also decreased by MSC-secreted CM. Furthermore, we found that doxorubicin resistance of TNBC was mediated by IL-8 presented in the MSC-secreted CM. These findings may enrich the list of potential targets for overcoming drug resistance induced by MSCs in TNBC patients.

1. Introduction

Mesenchymal stem cells (MSCs), also called multipotent mesenchymal stromal cells, are nonhematopoietic cells that reside mainly in the bone marrow and in adipose tissue [1–3]. They have stem cell-like characteristics and are able to differentiate into osteogenic, adipogenic, and chondrogenic lineages when placed in the appropriate environments [4]. MSCs are featured as plastic adherent cells that express stromal cell markers (CD73, CD105, CD44, CD29, and CD90) in the absence of hematopoietic markers (CD34, CD45, and CD14) and endothelial markers (CD34, CD31, and vWF) [5, 6]. MSCs are characteristically recruited to injured areas or hypoxic tumor microenvironments. The homing of MSCs to tumors was among the earliest phenomenon of MSC-cancer interactions to be reported [7, 8]. In the tumor microenvironment, upon interaction with MSCs, tumor cells exhibit altered biological functions of certain gene clusters. Accumulating evidence has demonstrated that MSCs play complicated roles in tumor development and progression, by increasing stemness of tumor cells, mediating tumor cell migration, promoting angiogenesis, supporting immune responses, and inducing drug resistance [9, 10]. Therefore, comprehensive knowledge on the mechanism of interaction between cancer and MSCs is critical.

Triple negative breast cancer (TNBC) is an aggressive histological subtype with limited treatment options and a worse clinical outcome compared with other breast cancer subtypes [11]. The duration of response to chemotherapeutic regimens is usually short and commonly relapses rapidly. Doxorubicin, an anthracycline antibiotic, is considered to be one of the most effective agents in the treatment of TNBC.

Unfortunately, resistance to this agent is common, leading to an unsuccessful outcome in many TNBC patients. Resistance to current standard regimens limits the available options for previously treated patients to a small number of noncross resistant regimens [12]. This makes TNBC an important issue which deserves further fundamental research.

Resistance to therapy is one of the major obstacles in cancer treatment. The mechanisms involved in classic chemotherapy resistance include enhanced activity of positive regulators of cell proliferation, loss of tumor suppressors, inactivation of cell death, or enhancement of survival functions [10]. Besides the classically defined causes of drug resistance, tumor microenvironment can also promote drug resistance by preventing drugs accumulation in tumor cells [9, 13]. In some drug-resistant cells, drug efflux is mediated by adenosine triphosphate- (ATP-) dependent membrane transporters termed adenosine triphosphate-binding cassette (ABC) transporters, which can drive the substrates across biological membranes against a concentration gradient [14]. Among dozens of human ABC transporters, three well-known ABC transporters account for most of the drug resistance phenomenon, namely, ABCB1/p-glycoprotein (P-gp), ABCC1/multidrug resistance-associated protein 1 (MRP 1), and ABCG2/breast cancer resistance protein (BCRP) [14, 15]. Chemoresistance to doxorubicin may be attributed to P-gp, MRP1, or BCRP, as doxorubicin is substrate of these ABC transporters [16].

In our present study, noticeable doxorubicin resistance of TNBC was observed by exposure of TNBC to MSC-secreted conditioned medium. Therefore, the aim of this study was to investigate the underlying mechanism of doxorubicin chemoresistance induced by MSC in TNBC. Understanding the tumor-promoting factors secreted by MSCs or the mechanism activated by MSCs in tumor cells may enrich the list of potential targets for molecular therapy and overcoming tumor drug resistance in triple negative breast cancer.

2. Materials and Methods

2.1. Materials. Rabbit anti-BCRP and anti-MRP antibodies were purchased from Santa Cruz (Santa Cruz, CA). Rabbit anti-P glycoprotein was purchased from Gene-Tex (Irvine, CA). Anti-mouse and anti-rabbit horseradish peroxidase- (HRP-) linked antibodies were purchased from Cell Signaling (Danvers, MA). Mouse anti-β-actin antibody, dimethyl sulfoxide, formaldehyde, ko143, crystal violet, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sulforhodamine B (SRB), and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Doxorubicin was purchased from Tocris Bioscience (Minneapolis, MN).

2.2. Cell Culture. Human triple negative breast cancer cells MDA-MB-231 purchased from American Type Culture Collection (Manassas, VA) were maintained in L-15 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin and were incubated at $37°C$ without CO_2. Human adipose-derived mesenchymal

stem cells (MSC-ad) purchased from ScienCell Research Laboratories (Carlsbad, CA) were grown on poly-L-lysin coated flask ($2 \mu g/cm^2$) and maintained in mesenchymal stem cell medium (MSCM) supplemented with 5% FBS, 1% mesenchymal stem cell growth supplement (MSCGA), and 1% penicillin/streptomycin solution. MSC-ad was incubated at $37°C$ in a humidified incubator under 5% CO_2 and 95% air. Confluent cultures were passaged by trypsinization.

2.3. Collection of Conditioned Medium from Human Adipose-Derived Mesenchymal Stem Cells (MSC-ad). MSC-ad human adipose-derived mesenchymal stem cells were cultured on coated flasks as described above. Subconfluent culture was refreshed by fully supplemented mesenchymal stem cell medium (MSCM) and cultured for 48 hours before medium was collected as MSC-ad conditioned medium (MSC-ad CM). The conditioned medium was filtered to remove cellular materials and supernatants were aliquoted and stored at $-20°C$ before use [17].

2.4. Crystal Violet Staining. Cell viability was determined by staining with crystal violet according to our previous report [18]. After the indicated period of treatment, cells were washed with PBS twice and then fixed with 12% formaldehyde. After 10 minutes incubation at room temperature, formaldehyde was aspirated and cells were air dried for 20 minutes, followed by staining with 1% crystal violet in 50% methanol for 5 minutes. Stained cells were washed with tap water and subjected to spectrophotometric quantitation (OD 540 nm) using Thermo Multiskan Spectrum plate reader.

2.5. MTT Assay. Live cells were measured by using the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to our previous study [19]. Culture medium was aspirated after indicated treatment and cells were washed with PBS twice. MTT solution (0.5 mg/mL in PBS) was then added in each culture well and cells were incubated at $37°C$. After incubation for 1 hour, MTT solution was removed and cells were lysed by DMSO. The absorbance was measured at 550 nm by Thermo Multiskan Spectrum plate reader.

2.6. Sulforhodamine B (SRB) Assay. The SRB assay is based on the measurement of cellular protein content according to our previous study [20]. Culture medium was aspirated after indicated treatment and cells were fixed with 10% trichloroacetic acid for 10 minutes. 0.4% (w/v) SRB in 1% acetic acid was then added in each culture well and stained for 30 minutes. Unbound SRB was washed out by 1% acetic acid and SRB-bounded cells were dissolved by 10 mM Tris solution. The absorbance was measured at 515 nm by Thermo Multiskan Spectrum plate reader.

2.7. Western Blot Analysis. After washing with ice-cold PBS, cells were lysed with radioimmunoprecipitation (RIPA) assay buffer on ice for 30 minutes. After centrifugation at 14,000 g for 20 minutes, the supernatant was used for Western blot or stored at $-20°C$ until use. Protein concentration was

measured by BCA assay kit (Pierce, Rockford, IL) with BSA as standard. Equal protein was separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were incubated for 2 hours with 7.5% dry skim milk in PBS-Tween 20 buffer to block nonspecific binding and then incubated with primary antibodies overnight at 4°C. After washing with PBS-Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies for another 1 hour. The blots were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology) using classic blue autoradiography film (MIDSCI, St. Louis, MO) [21]. Quantitative data were obtained using a densitometer and Image J software (National Institute of Health, Bethesda, MA).

2.8. Assay of Doxorubicin Efflux by Flow Cytometry.

To estimate doxorubicin efflux, cells were incubated with indicated concentrations of doxorubicin for indicated time periods at 37°C avoiding light exposure. After incubation, cells were rinsed with PBS buffer twice and incubated with fresh MSCM culture medium without doxorubicin for indicated periods allowing doxorubicin efflux from cells. After the wash periods, cells were detached by trypsin and then subjected to flow cytometry. When assessing the BCRP-mediated doxorubicin efflux, Ko143 was added during doxorubicin treatment and wash periods [22, 23]. The intracellular doxorubicin content was analyzed by Cytomics FC500 flow cytometer (Ex. 488 nm, Em. 575 nm) using CXP software (Beckman Coulter).

2.9. Cytokine Array.

Collection of conditioned medium was described above and 1 mL of sample medium was subjected to Human Cytokine Array Panel A purchased from R&D Systems (Minneapolis, MN). By following manufacturer's instruction, blots were visualized at the end by exposing membranes to autoradiography film for at least 5 minutes (MIDSCI, St. Louis, MO).

2.10. Statistics.

Values were expressed as mean ± S.E.M. of three independent experiments ($n = 3$). Results were analyzed by student's t-test and significance was defined as $P < 0.05$.

3. Results

3.1. Adipose-Derived Mesenchymal Stem Cells-Secreted Conditioned Medium Reduced Doxorubicin Sensitivity in MDA-MB-231 Human Triple Negative Breast Cancer Cells.

Firstly, MDA-MB-231 cells were treated by different fresh culture media (L15 or MSCM) or MSC-ad conditioned medium (CM) for 24 hours and doxorubicin was then added for another 24 hours before cell viability was assayed. As shown in Figure 1(a), 200 nM doxorubicin induced significant cell death after 4 hours' treatment on MDA-MB-231 cells in L15 medium. Similar results were obtained when MDA-MB-231 cells were in MSCM (fresh MSC culture medium) (Figure 1(b)). By the collected conditioned medium from

MSC-ad, we found that MDA-MB-231 cells showed decreased cell death induced by doxorubicin. Examined by crystal violet staining, 200 nM doxorubicin in MSCM (fresh MSC culture medium) decreased cell viability to 0.58 ± 0.039-fold to control; however, doxorubicin in CM of MSC-ad only decreased cell viability to 0.84 ± 0.036-fold (Figure 1(c)). Similar results were also exhibited by MTT assay and SRB assay (data not shown). These data indicated that changing from L15 medium to MSCM medium did not affect doxorubicin sensitivity and CM from MSC-ad significantly reduced doxorubicin sensitivity in MDA-MB-231 triple negative breast cancer cells.

3.2. Adipose-Derived Mesenchymal Stem Cells-Secreted Conditioned Medium Increased BCRP Protein Expression and Decreased Intracellular Doxorubicin Accumulation in MDA-MB-231 Human Triple Negative Breast Cancer Cells.

Reduced drug sensitivity may result from altered expression of ABC transporters, which can efflux substrate drugs across biological membranes against concentration gradient [14]. In order to explore the mechanism of reduced cytotoxic effect of doxorubicin caused by MSC-ad CM, we attempted to examine protein expression of ABC transporters after MSC-ad CM treatment. As shown in Figure 2, P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) were examined. It is worth mentioning that the MRP antibody used is suitable for detection of MRP1, MRP2, and MRP3. No significant change was observed in P-gp and MRP protein expressions (Figures 2(a) and 2(b)); however, 2.01 ± 0.09-fold increase of BCRP protein expression was displayed by treating MSC-ad CM on MDA-MB-231 cells (Figure 2(c)).

On top of the increased BCRP protein expression, we further investigated whether the intracellular doxorubicin accumulation was affected by MSC-ad CM in MDA-MB-231 cells. In order to obtain an optimal experimental condition, tests of different drug concentrations and wash periods were demonstrated. Before doxorubicin treatment, cells were refreshed in MSCM medium for 24 hours. Doxorubicin fluorescence was markedly increased dose dependently from 0.02 to 2 μM (1 h) without the wash period in MDA-MB-231 cells (Figure 3(a)). Cells were incubated with doxorubicin for 1 hour avoiding light exposure and then washed and incubated with doxorubicin-free medium for indicated period to estimate doxorubicin efflux. The wash period allows doxorubicin to efflux during the period. By different duration of wash period, doxorubicin fluorescence was decreased as wash period increased from 0 to 4 hours, which suggested an increasing doxorubicin efflux and decreasing doxorubicin accumulation (Figure 3(b)). In order to explore the BCRP-mediated doxorubicin efflux, Ko143 was added as a BCRP specific inhibitor [23–25]. As shown in Figure 3(c), after pretreatment of MSC-ad CM for 24 hours, doxorubicin accumulation was decreased compared with doxorubicin in MSCM medium. Noticeably, Ko143 antagonized the effect of MSC-ad CM and resulted in an increased amount of doxorubicin accumulation significantly. These data suggested that MSC-ad CM induced BCRP protein expression without

(a)

(b)

(c)
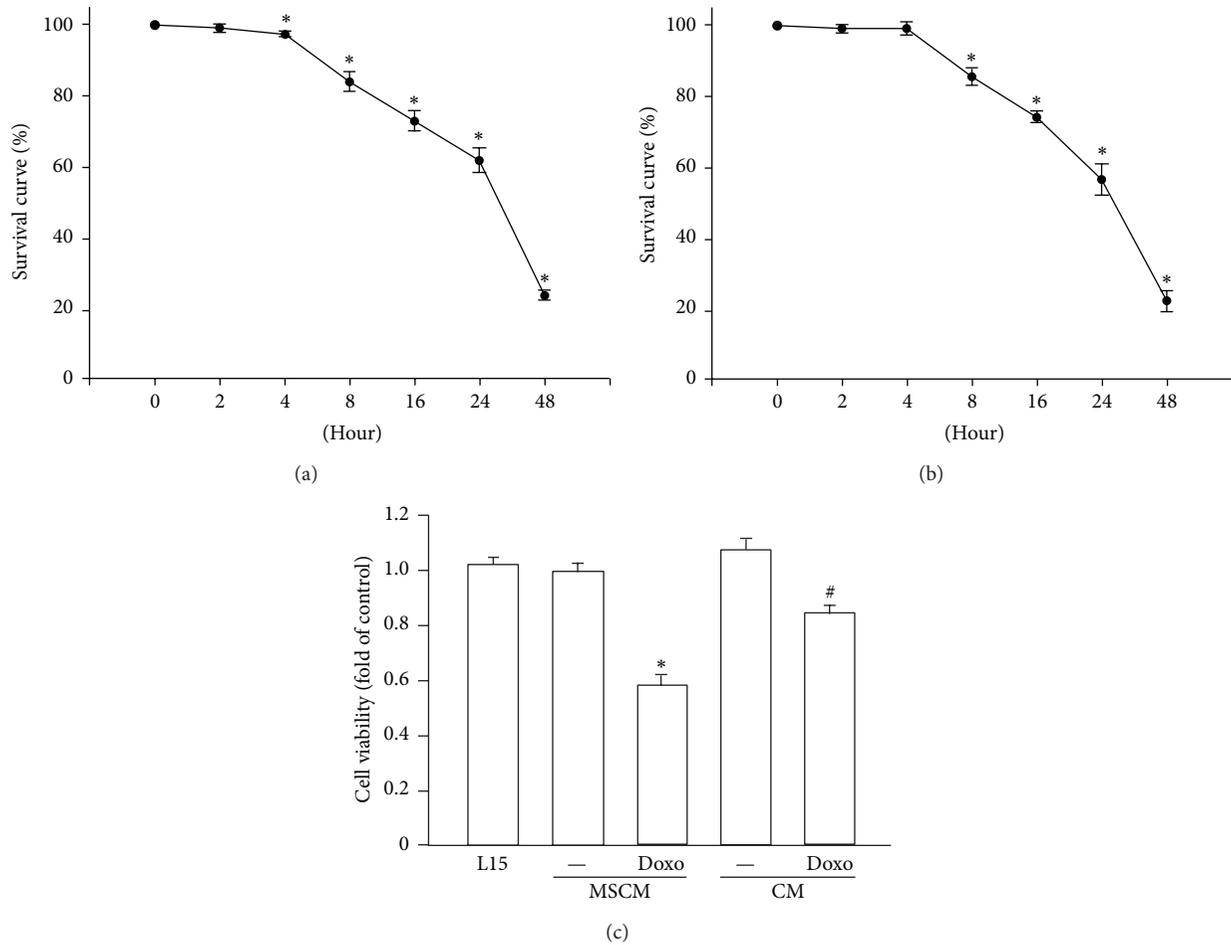
FIGURE 1: Adipose-derived mesenchymal stem cells-secreted conditioned medium induced doxorubicin resistance in MDA-MB-231 cells. Cells were treated by 200 nM doxorubicin with L15 (a) or MSCM medium (b) in a time-dependent manner and cell viability was examined by performing crystal violet staining. (c) Cells were treated by 200 nM doxorubicin with MSC-ad conditioned medium for 24 hours and cell viability was also examined by performing crystal violet staining. Graphs showed mean ± SEM of three independent experiments. $^*P < 0.05$ to doxorubicin-untreated group; $^#P < 0.05$ to doxorubicin in MSCM group. CM, conditioned medium from adipose-derived mesenchymal stem cells (MSC-ad); MSCM, mesenchymal stem cell medium (fresh MSC culture medium).

affecting P-gp and MRP and consequently decreased intracellular doxorubicin accumulation in MDA-MB-231 triple negative breast cancer cells.

3.3. Adipose-Derived Mesenchymal Stem Cells-Secreted IL-8 Is Responsible for Doxorubicin Resistance in MDA-MB-231 Human Triple Negative Breast Cancer Cells.

Accumulating evidence suggests that MSCs secreted various cytokines which are associated with tumor development and progression [9, 10]. Therefore, we assumed that important cytokines had been released by MSC-ad and consequently caused the observed doxorubicin resistance in MDA-MB-231 cells. The results obtained from cytokine array analysis of MSC-ad CM and MSCM showed that IL-6, IL-8, and Serpin E1 had been secreted by MSC-ad after culturing for 48 hours. Among these cytokines, IL-8 had the most pronounced amount of secretion (Figure 4(b), dot number 2).

It has been reported that IL-8 mediates drug resistance against certain anticancer agents [26, 27]. We hypothesized that IL-8 may also be responsible for the observed doxorubicin resistance in MDA-MB-231 cells. From the study of IL-8 on BCRP protein expression, we found that there was a 1.77 ± 0.07-fold increase of BCRP expression at 100 ng/mL human recombinant IL-8 stimulation and IL-8-induced BCRP expression was under a dose-dependent manner (Figure 5(a)). In order to confirm the contribution of IL-8 in MSC-ad CM in BCRP expression, IL-8 neutralizing antibody was added. MSC-ad CM-induced BCRP expression was antagonized by IL-8 neutralizing antibody (5 μg/mL) from 2.13 ± 0.13-fold down to 1.47 ± 0.05 that of control (Figure 5(b)). IgG isotype control antibody was used as negative control. Furthermore, cell viability under doxorubicin treatment was also determined. IL-8 (100 ng/mL) alone did not alter cell viability; however, doxorubicin-induced cytotoxicity was reduced in the presence of 100 ng/mL IL-8 and exhibited that 0.53 ± 0.03-fold cell viability was significantly

(a)

(b)

(c)
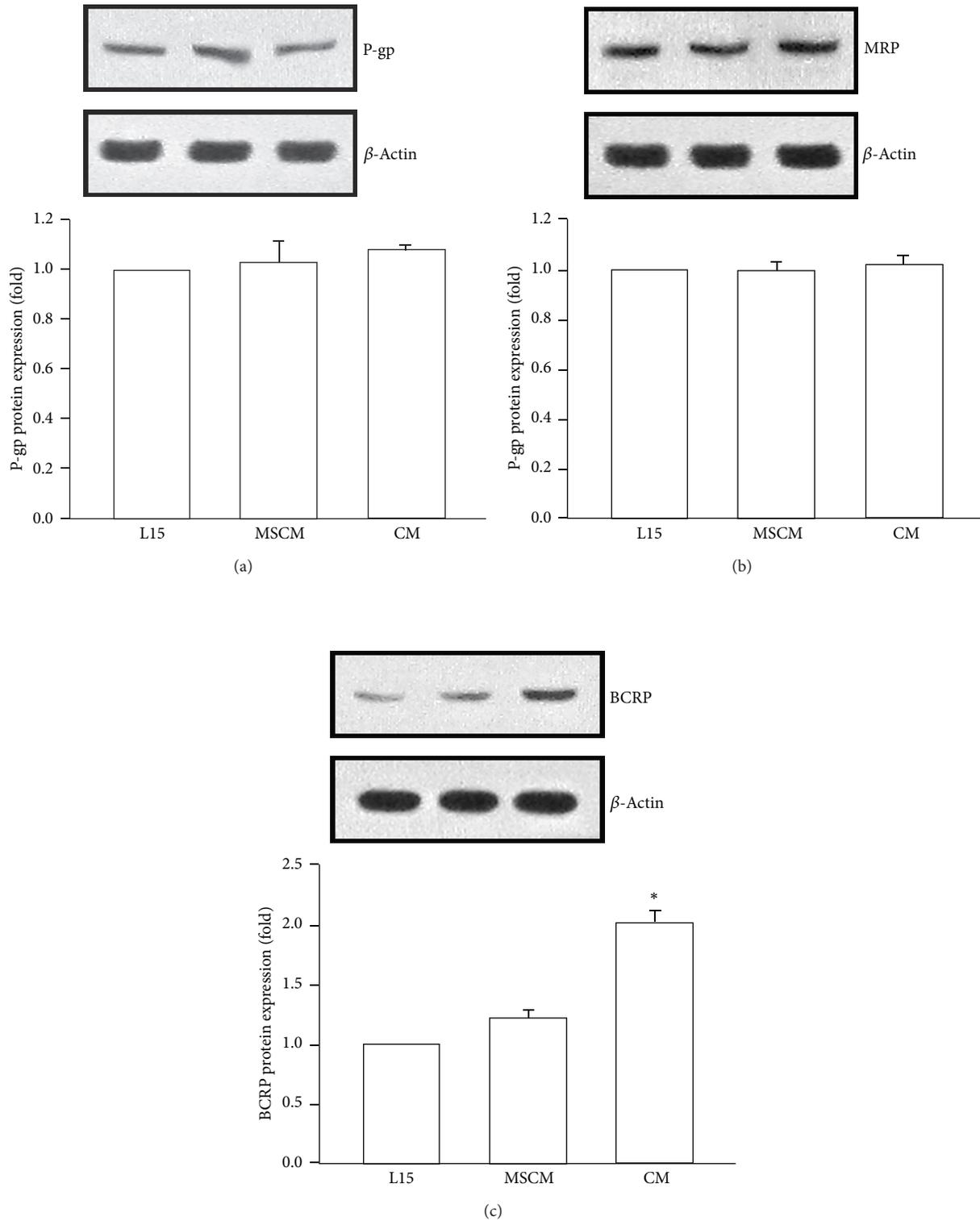
FIGURE 2: Adipose-derived mesenchymal stem cells-secreted conditioned medium increased BCRP protein expression in MDA-MB-231 cells. Cells were treated with L15 (control medium), MSCM, or MSC-ad conditioned medium for 24 hours and protein expression of (a) P-gp, (b) MRP, and (c) BCRP was examined by Western blotting. Graphs showed mean ± SEM of three independent experiments. $^{*}P < 0.05$ to MSCM group. CM, conditioned medium from adipose-derived mesenchymal stem cells (MSC-ad); MSCM, mesenchymal stem cell medium (fresh MSC culture medium).
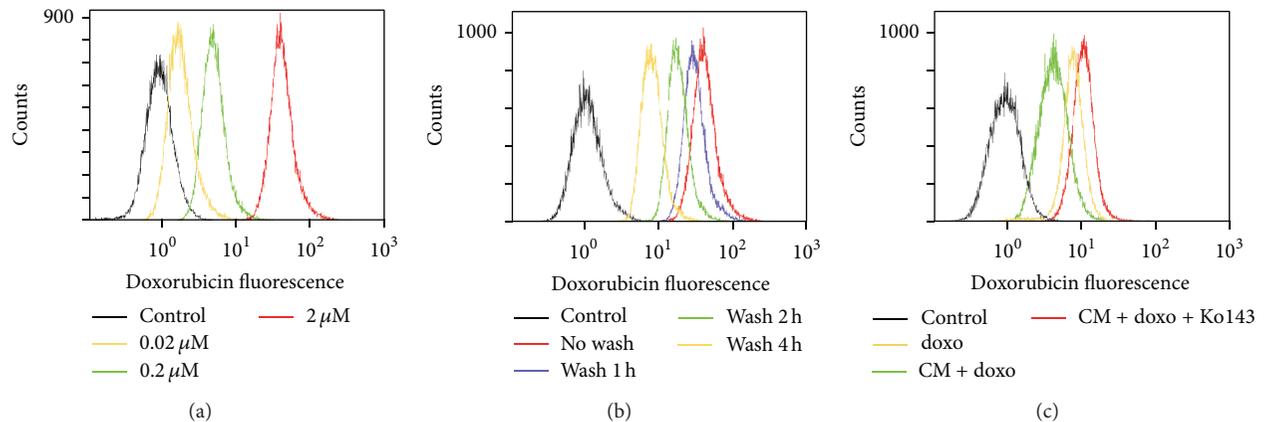
FIGURE 3: Adipose-derived mesenchymal stem cells-secreted conditioned medium decreased intracellular doxorubicin accumulation in MDA-MB-231 cells. Intracellular doxorubicin accumulation was measured by intensity of doxorubicin fluorescence using flow cytometry. (a) Cells were treated with different concentrations of doxorubicin for 1 hour without wash period. (b) Cells were treated with $2\,\mu$M doxorubicin for 1 hour and then washed for 1, 2, or 4 hour(s). (c) Cells were treated with $2\,\mu$M doxorubicin for 1 hour with or without conditioned medium and then washed for 4 hours. When using Ko143 as BCRP specific inhibitor, Ko143 was present during both doxorubicin-treated period and wash period. Each histogram image was a representative from three independent experiments ($n = 3$). Doxo, doxorubicin; CM, conditioned medium from adipose-derived mesenchymal stem cells (MSC-ad).
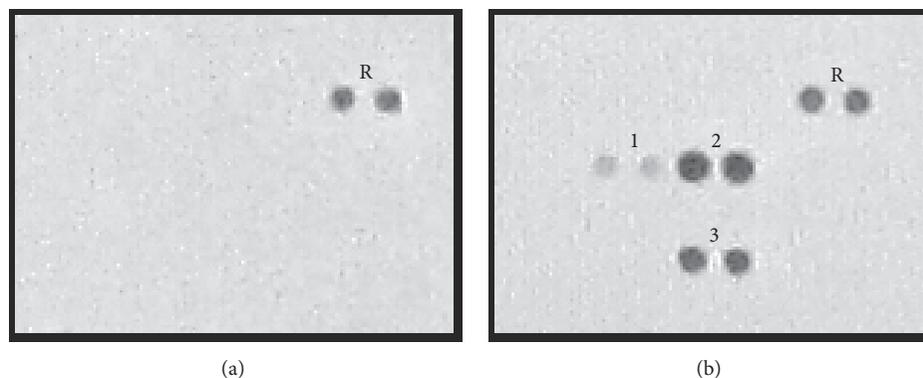
FIGURE 4: Cytokine expression of adipose-derived mesenchymal stem cells-secreted conditioned medium. Analysis of human cytokine expression of (a) MSCM (fully supplemented MSC culture medium) only and (b) MSC-ad conditioned medium by human cytokine array. R, reference spot; 1, IL-6; 2, IL-8; 3, Serpin E1.

elevated to 0.85 ± 0.03 that of control (Figure 6(a)). As shown in Figure 6(b), MSC-ad CM-induced doxorubicin resistance caused cell viability to 0.82 ± 0.04 that of control. However, cell viability was markedly down to 0.63 ± 0.02 that of control in the presence of IL-8 neutralizing antibody ($5\,\mu$g/mL) in MSC-ad CM (Figure 6(b)). These data implicated that IL-8 secreted by MSC-ad led to increased BCRP protein expression and was responsible for reduced doxorubicin sensitivity in MDA-MB-231 triple negative breast cancer cells.

4. Discussion and Conclusion

In tumor microenvironment, cytokines have been secreted by cancer cells, macrophages, endothelial cells, and mesenchymal cells [28–31]. The secreted cytokine cocktail includes SDF-1, IL-1β, IL-3, IL-6, IL-8, TNF-α, NO, G-CSF, M-CSF, GM-CSF, and many others, and by various signaling

pathways to protect cancer cells against chemotherapy [9]. Numerous *in vitro* and *in vivo* studies reported that cytokines are capable of modulating the expression and function of different drug transporters including P-gp, MRPs, and BCRP [26, 32, 33]. Among various types of cytokines in the tumor microenvironment, IL-8 is one of the major cytokines produced by cancer cells and stroma cells. Accumulating studies have reported that IL-8 signaling is involved in proliferation, survival, angiogenesis, and metastatic migration of cancer cells in solid tumors including ovarian, intestine, prostate, and glioma [34–36]. Increasing evidence has also suggested potential autocrine or paracrine effects of IL-8 on drug resistance in human cancers [37–39]. For example, IL-8, produced by tumor cells as an autocrine growth factor, promotes tumor growth, metastasis, angiogenesis, and chemoresistance against oxaliplatin in IL-8-overexpressing human colorectal cancer cells both *in vitro* and *in vivo* [37]. Another study has shown that autocrine production

(a)

(b)
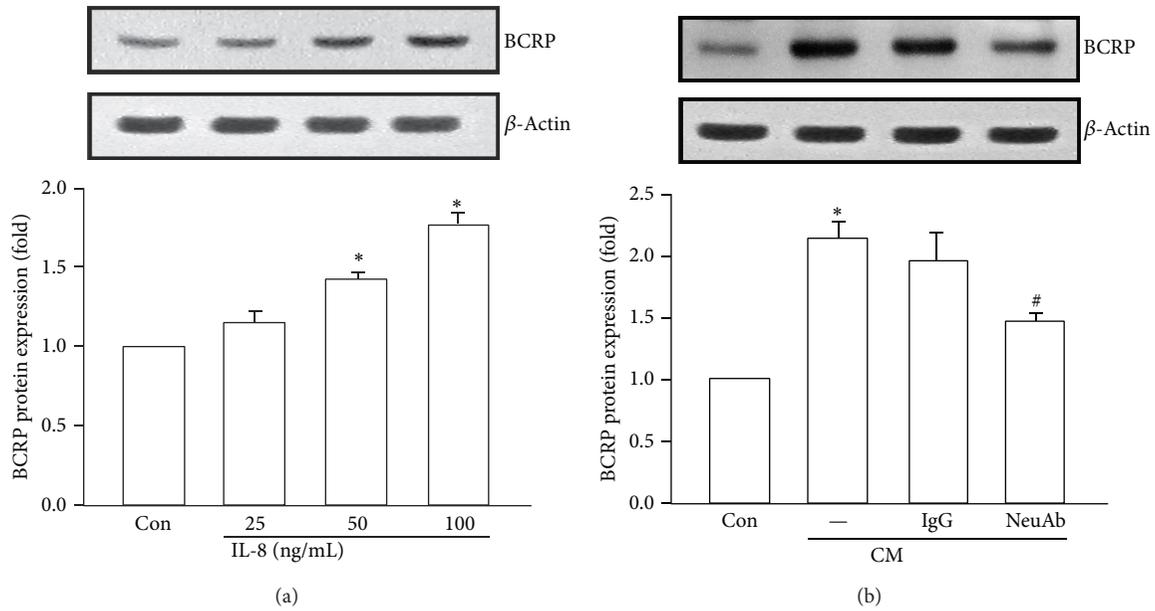
FIGURE 5: IL-8 induced BCRP protein expression in MDA-MB-231 cells. (a) Human recombinant IL-8 dose-dependently elevated BCRP protein expression after 24 hours of examination by Western blotting. (b) IL-8 neutralizing antibody (5 μg/mL) antagonized MSC-ad conditioned medium-induced BCRP protein expression. IgG isotype control antibody was used as negative control. Graphs showed mean ± SEM of three independent experiments. $^*P < 0.05$ to control group; $^\#P < 0.05$ to CM-treated group. CM, conditioned medium from adipose-derived mesenchymal stem cells (MSC-ad); NeuAb, IL-8 neutralizing antibody.

(a)

(b)
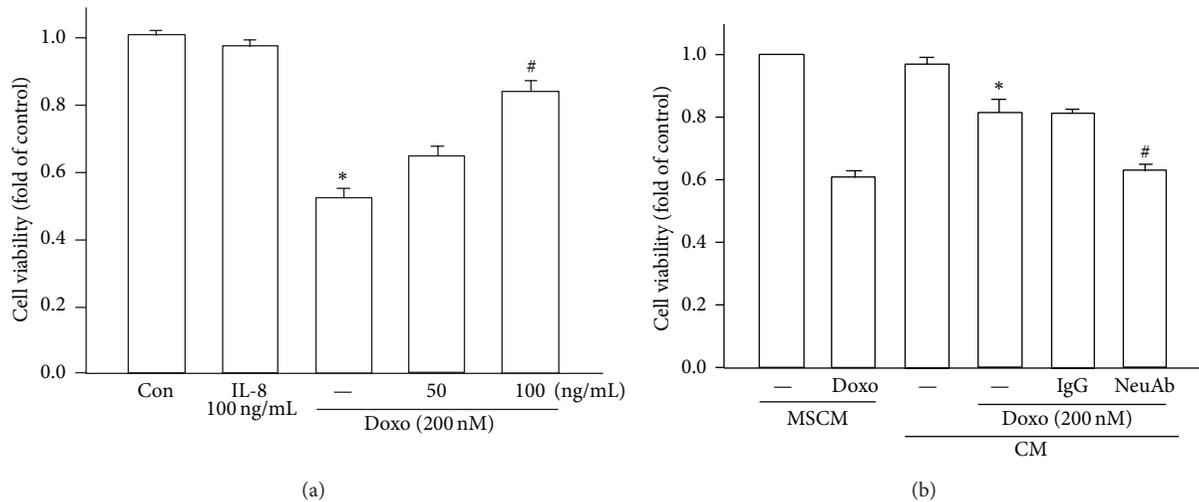
FIGURE 6: IL-8 induced drug resistance against doxorubicin in MDA-MB-231 cells. (a) Cells were treated by 200 nM doxorubicin for 24 hours with or without pretreatment of human recombinant IL-8 (50 or 100 ng/mL) for 24 hours and cell viability was examined by performing crystal violet staining. $^*P < 0.05$ to control group; $^\#P < 0.05$ to doxorubicin-treated group. (b) IL-8 neutralizing antibody (5 μg/mL) antagonized MSC-ad conditioned medium-induced doxorubicin (200 nM) resistance. IgG isotype control antibody was used as negative control. $^*P < 0.05$ to MSCM with doxorubicin-treated group; $^\#P < 0.05$ to CM with doxorubicin-treated group. Graphs showed mean ± SEM of three independent experiments. Doxo, doxorubicin; MSCM, mesenchymal stem cell medium (fresh MSC culture medium); CM, conditioned medium from adipose-derived mesenchymal stem cells (MSC-ad); NeuAb, IL-8 neutralizing antibody.

of IL-8 by ovarian cancer cells confers increased expression of apoptosis inhibitory proteins (Bcl-2, Bcl-XL, and XIAP) and P-gp, leading to cisplatin and paclitaxel resistance [38]. Furthermore, IL-8 plays a role in chemoresistance to temozolomide in melanoma side population cells [39]. However,

limited studies have discussed the relation between IL-8 and breast cancer resistance protein (BCRP). BCRP, also called ABCG2, was first discovered in doxorubicin-resistant breast cancer MCF-7 cells [40]. BCRP is widely expressed in various normal tissues including mammary gland, intestine, kidney,

liver, ovary, testis, placenta, endothelium, and hematopoietic stem cells [33, 41, 42]. The overexpression of BCRP is commonly found in human solid tumors such as breast, colon, ovary, and gastric cancers and accumulating evidence indicates that BCRP expression may be associated with multidrug-resistant phenotype in these cancer cells against various chemotherapeutic agents including anthracyclines, mitoxantrone, and the camptothecins by enhancing drug efflux [24, 40, 43]. In our present study, human recombinant IL-8 was found to induce BCRP protein expression, leading to doxorubicin resistance in triple negative breast cancer cells.

During tumor progression, recruitment of mesenchymal stem cells (MSCs) to tumors is reported due to the presence of soluble factors secreted in the tumor microenvironment [10]. Tumor cells secrete cytokines and growth factors to promote MSCs migration and survival [44, 45]. Hypoxic condition in the tumor microenvironment also results in the generation of cytokines and chemokines that are involved in MSCs migration to tumors [46]. When induced by soluble factors of tumor cells to migrate to the area surrounding the tumor, MSCs are involved in supporting the progression and malignant properties of tumor cells. The contribution of MSCs to drug resistance in tumor cells has also been increasingly reported. For instance, head and neck squamous carcinoma cells are resistant to paclitaxel when cocultured with bone marrow-derived MSCs [47]. MSCs can also utilize autophagy to recycle macromolecules and synthesize antiapoptotic factors to facilitate growth and survival of surrounding tumor cells [48]. In colorectal carcinoma, NRG1 released by MSCs activates PI3K/AKT pathway to stimulate growth of tumor cells [49]. Platinum-based chemotherapy in breast cancer also induces MSCs to secrete unique fatty acids that confer chemoresistance [50]. It has also been reported that MSCs are able to protect ovarian cancer cells from hyperthermia-induced cell death via SDF-1α/CXCR4 signaling [51]. In our presented study, conditioned medium collected from adipose-derived MSCs enhanced BCRP protein expression, leading to reduced doxorubicin sensitivity, and the secreted IL-8 is responsible for the observed phenomenon in triple negative breast cancer cells.

In some cases, patients encounter a poorer quality of life and psychological impacts after surgical removal of breast cancer, especially in young women. Hence, plastic surgery procedures for breast reconstruction concur to reduce cosmetic and psychological problems [52, 53]. In order to have a better maintenance of transplanted fat in reconstructed breast, adipose-derived mesenchymal stem cells (MSC-ad) are now added as new stem cell-enriched fat grafting techniques [54, 55]. Although breast reconstruction is safe when the remaining breast cancer cells are inactive or resting, it has not been clear whether these MSC-ad are safe for breast cancer patients because these cells may send signals that promote reactivation of the tumor cells, if there was any left [52, 53, 56–58]. According to our finding, if breast cancer recurred, MSC-ad-secreted factors may cause chemoresistance of surrounding cancer cells and place an obstacle for further treatment on breast-reconstructed patients. At the present, reconstructive therapy utilizing adipose-derived MSCs-enriched fat grafting

should be considered more carefully in patients previously treated for breast cancer [59, 60].

Altogether, our study indicated that conditioned medium collected from MSC-ad increased BCRP protein expression without affecting P-gp and MRP and consequently resulted in reduced intracellular doxorubicin accumulation in MDA-MB-231 human triple negative breast cancer cells. Moreover, at least IL-8 secreted in the MSC-ad conditioned medium is responsible for the observed doxorubicin resistance. This finding provides better understanding of the role of MSCs in tumor microenvironment concerning tumor chemoresistance and shed light on discovering novel therapeutic strategies to circumvent MSCs-related drug resistance in triple negative breast cancer.

List of Abbreviations

BCRP: Breast cancer resistance protein
CM: Conditioned medium
MRP: Multidrug resistance-associated protein
MSC-ad: Adipose-derived mesenchymal stem cell
P-gp: P-glycoprotein
TNBC: Triple negative breast cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Biomedical Properties of a Natural Dietary Plant Metabolite, Zerumbone, in Cancer Therapy and Chemoprevention Trials

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Zerumbone (ZER) is a naturally occurring dietary compound, present in many natural foods consumed today. The compound derived from several plant species of the Zingiberaceae family that has been found to possess multiple biomedical properties, such as antiproliferative, antioxidant, anti-inflammatory, and anticancer activities. However, evidence of efficacy is sparse, pointing to the need for a more systematic review for assessing scientific evidence to support therapeutic claims made for ZER and to identify future research needs. This review provides an updated overview of in vitro and in vivo investigations of ZER, its cancer chemopreventive properties, and mechanisms of action. Therapeutic effects of ZER were found to be scientifically plausible and could be explained partially by in vivo and in vitro pharmacological activities. Much of the research outlined in this paper will serve as a foundation to explain ZER anticancer bioactivity, which will open the door for the development of strategies in the treatment of malignancies using ZER.

1. Introduction

Medical herbs and plant foods such as fruits, vegetables, and spices contain many biologically active phytochemicals that have various health-promoting effects [1]. The Zingiberaceae family found in tropical and subtropical regions of the world and approximately 161 species from 18 genera of this family are found in Peninsular Malaysia [2]. *Zingiber zerumbet* (L.) Smith tree (Figure 1(a)), belonging to this family, is an edible ginger, originating in South-East Asia, and has been cultivated for thousands of years as a spice and for medical purposes [3]. Although this plant is known to be indigenous to India and the Malay Peninsula, it is nonetheless distributed in many other countries including Indonesia, China, Bangladesh, Vietnam, Japan, Burma, Nepal, Sri Lanka, Jamaica, and Nigeria and other parts of the globe [4]. This herbal plant is popularly referred to as the pinecone, wild ginger, Asian ginger, or shampoo ginger. It is also called by many other names in different countries, such as *lempoyang* in Malaysia and Indonesia; *parsu kedar, ghatian, and yaiimu* in India [5], *jangliadah* in Bangladesh [6], *hong qui jiang* in China, *haeo dam* in Northern Thailand, *awapuhiin* in Hawaii, and *zurunbah* in the Middle East [3]. Generally, the rhizome and the leaves are used for spice, tea, beverage, and medical purposes, while the milky, mucilaginous substance of the inflorescences (pinecones) (Figure 1(b)) is famously used as a
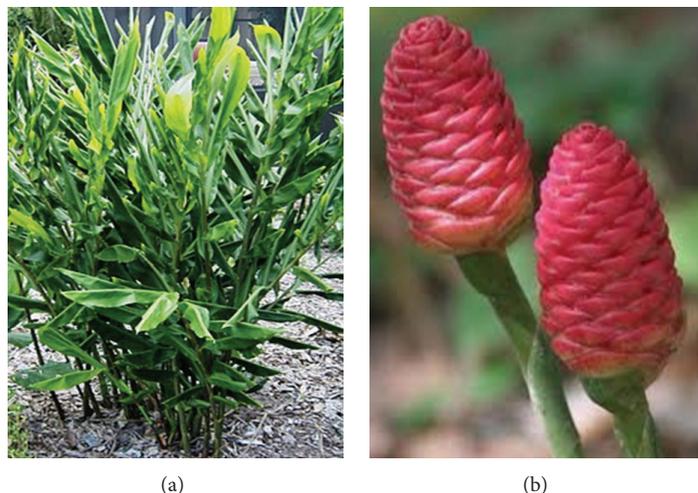
FIGURE 1: *Zingiber zerumbet* tree (a) and inflorescences (b).
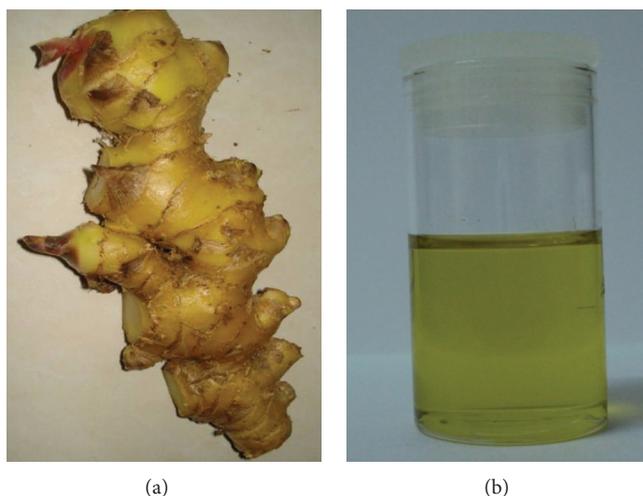
FIGURE 2: *Zingiber zerumbet* rhizome (a) and essential oil (b).

shampoo and natural hair conditioner, especially in Asia and Hawaii [7, 8].

Zingiber zerumbet contains several types of phytochemical and is considered as one of the most widely used traditional dietary condiments in various cuisines and beverages throughout Asia, although the essential oil is also used as perfume and in other toiletry articles [9]. Besides its extensive use as a spice, the rhizome particularly has been used in traditional oriental medicine for many human disorders, especially in the treatment of a variety of digestive conditions [10, 11]. The rhizome and oils from the leaves of *Zingiber zerumbet* have been subjected to close chemical scrutiny for their medicinal value [12].

Ginger is generally recognized as safe and is used traditionally in local folk medicine for treatment of nausea, hangovers, asthma, morning and motion sickness, loss of appetite, dyspepsia, diarrhea, colic, cramp, stomach upset, sprain, worm infestation in children, cough and cold, flu, sinusitis, catarrh, congestion, sore throat, migraine headache, toothache, diabetes, bruising, carbuncles, fracture, swelling, rheumatism, arthritis, and chills and fever [13–15].

Presently, rhizome's extract has been extensively studied for its effectiveness in a broad range of biological activities including antimicrobial [16], antipyretic [17], antispasmodic and anticonvulsant [3], antiulcer [18], antioxidant [19], antidiabetic [20], antitumor [21], anticancer [22, 23], anti-inflammatory [24, 25], antinociceptive and analgesic [26, 27], antiallergenic [28], antiangiogenic [29], antidipogenetic [30], antiplatelet aggregation and anticoagulant [31], and hepatoprotective effects [32]. Other studies have shown that consuming the rhizome also exhibits hypolipidemic effect by reducing intestinal cholesterol absorption, which makes it useful for treating heart diseases [33, 34].

The essential oil of *Zingiber zerumbet* rhizome (Figure 2(a)) contains approximately 86% sesquiterpenoids [35] while the leaf and rhizome oils (Figure 2(b)) of this plant

(a)   (b)
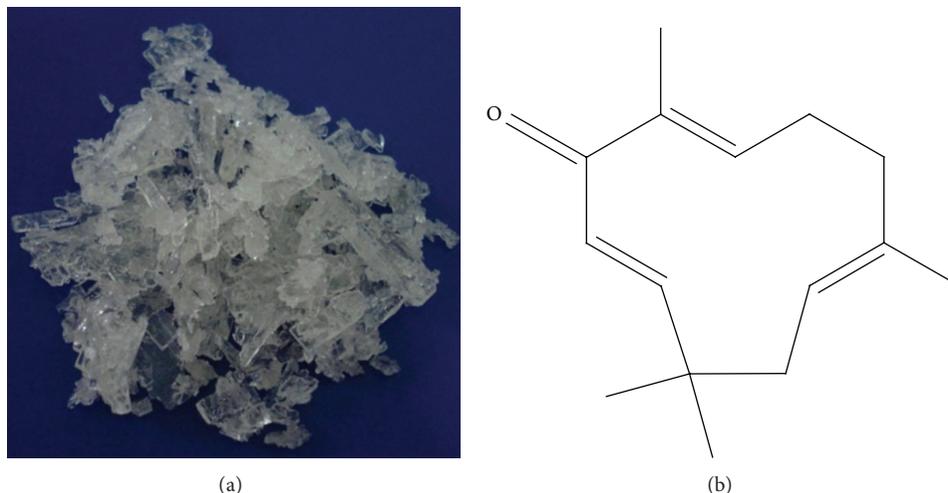
FIGURE 3: Zerumbone pure crystals (a) and chemical structure (b).

contain a complex mixture of 29 and 30 compounds, respectively [6]. Many of these compounds are in trace amounts with great variations in their chemical compositions.

Zerumbone (Figure 3(a)) was first isolated from the essential volatile oil of rhizomes of *Zingiber zerumbet* in 1956 [36], while its chemical structure (Figure 3(b)) was determined in 1960 and later characterized by NMR and X-ray [37]. Zerumbone possesses three double bonds, two conjugated and one isolated, as well as double conjugated carbonyl group in the 11-membrane ring structure [38]. The chemical characteristics of ZER are presented in Table 1 [39–43].

2. Plant Sources of Zerumbone

Early investigations in different parts of the world showed that 12.6 to 73.1% of ZER in *Zingiber zerumbet* is in the rhizome oils [44]. The Kerala state in the South Indian accessions reported that in *Zingiber zerumbet* 76.3 to 84.8% of its ZER content is also in the rhizome oils [44]. On the other hand, a silviculture farm in India reported that 1.81% ZER content was found in the rhizome, 0.16% in the root, 0.09% in the leaf, and 0.03% in the flower of *Zingiber zerumbet* [5]. The Penang Malaysian accession recorded the content of ZER in the plant at 68.9% [44]. Another study conducted in the state of Selangor, Malaysia, showed that the ZER content of *Zingiber zerumbet* is 1.3 g/kg rhizome [23]. The oils of *Zingiber zerumbet* from Tahiti Island and Vietnam were also found to be rich in ZER at 65.3 and 72.3, respectively [45, 46]. In Vietnam, ZER was also isolated from the rhizomes of the Vietnamese *Curcuma zedoaria* (Berg.) Roscoe [47]. Other reports on the ginger plant include that by Chane-Ming et al. [48] and Bhuiyan et al. [6] each showing the rhizome to contain approximately 37% of the plant ZER content. The differences in ZER content in the plant are not due to geographic or ecological variations but instead because of differences in ZER chemotype [3].

Other ginger plant species with ZER among their constituents include the *Zingiber amaricans* [49], *Zingiber ottensii* Valeton [50], *Zingiber aromaticum* (17.72%) [51], *Zingiber cassumunar* Roxb. (1%) [52], *Zingiber ottensii* [53], and *Zingiber montanum* [18]. Various other plants also contain ZER; among them are *Curcuma amada* Roxb. [35] from India, *Alpinia galanga* from Sri Lanka [54], and *Xylopia aethiopica* from Ibadan, Southwest Nigeria [55].

3. Anticancer Properties of Zerumbone

Several researchers have reported that ZER has both in vitro (Table 2) and in vivo (Table 3) anticancer properties at different concentrations and doses [56]. Zerumbone possesses antiproliferative properties towards several cancer cell lines with minimal effect on normal cells [57–59]. Among the effects of ZER is induction of high intracellular redox potential that can inhibit proliferation of cancer cells [60]. The cytotoxic effect of ZER on the cancer cells appears to be attributed to the versatile α,β-unsaturated carbonyl group in its structure, which plays an important role in the interaction of the compound with the most biologically active molecules. Clearly the carbonyl group is important for biological activity because α-humulene, also found in ginger, lacking in this functional group is virtually and consistently pharmacological inactive [61]. The α,β-unsaturated carbonyl group in ZER effectively removes the intracellular glutathione (GSH) through the formation of Michael adducts, thus enhancing the potential of intracellular redox (E), resulting in the inhibition of spread of cancerous cells. However, the average intracellular redox potential of normal cells differs from that of cancerous cells; this difference could be the reason for ZER not inducing proliferation of normal cells [60, 61]. Because there is a close link between tumor promotion, inflammation, and oxidative stress, the anti-inflammatory and/or antioxidant compounds could also act as an anticarcinogenic agent [62]. Although the stimulation of neoplastic cell death by ZER was reported to be through

Table 1: Characteristic features of zerumbone.

Characters	Description
Natural occurrence	*Zingiber* species
Chemical class	Sesquiterpene
Chemical formula	(2E, 6E, 10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one
Molecular formula	$C_{15}H_{22}O$
Chemical structure	Three-double bond (two conjugated and one isolated), α,β-unsaturated carbonyl group, and a double conjugated carbonyl group in 11-membered ring structure
Molecular weight	218.3 dalton
Flashing point	272°F
Boiling point	321-322°C at 760 mmHg
Melting point	65.3°C
Vapor pressure	0.000295 mm/Hg at 25°C
Purity	92–100%
Appearance	Solid white crystals or powder
Short term storage	+4°C
Stability	Stable for at least 2 years when stored at −20°C
Solubility	Completely soluble in ethanol, DMSO, while solubility in water is approximately 1.296 mg/L at 25°C
Extraction	Mainly isolated from fresh rhizomes by hydrodistillation (steam distillation) and recrystallization methods
Usage	For researches and medical purposes, not for flavor or fragrance

the mitochondrial pathway of apoptosis [47], it also exhibits antiproliferative and anti-inflammatory activities through the modulation of NF-κB activity. Zerumbone inhibits NF-κB in association with the sequential suppressions of IκBα kinase activity, phosphorylation, and degradation. This compound also inhibits NF-κB-dependent reporter gene expression activated by TNF, TNFR1, TRADD, TRAF2, NIK, and IKK but not by the p65 subunit of NF-κB. Zerumbone also downregulates NF-κB-regulated gene products, including cyclin D1, COX-2, MMP-9, ICAM-1, c-myc, survivin, IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, Bfl-1/A1, TRAF1, and FLIP. These effects lead to the potentiation of apoptosis induced by cytokines and chemotherapeutic agents. The inhibition of these NF-κB-regulated genes expression is in association with the suppression of TNF-induced cancer invasiveness. Thus, it is hypothesized that inhibition of NF-κB and NF-κB-regulated gene expression induced by carcinogens may also represent the molecular basis for cancer prevention and treatment by ZER [63]. Furthermore, it was shown that ZER is a novel inhibitor of CXC chemokine receptor-4 (CXCR4) expression,

which mediates homing of tumor cells to specific organs during metastasis, suggesting the potential of the compound in the suppression of metastasis [64]. This receptor has been identified in various tumors including those in the breast, ovary, prostate, gastrointestinal tract, head, neck, bladder, brain, and skin.

3.1. Blood Cancer (Leukemia). It has been shown that ZER effectively suppresses the tumor promoter 12-O-tetradecanoylphorbol-13-acetate- (TPA-) induced superoxide anion (O_2^-) generation from NADP oxidase in dimethyl sulfoxide- (DMSO-) differentiated human acute promyelocytic leukemia (HL-60) cells [60]. One study determined the effect of diethyl ether extract of *Zingiber zerumbet* fresh rhizome on cultured P-388D1 cells and in P-388D-bearing CDF mice. This study showed that the extract could induce DNA fragmentation in P-388D1 cells in vitro and significantly prolonged the life of P-388D1-bearing CDF mice. The same result was obtained when the activity of ZER isolated from the same extract was examined in vitro and in vivo [69]. The study further found that ZER inhibited the growth of HL-60 cells, in time- and concentration-dependent manner. HL-60 cell cycle analysis after treatment with ZER showed induction of G2/M arrest and decreased cyclin B1/CDK1 protein level. Using CEM-ss cells as targets, it was shown that ZER increased the number of TUNEL-positive cells and cellular caspase-3 level; the hallmarks of apoptosis [65]. The anticancer effects of ZER seem boundless when it was shown that it inhibits the proliferation of NB4 cell line, derived from acute promyelocytic leukemia cells, through the induction of G2/M phase cell cycle arrest associated with a decline of cyclinB1 protein and phosphorylation of ATM/Chk1. The study indicated that ZER induction of NB4 cell apoptosis was initiated by the expression of Fas (CD95)/Fas ligand (CD95L), concomitant with the activation of caspase-8. At the same time, they found that ZER induced cleavage of Bid, Bax, and Mcl-1 proteins, phosphorylation of Cdc25C and Cdc2 at the Thr48 and Thr14/Tyr15 residues, respectively, degradation of the proteolytic poly-(ADP-ribose) polymerase (PARP), and triggering of cytochrome c release into the cytoplasm [69]. On leukemic cells, ZER is cytotoxic to human myeloid (KBM-5) [67], mouse myelomonocytic (WEHI-3B) [126], and human acute lymphoblastic leukemic (Jurkat) cell lines [66]. Zerumbone also regulates expression of apoptotic biomarkers in BALB/c mice model of acute myelocytic leukemia via the mitochondrial intrinsic pathway [70].

3.2. Skin Cancer. Zerumbone suppressed 7,12-dimethylbenz[α]anthracene- (DMBA-) and TPA-induced initiation and promotion of skin tumors in female ICR mice. Using RT-PCR, it was shown that ZER enhances expression of manganese superoxide dismutase (MnSOD), glutathione peroxidase-1 (GPx-1), glutathione S-transferase-P1, and NAD (P) H quinine oxidoreductase (NQO1) mRNA in the epidermis while diminishing TPA-induced COX-2 protein expression and phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) [127]. The phorbol ester-induced papilloma formation in mouse skin can also be inhibited by

TABLE 2: In Vitro biological effects of zerumbone.

Organ	Cell line	Biological effect of ZER
Blood	Human acute lymphocytic leukemia (CEM-ss) [65]	Induces apoptosis and DNA internucleosomal degradation activate caspase-3
	Human acute lymphoblastic leukemia (Jurkat) [66]	Induces G2/M cell cycle arrest Induces intrinsic apoptotic pathway via activation of caspase-3 and caspase-9, cytochrome c release from mitochondria, and PARP cleavage
	Human chronic myeloid leukemia (KBM-5) [63]	Induces cytotoxicity
	Human acute promyelocytic leukemia (HL-60) [61, 67]	Suppresses TPA-induced superoxide anion generation from NADPH oxidase Induces G2/M cell cycle arrest in time- and concentration-dependent manner Decreases cyclin B1/CDK1 protein level
	Human acute promyelocytic leukemia (NB4) [67]	Induces G2/M cell cycle arrest associated with decline of cyclin B1 protein and phosphorylation of ATM/Chk1, induced apoptosis via expression of Fas (CD95)/Fas ligand (CD95L), with the activation of caspase-8
	Human acute myelocytic leukemia (U937) [67]	Antagonizes action of DDT and TCDD by upregulating the expressions of COX-2 and VEGF mRNA
	Human acute lymphoblastic leukemia (MOLT4), human acute lymphocytic leukemia (OKM-2T), and human chronic myelocytic leukemia (K562 and KT-1) [67]	No cytotoxicity at concentration of 10 μM
	Human peripheral blood multiple myeloma (U266) [68]	Suppresses CXCR4 expression
	Murine lymphoid neoplastic (P-388D1) [69]	Causes DNA fragmentation and growth inhibition
	Murine acute myelocytic leukemia (WEHI-3B) [70]	Induces G2/M cell cycle arrest and apoptosis
	Normal human umbilical vein endothelial cell (HUVEC) [67]	Does not inhibit proliferation at concentration of 10 μM
	Normal human primary mononuclear cells (PBMCs) [71, 72]	No cytotoxicity (1–100 μg/mL) Cytotoxic at high doses (40–80 μM)
	Mice thymocytes and splenocytes human PBMC [73]	Stimulates time- and dose-dependent proliferation of mice cells and human PBMC Upregulates human cytokine (interleukin, IL-2 and IL-12) immunomodulatory
	Human peripheral blood lymphocytes (PBL) Al [74]	Cytotoxic but not clastogenic at 40 and 80 μM, Does not induce chromosomal aberration and micronuclei formation
	Lymphoblastoid (Raji) cells	Suppresses tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate- (TPA-) induced activation of Epstein-Barr virus
	Human monocyte-like cells (THP-1) [75]	Suppresses TPA-induced LOX-1 mRNA expression Attenuates expression of SR-A, SR-PSOX, and CD-36 and led to block DiI-AcLDL uptake Inhibits AP-1 and NF-κB transcriptional activity
	Normal murine macrophages 9RAW264.7) [68]	Markedly diminishes inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression Suppresses free radical generation and inhibits tumor necrosis factor (TNF)-α release Induces phase II drug metabolizing enzymes GSTP1 and NQO1 mRNA expressions
	Immortalized mouse embryonic fibroblasts (SV40) [76]	Not cytotoxic
	Human whole blood [31]	Inhibits platelet aggregation induced by arachidonic acid (AA), collagen, and ADP

TABLE 2: Continued.

Organ	Cell line	Biological effect of ZER
Skin	Human melanoma (WM1552C) [77]	Induces apoptosis and autophagy
	Murine melanoma (B16-F0) [77]	Induces apoptosis and autophagy
	Normal human dermal fibroblast (2F0-C25) [77]	Not cytotoxic at a concentration of 13 μM
	Murine epidermal cells (JB6 Cl41) [78]	Induces heme oxygenase-1 expression through activation of Nrf2
Liver	Human liver adenocarcinoma (HepG2) [79]	Induces apoptosis via up- and downregulation of Bax/Bcl-2 proteins independent of functional p53 activity Induces DNA fragmentation
	Human hepatoma (HTC) [80, 81]	Cytotoxic
	Murine hepatoma cells (Hepa1c1c7) [82–84]	Marked upregulation of multiple HSPs, such as HSP40 and HSP70HSPs Increases proteasome activity with upregulation of β5, a major proteasome functional protein Upregulates expressions of several proautophagic markers, including p62 and microtubule-associated protein 1 light-chain 3 (LC3)-II Suppresses cellular protein modifications by 4-hydroxy-2-nonenal (HNE) Confers resistance to toxicity of HNE via p62 induction Induces ubiquitination and aggregation of cellular proteins Activates ubiquitin-proteasome system and autophagy
	Normal human liver cells (Chang) [79]	Inhibits cell growth with an IC$_{50}$ value of 10.96 ± 0.059 μg/mL
	Normal rat liver epithelial cells (RL34) [85]	Activates phase II drug metabolizing enzymes, such as GST (glutathione S-transferase), epoxide hydrolase, and hemeoxygenase via the transcription factor Nrf2 dependent pathway
	Normal human liver cells (WRL-68) [86]	Not cytotoxic
Cervical	Human cervical cancer (HeLa) [87–89]	Causes growth inhibition and induces apoptosis Decreased level of IL-6 secretion and membrane bound IL-6 receptor Induces G2/M cell cycle arrest
Colon	Human colonic adenocarcinoma (Caco-2, Colo320DM, and HT-29) [61]	Markedly induces expressions of interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α
	Human colonic adenocarcinoma (LS174T, LS180, COLO205, COLO320DM) [61]	Inhibits cell proliferation in dose-dependent manner
	Normal human colon fibroblast (CCD-18Co) [61]	Not cytotoxic at a concentration of 13 μM
Colorectal	Human colorectal carcinoma (HCT116) [90, 91]	Enhances TRAIL-induced apoptosis Causes activations of caspase-8, caspase-9, caspase-3 and PARP in combination with TRAIL Induces expression of TRAIL receptors DR4 and DR5 Downregulates expression of antiapoptotic protein c-FLIP Causes activation of ERK in time-dependent manner
	Human colon carcinoma (HCT-116) [76]	Induces apoptosis
Bile duct	Poorly differentiated adenocarcinoma (KKU-100), squamous cell carcinoma (KKU-M139), moderately differentiated adenocarcinoma (KKU-M156), adenosquamous carcinoma (KKUM213), and moderately differentiated adenocarcinoma (KKU-M214) [92]	ZER derivatives (5, 10, 14, and 20) showed antiproliferative activity
Breast	Human breast adenocarcinoma cell lines (MCF-7 and MDA-MB 231) [68, 90] Human breast benign cell line (MCF-10A) [76]	G2/M phase cell cycle arrest Downregulates cyclin B1, cyclin-dependent kinase 1, Cdc25C, and Cdc25B and Bax/Bak-mediated apoptosis Induces significant expression of DR4 Activation of Bax and Bak Not cytotoxic

TABLE 2: Continued.

Organ	Cell line	Biological effect of ZER
Ovarian	Human ovarian cancer (Caov-3) [59]	Causes growth inhibition and induces apoptosis Decreases level of IL-6 secretion and membrane bound IL-6 receptor Induces G2/M cell cycle arrest
	Normal Chinese hamster ovarian cells (AS52) [61]	Suppresses tumor promoter 12-O-tetradecanoylphorbol-13-acetate-(TPA-) induced superoxide anion (O_2^-) generation from xanthine oxidase (XO)
	Normal Chinese hamster ovary cells (CHO) [93]	High concentrations produce genotoxic and cytotoxic effects (40–80 μM)
Pancreatic	Human pancreatic carcinoma (PaCa) [94]	Novel inhibitor of Jak2/Stat3, which inhibits promigratory gene expression, growth, and migration of pancreatic cancer cells
	Human pancreatic cancer (PANC-28, MIA PaCa-2, and AsPC-1) [64]	Inhibits CXCL12-induced invasion of pancreatic tumor cells
	Human pancreatic carcinoma (PANC-1 and SW1990) [95]	Time-dependent inhibition of cell viability induces apoptosis
	Human pancreatic carcinoma (PaCa) [96]	Inhibits PaCa-associated angiogenesis through the inhibition of NF-κB and NF-κB-dependent proangiogenic gene products
Lung	Human nonsmall cell lung carcinoma (H1299 cells) [63, 90]	Enhances TNF-induced cytotoxicity and potentiates apoptosis Inhibits TNF-induced IκBα protein degradation and phosphorylation Inhibits TNF-induced phosphorylation of p65 protein Suppresses TNF-induced invasion activity
	Human small cell lung carcinoma (NCI-H187) [97]	Inhibits monomeric form of the HSP 27 protein ZER derivative (parent alcohol 8) induces strong cytotoxicity
	Human embryonic kidney carcinoma cell line (A293 cells) [63]	Inhibits cell growth
Kidney	Bovine normal kidney cell line (MDBK) [79]	Inhibits cell growth with an IC$_{50}$ value of 10.02 ± 0.03 μg/mL
	Human kidney embryonic cells (HEK 293) [98]	ZER derivative (parent alcohol 8) could protect irradiation induced cell apoptosis and DNA damage, at least partly, via activation of Keap1/Nrf2/ARE pathway
	Normal African green monkey kidney cells (Vero) [97]	Nonsignificant cytotoxicity with IC50 of 30 μM.
Brain	Human brain malignant glioma (GBM8401) [99]	Induces human glioblastoma multiforme cell apoptosis via inhibition of the IKKα-Akt FOXO1 cascade and activation of caspase-3
	Human brain malignant glioma (U87MG) [99]	Significantly decreases cell viability at the concentration of 30 and 50 μM
Prostate	Human adenocarcinoma (DU145) [90]	Induces cytotoxicity and significant PARP cleavage Effectively blocks Jak2/STAT3-mediated signaling pathways Induces nonsignificant expression of DR4
	Human adenocarcinoma (PC3) [90]	Induces nonsignificant expression of DR4
Stomach	Human gastric adenocarcinoma (AGS) [100]	Inhibits tumor angiogenesis via reduction of VEGF production and NF-κB activity
Oral	Human oral cancer (KB) [97]	ZER derivative (parent alcohol 8) induces strong cytotoxicity
Headand neck	Human squamous cell carcinomas (SCC4) [64]	Suppresses CXCR4 expression and cancer invasion and metastasis
	Human squamous cell carcinoma (LICR-LONHN5) [63]	Inhibits activation of NF-κB and NF-κB regulated gene expression Suppresses IκBα kinase activity, phosphorylation, and degradation Suppresses p65 phosphorylation, nuclear translocation, and acylation
Pharynx	Human squamous cell carcinoma (FaDu) [63]	Inhibits NF-κB and IκBα kinase activation Suppresses antiapoptotic and metastatic gene expression Upregulates apoptosis and downregulates cancer invasion

TABLE 2: Continued.

Organ	Cell line	Biological effect of ZER
Bone	Mouse macrophage (RAW 264.7) [68]	Inhibits RANKL-induced NF-κB activation through inhibition of activation of IKBA kinase, IKBA phosphorylation, and IKBA degradation Suppresses RANKL-induced differentiation of an osteoclast precursor cells to osteoclasts Inhibits osteoclastogenesis induced by RANKL and tumor (RAW264.7) cells after incubation in the presence of MDA-MB-231 cells or U266 cells for 24 h, then exposed to ZER for 5 days, and finally stained for TRAP expression) Potential therapeutic agent for osteoporosis and cancer-associated bone loss

ZER [110]. Recently, it was found that ZER induces heme oxygenase-1 expression in female HR-1 hairless mouse skin and cultured murine epidermal (JB6 Cl4) cells, through the activation of Nrf2 [78]. More recently, ZER was found to induce apoptosis and autophagy in human (WM1552C) and murine (B16-F0) melanoma cell lines [128]. Zerumbone also significantly reduced tumor mass and lung metastasis in B16-F0 bearing C57 BL/6 male mice through the activation of Akt and MAPK and suppression of NF-κB activation [77].

3.3. Liver Cancer.

Zerumbone was also found to inhibit the proliferation of nonmalignant Chang liver cell line [129], while being innocuous to the normal human liver (WRL-68) cells [86]. DNA fragmentation and apoptosis induced by ZER is by way of up- and downregulation of Bax/Bcl-2 proteins independent of functional p53 activity in the liver adenocarcinoma (HepG2) cell lines. In vivo, ZER inhibits diethyl nitrosamine (DEN) and dietary 2-acetylaminofluorene- (AAF-) induced Sprague Dawley rat hepatocarcinogenesis. This effect was suggested to be through the reduction of oxidative stress, inhibition of cancer cell proliferation, and induction of mitochondria-regulated apoptosis of liver cancers [105].

3.4. Cervical Cancer.

Zerumbone is known to exhibit an antiproliferative effect on human cervical cancer (HeLa) cell line [87]. In diethylstilboestrol- (DES-) induced mice cervical interepithelial neoplasia (CIN), ZER caused overexpression of proapoptotic protein, Bax [88, 130].

When ZER and cisplatin were used in combination, the cervical cancer in BALB/c mice was suppressed through the modulation of serum interleukin-6 [131]. One experiment was conducted on pregnant BALB/c rats treated with DES to develop cervical intraepithelial neoplasia. When the progenies were treated with different doses of ZER, histological examination revealed that ZER had inhibited the cervical dysplasia from developing into more severe dysplasia [89].

3.5. Colon Cancer.

Zerumbone was shown to inhibit the proliferation of human colonic adenocarcinoma (LS174T, LS180, COLO205, and COLO320DM) cell lines in a dose-dependent manner, while the growth of normal human colon (CCD-18Co) fibroblasts and normal human dermal (2F0-C25) cells was less affected [90, 110]. The effect of ZER on human colorectal cancer (HCT116) cells was via potentiation of TRAIL-induced apoptosis [90, 91] as indicated by the expression of TRAIL death receptor (DR) 4 and 5. The subsequent effects were activations of caspase-8, caspase-9, and caspase-3 and PARP and downregulation antiapoptotic protein c-FLIP expression and activation of ERK in a time-dependent manner. The RT-PCR assay showed that ZER markedly induced the expressions of IL-1α, IL-1β, IL-6, and TNF-α in human colon adenocarcinoma (Caco-2, Colo320DM, and HT-29) cell lines, in concentration- and time-dependent manners [110]. Developing azoxymethane-(AOM-) induced rat colonic aberrant crypt foci (ACF) in male F344 rat can be significantly inhibited by ZER treatment through suppression of COX-2 expression, cell spreading activity of colonic mucosa, and induction of phase II detoxification enzymes [104]. Similarly, using ACF as a preneoplastic marker, ZER was shown to suppress AOM-induced colon cancer in male Sprague Dawley rats [101]. Zerumbone inhibited the multiplicity of colonic adenocarcinoma induced by AOM, potentiated apoptosis, and suppressed NF-κB and HO-1 expressions in male ICR mice [102].

3.6. Bile Duct Cancer.

Amine 5 derived from ZER showed potent antiproliferative activity against cholangiocarcinoma (CCA) cell line and poorly differentiated adenocarcinoma (KKU-100). However, amine 5 and other ZER derivatives (10, 14, and 20) (Figure 4) showed lesser cytotoxicity toward other CCA cell lines including squamous (KKU-M139) cell carcinoma, moderately differentiated adenocarcinoma (KKU-M156), adenosquamous carcinoma (KKUM213), and moderately differentiated adenocarcinoma (KKU-M214) [92].

3.7. Breast Cancer.

In breast cancers, ZER caused G2/M phase cell cycle arrest associated with downregulation of cyclin B1, Ddk1, Cdc25C, and Cdc25B and Bax/Bak-mediated apoptosis in human breast cancer (MDA-MB-231 and MCF-7) cells and retarded growth of MDA-MB-231 xenografts in vivo [76]. In addition, its derivative, parent alcohol 8 (2E,6Z,10E)-13-Hydroxy-2,9,9-trimethylcycloundeca-2,6,10-trienone (Figure 5(a)) significantly displayed antiproliferative effect towards human breast cancer (MCF-7) cell

TABLE 3: In Vivo biological effects of zerumbone.

Organ	Animal model	ZER route	Biological effect of ZER
Cervix	Female BALB/c mice [88, 89]	Intraperitoneal injection	Suppresses cervical intraepithelial neoplasia in female Balb/c mice prenatally exposed to diethylstilbestrol (DES) Reduces the expression of cell proliferation marker PCNA in dose dependent manner Causes overexpression of proapoptotic protein Bax Suppresses Bcl-2 specific mRNA expression Inhibits progression of cervical dysplasia from becoming more severe dysplasia (CIN 3) and suppresses level of serum IL-6
Colon	Male Sprague Dawley rats [101]	Oral dose	Suppresses azoxymethane- (AOM-) induced colon cancer using aberrant crypt foci (ACFs) as a preneoplastic marker
	Male ICR mice [102]	Oral dose	Inhibits multiplicity of colonic adenocarcinomas induced by azoxymethane (AOM) Suppresses colonic inflammation in dose-dependent manner Inhibits cancer proliferation, potentiates apoptosis, and suppresses NF-κB and HO-1 expressions
	Female ICR mice [103]	Oral dose	Suppresses acute ulcerative colitis (UC) induced by dextran sodium sulfate (DSS) Significantly lowers levels of inflammatory biomarkers IL-1β, TNF-α, and PGE$_2$ in colonic mucosa Suppresses expression of inflammatory cytokines, TNF, and IL-1β in LPS/IFN-γ
	Male F344 rats [104]	Oral dose	Reduces development AOM-induced colonic aberrant crypt foci Reduces expression of COX-2 and prostaglandins in colonic mucosa Reduces number of AgNORs in colonic crypt cell nuclei
Liver	Male Sprague Dawley rats [105]	Intraperitoneal injection	Protects rat liver from carcinogenic effects of DEN and AAF Lowers serum ALT, AST, AP, and AFP concentrations Lowers concentration of GSH in hepatic tissue Lowers expression of PCNA in the rat liver Increases Bax and decreases Bcl-2 protein expression in the liver
	Male Sprague Dawley rats [106, 107]	Oral dose	Suppresses fatty liver formation induced by overdosage of ethanol Prevents necrosis of liver tissues after administration of overdosage of paracetamol Reduces levels of liver ALT, AST, and ALP at 24 h after administration of overdosage of paracetamol
	Male golden Syrian hamsters [108]	Oral dose	Attenuates nonalcoholic fatty liver disease Improves insulin sensitivity, decreases lipogenesis, and increases lipid oxidation
	Male Sprague Dawley rats [82]	Oral dose	Upregulates heat shock protein expressions in the liver Confers thermoresistant phenotype
Lung	Female A/J mice [102]	Oral dose	Significantly inhibits multiplicity of lung adenomas induced by 4-(Nmethyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) Inhibits cancer proliferation, potentiates apoptosis, and suppresses NF-κB and HO-1 expressions

TABLE 3: Continued.

Organ	Animal model	ZER route	Biological effect of ZER
Breast	Female Sprague Dawley rats [109]	Intraperitoneal injection	Inhibits tumor growth via Wnt pathway in LA-7 bearing rats
	Female severe combined immune deficient (SCID) mice [76]	Intraperitoneal injection	Retards growth of orthotopic MDA-MB-231 xenografts in association with apoptosis induction and suppression of cell proliferation (Ki-67 expression)
	Female BALB/c nu/nu mice [68]	Intraperitoneal injection	Decreases osteolytic bone metastasis in MDA-MB-231 bearing athymic nude mice dose dependently
Blood	WEHI-3B bearing male BALB/c mice [70]	Oral dose	Induces apoptosis via the mitochondrial intrinsic pathway Increases expression of Bax, Cyt-c, and PARP and decreases the expression of Bcl-2
	CDF mice [69]	Intraperitoneal injection	Significantly prolongs life of P-388D1-bearing CDF mice
	C57 BL/6 male mice [77]	Intraperitoneal injection	Significantly reduces tumor mass and lung metastasis in B16-F0 bearing mice through the activation of Akt and MAPK and inhibition of NF-κB activity
Skin	ICR mice [110]	Topical application	Suppresses 7,12-dimethylbenz[α]anthracene (DMBA) and TPA-induces initiation and promotion of skin tumor formation Enhances expression of antioxidative and phase II xenobiotics metabolizing enzymes manganese superoxide dismutase (MnSOD), glutathione peroxidise-1 (GPx-1), glutathione S-transferase-P1 (GST-P1), and NAD (P) H quinine oxidoreductase (NQO1) mRNA in the epidermis Suppresses TPA-induced COX-2 expression and phosphorylation of ERK1/2 Suppresses TPA-induced leukocyte maturation and dermal infiltration as well as activation stages of skin tumors
	Female HR-1 hairless mice [78]	Topical application	Induces HO-1 expression through activation of Nrf2
Paw	Mice [24]	Intraperitoneal injection	Inhibits carrageenan-induced paw edema dose dependently Suppresses granulomatous tissue formation in cotton pellet-induced granuloma test
Eye	ICR mice [111, 112]	Oral dose	Protects mouse cornea from ultraviolet B- (UVB-) induced inflammatory photokeratitis Inhibits NF-κB, iNOS, and TNF-α expressions Abrogates nuclear translocation of NF-κB Reduces malonyldialdehyde (MDA) accumulation and increases GSH and glutathione reductase levels Protects mice cornea from UVB-induced cataractogenesis
Pancreas	Male Wistar rats [113]	Oral dose	Suppresses cholecystokinin octapeptide- (CCK-8-) induced acute pancreatitis Significantly reduces serum amylase and lipase activities Reduces cytosolic IL-6 and TNF-a and increases cytosolic IáťŃBα concentration Reduces iNOS and Mn- and Cu/Zn-superoxide dismutase activities Significantly reduces pancreatic weight to body weight ratio

TABLE 3: Continued.

Organ	Animal model	ZER route	Biological effect of ZER
Bone	Male SPF Wistar rats [114]	Intravenous injection	Attenuates severity of acute necrotizing pancreatitis induced by sodium taurocholate and pancreatitis-induced hepatic injury, via inhibition of NF-κB activity and downregulation of ICAM-1 and IL-1β expressions
	Male Sprague Dawley rats [115]	Oral dose	Reduces inflammatory process in collagen-induced osteoarthritis (OA) Significantly reduces number of major histocompatibility complex type II cells (MHC) expression in the affected synovial membrane Reduces the number of antigen presenting type A cells presented during arthritis
	Male Sprague Dawley rats [116, 117]	Oral dose	Produces chondroprotective effects in MIA-induced knee osteoarthritis Improved immunoreactivity of neuropeptides Improves density of protein gene products (PGP), calcitonin gene-related peptide (CGRP), and neuropeptides-Y (NPY) immunoreactive nerve fibers Reduces the level of PGE$_2$Produces induction of cytochrome P450 and cytosolic GST
	Male ICR mice [118]	Intraperitoneal injection	Produces pronounced antinociception against chemical models of nociception through L-arginine-nitric oxide-cGMP-PKC-K+ ATP channel pathways, the TRPV1, and kinin B2 receptors
	Male BALB/c mice [119]	Intraperitoneal injection	Produces significant peripheral and central antinociceptive effects when assessed in acetic acid-induced abdominal writhing and hot-plate test models
Miscellaneous	Female and male BALB/c mice [120]	Oral dose	No toxic effects to liver and renal tissues Does not cause significant change in hematological and serum biochemical parameters
	Female and male ICR mice [121]	Intraperitoneal injection	Does not cause mortality or change in the general condition, growth, organ weights, hematology, serum biochemistry, or histopathology after a single dosage of 500 mg/kg or multiple dosage of f 5, 25, and 50 mg/kg for a period of 28 days
	Female Sprague Dawley rats [122]	Single intraperitoneal injection	Not toxic to liver and renal tissues at dose of 100–200 mg/kg Produces severe renal and hepatic damage at a dose of 500 mg/kg with increased serum creatinine, BUN, liver enzymes (ALT, ALP, and GGT), and MDA concentrations Does not cause mortality at 100, 200, 500, and 1000 mg/kg Causes 20 and 40% death for animals receiving 1500 and 2000 mg/kg, respectively Causes 100% death in animals receiving 2500 and 3000 mg/kg
	Male Sprague Dawley rats [71, 74]	Intraperitoneal injection	Induces significant increase in the frequency of micronuclei in polychromatic erythrocytes (PCEs) at dose 1000 mg/kg after 24-hour injection Inhibits cell proliferation and causes cytotoxicity in the rat bone marrow
	Female Sprague Dawley rats [123]	Intraperitoneal injection	Beneficial in cisplatin-induced renal dysfunction, toxicity, and organ damage via preservation of antioxidant glutathione and prevention of lipid peroxidation Attenuates cisplatin, decreases renal GSH, and increased MDA levels

| Organ	Animal model	ZER route	Biological effect of ZER
	Male New Zealand white rabbits [124]	Oral dose	Significantly averts and decreases early atheroma plague formation and development via reduction in monocytes and/or macrophages migration, aggregation, and smooth muscle cells proliferation in rabbits fed on cholesterol-rich diet
			Repairs endothelial dysfunction resulting from hyperlipidemia in rabbit atherosclerosis model
	Male golden Syrian hamsters [125]	Oral dose	Improves dyslipidemia by modulating the genes expression involved in the lipolytic and lipogenic pathways of lipids metabolism
			Decreases hepatic mRNA levels of fatty acid synthase, malic enzyme, sterol-regulatory element binding protein, and 3-hydroxy-3-methyl-glutaryl-CoA reductase
	Male Wistar rats [20]	Oral dose	Ameliorates streptozotocin-induced diabetic nephropathy (DN) by reducing the hyperglycemia-induced inflammatory response
			Decreases infiltration of macrophages, IL-1, IL-6, and TNF-α produced by p38 mitogen-activated protein kinase activation
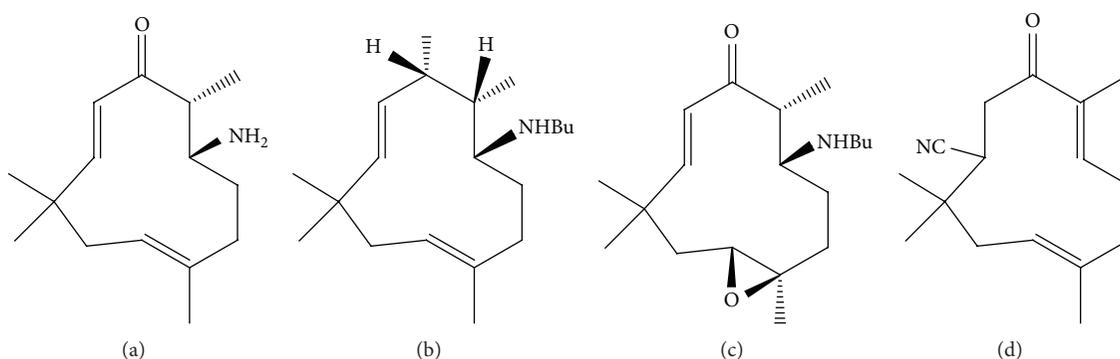
Figure 4: Zerumbone derivatives. (a) (±)-*[6E,10E]-3amino-2,6,9,9-tetramethylcloundeca-6,10-dienone* (5), (b) (±)-*[6E,10E]-3-butylamino-2,6,9,9-tetramethylcloundeca-6,10-dienol* (10),(c) (±)-*[10E]-3-butylamino-6,7-epoxy-2,6,9,9-tetramethylcloundeca-10-enone* (14), and (d) (±)-*[2E,6E]-10-cyano-2,6,9,9-tetramethylcloundeca-2,6-dienone* (20).

line [97]. The inhibition of mammary tumor growth in LA7-bearing Sprague Dawley rats was via Wnt/β-catenin signaling pathway [109].

3.8. Ovarian Cancer. The antiproliferative effect of ZER towards human ovarian cancer (Caov-3) cell line is dose dependent and time dependent. Zerumbone also effectively suppressed tumor promoter TPA-induced superoxide anion (O_2^-) generation from xanthine oxidase (XO) in Chinese hamster ovary (AS52) cells (CHO) [132], while even at high concentrations it does not adversely affect normal cultured CHO [93].

3.9. Pancreatic Cancer. Zerumbone is a novel inhibitor of Jak2/Stat3, which inhibits promigratory gene expression, growth, and migration of human pancreatic carcinoma (PaCa) [94]. It also inhibits CXCL12-induced spread of pancreatic (PANC-28, MIA PaCa-2, and AsPC-1) tumors [64].

The antipancreatic cancer effect of ZER is facilitated by the inhibition of cancer angiogenesis through the inhibition of NF-κB and NF-κB-dependent proangiogenic gene products [96]. The inhibition and apoptosis of human pancreatic carcinoma cell lines (PANC-1 and SW1990) were via p53 signaling pathway [95].

3.10. Lung Cancer. The nonsmall lung adenocarcinoma (H1299) cell can be suppressed by ZER, while its derivative, the parent alcohol 8 (2E,6Z,10E)-13-Hydroxy-2,9,9-trimethylcycloundeca-2,6,10-trienone, is one of the most potent cytotoxic compounds against human small cell lung carcinoma (NCI-H187) [97]. Zerumbone also effectively inhibited proliferation, multiplicity of lung adenomas induced by NNK, potentiated apoptosis, and suppressed NF-κB and HO-1 expressions in female A/J mice [133].

3.11. Renal Cancer. Human embryonic kidney carcinoma (A293) cell [64] and kidney epithelial (MDBK) cell line [129]
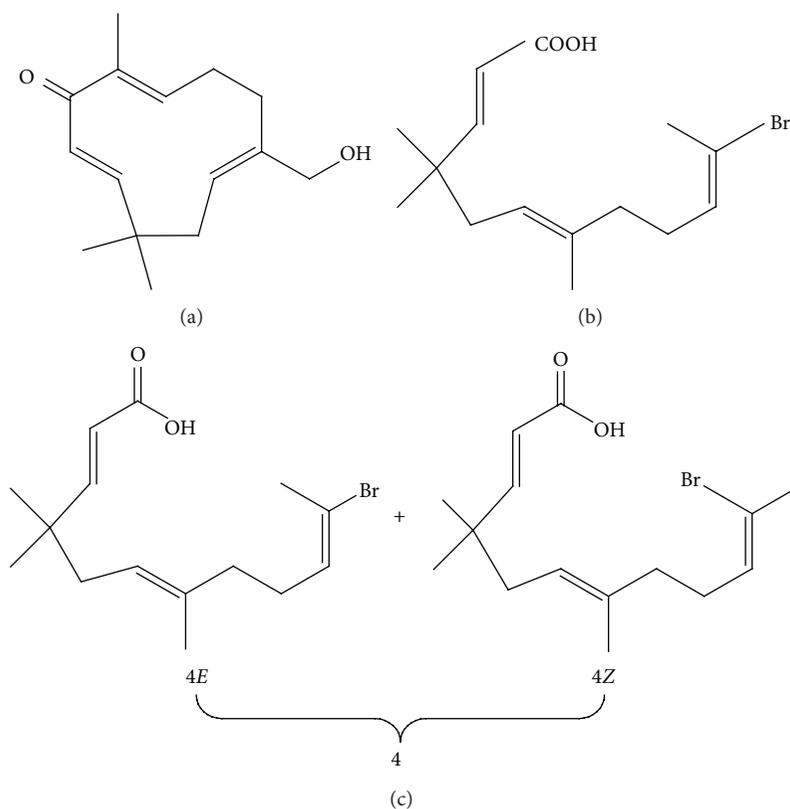
FIGURE 5: Zerumbone imidazole and ring opening derivatives. (a) Parent alcohol 8 *(2E,6Z,10E)-13-Hydroxy-2,9,9-trimethylcycloundeca-2,6,10-trienone,* (b) NH0891 ([*2E,6E,10E/Z0*]-11-bromo-4,4,7-trimethyl-2,6,10-dodecatrienoic acid), and (c) 4 (10*E*/10*Z* = 3/2).

proliferation was found to be inhibited by ZER treatment. Zerumbone could also protect irradiation-induced cell apoptosis and DNA damage, partly through the activation of the Keap1/Nrf2/ARE pathway in human kidney embryonic (HEK 293) cells [98]. The ZER derivative, parent alcohol 8 (2*E*,6*Z*,10*E*)-13-Hydroxy-2,9,9-trimethylcycloundeca-2,6,10-trienone, showed nonsignificant cytotoxicity toward normal monkey kidney (Vero) cell line [97].

3.12. Brain Cancer. Zerumbone can induce human glioblastoma multiforme (GBM8401) cell apoptosis via inhibition of the IKKα-Akt FOXO1 cascade [99].

3.13. Prostate Cancer. Zerumbone induced cytotoxicity and significant PARP cleavage in human prostate cancer (DU145) cell line through the inhibition of Jak2/STAT3-mediated signaling pathways [134].

3.14. Gastric Cancer. Zerumbone inhibits tumor angiogenesis in human gastric adenocarcinoma (AGS) cells of via reduction of VEGF production and NF-κB activity [135].

3.15. Oral Cancer. Parent alcohol 8 (2*E*,6*Z*,10*E*)-13-Hydroxy-2,9,9-trimethylcycloundeca-2,6,10-trienone is one of the most powerful compounds inducing cytotoxicity of human oral cancer (KB) cells [97].

3.16. Head and Neck Cancer. Expression of CXCR4 and invasion and metastasis of human tongue squamous (SCC4) cell carcinoma can occur with ZER treatment [64]. Similarly, ZER inhibited the NF-κB- and NF-κB-regulated gene expression induced by various carcinogens and inflammatory stimuli, such as TNF, okadaic acid, cigarette smoke condensate, phorbol myristate acetate, and H_2O_2. It also suppressed IκBα kinase activity, phosphorylation, and degradation and p65 phosphorylation, nuclear translocation, and acylation in human squamous (LICR-LONHN5) cell carcinoma line [63].

3.17. Pharyngeal Cancer. Zerumbone inhibited NF-κB and IκBα kinase, suppressed antiapoptotic and metastatic gene expression, upregulated apoptosis, and inhibits proliferation of human hypopharyngeal carcinoma (FaDu) cells [63].

4. Anti-Inflammatory Activity

Zerumbone has been shown to possess anti-inflammatory properties [25, 26]. Oral ZER treatment suppressed dextran sodium sulfate- (DSS-) induced acute ulcerative colitis (AUC) in female ICR mice. The anti-inflammatory effect of ZER was reflected by the significant lowering of the inflammatory biomarkers, IL-1β, TNF-α, and PGE2 [103]. In a female ICR mouse ultraviolet B (UVB) photokeratitis and cataractogenesis model, dietary ZER prevented corneal

damage by inhibiting NF-κB, iNOS, and TNF-α expression with concomitant reduction of malondialdehyde (MDA) and increase of glutathione (GSH) and GSH reductase (GR) levels [111, 112]. Moreover, ZER inhibited iNOS and COX-2 expression and release of TNF-α in a mouse macrophage (RAW264.7) cell line treated with lipopolysaccharide (LPS) and IFN-γ. Zerumbone also inhibited the NO/O$_2^-$ generation in inflammatory leukocytes [61, 103]. Oral feeding of ZER compound reduced the inflammatory process in collagen-induced osteoarthritis (OA) in Sprague Dawley rats. The treatment caused a significant reduction in the number of major histocompatibility complex (MHC) type II cells expressions in the affected synovial membrane and thus reducing accumulation of antigen presenting type A cells in arthritis [115]. In a rat knee osteoarthritis model, induced with monosodium iodoacetate (MIA), oral administration of ZER improved the densities of protein gene products (PGP), calcitonin gene-related peptide (CGRP), and neuropeptides-Y (NPY) immunoreactive nerve [116, 117].

In male Wistar rats, ZER suppressed cholecystokinin octapeptide- (CCK-8-) induced acute pancreatitis with significant reduction in serum amylase and lipase, cytosolic IL-6, iNOS, Mn- and Cu/Zn-SOD activities, and TNF-α concentration [113]. In these rats ZER treatment attenuates the severity of acute necrotizing pancreatitis and pancreatitis-induced hepatic injury via the inhibition of NF-κB and downregulation of ICAM-1 and IL-1β expressions [114].

5. Antioxidant Activity

The antioxidant activity of ZER has been reported to occur through the attenuation of reactive oxygen (RO) and generation of nitrogen species [136]. Thus, it is plausible that the potential of ZER as an agent against cancer-related inflammation may be mediated through its antioxidant activity. The ability of ZER to stimulate phase II detoxification enzymes was determined in the RL34 cells, a normal rat liver epithelial cell line. Induction of phase II enzymes is known to protect cells and tissues against toxicity and chemical carcinogenesis, particularly in the early phase. The effect of ZER on the stimulation of glutathione S-transferase is dose- and time-dependent and causes considerable increase in the level of the GSTP1-1 protein. Zerumbone also elicited significant induction in the nuclear localization of Nrf2, a transcription factor that binds to the antioxidant response element (ARE) of phase II enzyme genes, activating expression of phase II enzyme genes. Among the phase II enzyme involved in the activation are γ-glutamylcysteine synthetase (GCS), glutathione peroxidase (GPx), and HO-1. These enzyme systems, through their conjugation reactions, play important roles in the metabolic inactivation of pharmacologically active substances, thus minimizing cell damage [85].

6. Immunomodulatory Activity

Zerumbone has effect on the proliferation, cell cycle progression, and induction of cytokine (IL-2 and IL-12) of immune cells in vitro. This was shown by the proliferation of ICF mice thymocytes and splenocytes and human peripheral blood mononuclear cells (PBMC). Using flow cytometry, ZER treatment was shown to cause the highest population of PBMC to enter G2/M phase [73]. This study showed prominent upregulation of IL-2 and IL-12 in activated lymphocytes after ZER treatment.

7. Other Biomedical Properties of Zerumbone

7.1. Hepatoprotective Activity. Zerumbone was shown to have hepatoprotective properties in ethanol-induced liver injury in male Sprague Dawley rats, while ZER pretreatment extensively reduced fatty liver development in these rats [106]. Similar ZER has healing effects in paracetamol-induced hepatotoxicity in male Sprague Dawley rats as indicated by the corresponding reductions of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) blood concentrations in the treated rats [87].

7.2. Antiatherosclerotic Activity. Zerumbone is a phytochemical with potential for the regulation of atherosclerosis because it suppresses TPA-induced oxidized low density lipoprotein (LDL) receptor-1 (LOX-1) mRNA expression in THP-1 human monocyte-like cells and in differentiated colonic adenocarcinoma (Caco-2) cells. A key event in the development of atherosclerosis is the unregulated uptake of oxidized LDL via scavenger receptors (SR), which are integral membrane proteins. Zerumbone reduces the expression of several subclasses of the macrophage SR such as SR-A, SR-PSOX, and CD36, leading to the inhibition of uptake of DiI-acLDL, a modified LDL. Downregulation in the expression of SR by ZER was postulated to be partly attributed to the inhibition of transcriptional activities of activator protein-1 and NF-κB [75]. In rabbits fed cholesterol-rich diet, oral ZER treatment significantly decreased or averted early atheroma plague formation and development via reduction in monocytes and/or macrophages migration, aggregation, and smooth muscle cells proliferation. In a rabbit atherosclerosis model, ZER was also shown to repair endothelial dysfunction [124].

7.3. Antinociceptive Activity. Significant antinociceptive effects of intraperitoneal ZER were observed in adult male BALB/c mice. The results of this study indicated that ZER possesses considerable marginal and central antinociceptive effects at various dosages [27]. The production of antinociception in the mice model suggests significant involvement of L-arginine-nitric oxide-cGMP-PKC-K+ ATP channel pathways, the TRPV1 and kinin B2 receptors [118].

7.4. Antimicrobial Activity. Zerumbone and its derivatives such as 410E/10Z = 3/2 and NH0891 (Figures 5(b) and 5(c)) were found to be selective inhibitors of gram-positive bacteria, *Bacillus subtilis* 168 growth. It was suggested that the new haloolefinic acids synthesized by the cleavage of the C1-C2 bone of ZER inhibits growth of gram-positive bacteria by inhibiting YycG histidine kinase [137, 138]. Zerumbone

(a)   (b)
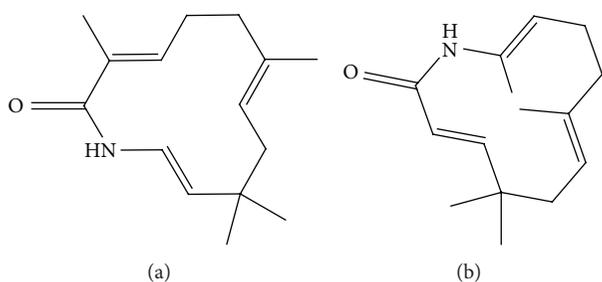
FIGURE 6: Zerumbone derivatives. (a) Azazerumbone 1, and (b) azazerumbone 2.

also inhibits *Salmonella choleraesuis,* a gram-positive bacteria while not affecting the viability of *Escherichia coli* [139]. Similarly, ZER and its synthetic analogues (azazerumbone 1 and azazerumbone 2) (Figure 6) exhibited strong protection against sodium azide-induced mutagenicity of *Salmonella typhimurium* (TA 98 and TA 1531) strains. Among the bacteria tested, *Bacillus cereus* was most sensitive to these analogues [140].

Other antipathogen effects of ZER include inhibition of human immunodeficiency virus (HIV) activity [33] and antifungal activity towards *Rhizoctonia solani,* the damping-off pathogen [52].

Zerumbone was reported to have antimalarial activities by inhibiting propagation of *Plasmodium falciparum* [141]. Exposure of the nematode *Caenorhabditis elegans* to ZER increased expression of HSP16.41 mRNA, suggesting that ZER can increase the survival of nematodes after heat-shock treatment.

In lipid metabolism, ZER improved dyslipidemia by modulating expression of genes involved in the lipolytic and lipogenic pathways of a diet-induced hyperlipidemic animal model [125]. This study suggests that ZER is beneficial to patients with hypercholesterolemia and hypertriglyceridemia. Another study showed that ZER attenuated nonalcoholic fatty liver disease, improved insulin sensitivity, decreased lipogenesis, and increased lipid oxidation in male golden Syrian hamster [108]. Zerumbone also seems to be beneficial in alleviating symptoms of renal dysfunction. Treatment of female Sprague Dawley rats with cisplatin-induced renal disease with ZER had reduced toxicity and organ damage via the preservation of antioxidant glutathione and prevention of lipid peroxidation [123].

Zerumbone induces genotoxic and cytotoxic effects on cultured human peripheral blood lymphocytes [71], CHO cells, and rat bone marrow polychromatic erythrocytes (PCEs) [74, 142]. In fact highly concentrated ZER could cause substantial increase in the frequency of micronuclei in these cells. This study suggests that there are safety issues in the development of ZER as a potential therapeutic compound, because very high doses of ZER may produce adverse effects.

Finally, there is evidence that ZER may be useful in the treatment of Alzheimer's disease. This was suggested by a recent study that showed ZER inhibits acetylcholinesterase [143]. The enzymolytic effect of ZER towards AChE (acetylcholinesterase) could be the basis for the development of ZER in the treatment of Alzheimer's disease.

8. Discussion

Many natural compounds possess various and significant biological activities. Thus traditionally these compounds are included in the diet of many Asian societies because they are not only nontoxic but also beneficial to health [144]. However, there is a dearth of scientific and clinical evidence supporting effectiveness, usefulness, and safety of herbal compound used in traditional medicine. Because of lacking evaluation of the toxicity and negative reactions of medicinal herbs, the use of natural compounds may prove unsafe.

Malaysia, with its tropical rainforests, is blessed with high biodiversity. The Malaysian forest is an enormous potential source of chemicals and metabolites that can be developed into new agents or novel drugs for treatment of chronic diseases [145]. The jungles of South East Asia have provided more than 6,500 different plants that have been used in the treatment of various illnesses particularly cancers [146]. The South East Asians seemed to have lower risks for development of cancers including colon, gastrointestinal, prostate, and breast cancers compared to Westerners [147]. It is probably the practice of regular consumption of natural plant products that contributes to the lower incidence of these debilitating diseases in the South East Asians.

Recently, in our laboratory, ZER was made soluble by incorporating in the cyclodextrin complex. The production of the ZER-cyclodextrin complex enabled ZER to be formulated as an encapsulated natural compound ready for use, either as an injectable solution or delivered orally as an anticancer product [148, 149]. The usefulness of encapsulated ZER complex as potential anticancer is worth future exploration through preclinical and human clinical trials to determine efficacy and safety of the product for human use. More recently we also encapsulated ZER into a nanostructured lipid carrier (NLC) using the high pressure homogenization (HPH) technique. The physiochemical properties, entrapment efficiency, storage stability, in vitro release, and cytotoxic effect of this formulation against human acute lymphocytic leukemia (Jurkat) cell line were studied and showed promising results. Our study also showed that ZER-loaded NLC can be further developed as a drug delivery system for cancer therapy [23, 66]. This new approach to using a natural metabolite in innovative delivery systems would seemingly be an alternative and new approach in the treatment of cancers [72].

This review has clearly indicated that ZER from *Zingiber zerumbet* Smith possesses various beneficial in vitro and in vivo biological activities. The findings from all the researches reviewed in this paper are conclusive evidences that ZER is a strong potential candidate for anticancer compound. There is need to conduct animal studies and human clinical trials to ascertain the efficacy, usefulness, and safety of this compound as an intended pharmaceutical drug.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

p53 Is a Key Regulator for Osthole-Triggered Cancer Pathogenesis

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Osthole has been reported to have antitumor activities via the induction of apoptosis and inhibition of cancer cell growth and metastasis. However, the detailed molecular mechanisms underlying the anticancer effects of osthole in human colon cancer remain unclear. In the present study, we have assessed osthole-induced cell death in two different human colon cancer cell lines, HCT116 and SW480. Our results also showed that osthole activated proapoptotic signaling pathways in human colon cancer cells. By using cell culture insert system, osthole reduced cell motility in both human colon cancer cell lines. This study also provides evidence supporting the potential of osthole in p53 activation. Expression of p53, an apoptotic protein, was remarkably upregulated in cells treated with osthole. Importantly, the levels of phosphorylation of p53 on Ser15 (p-p53) and acetylation of p53 on Lys^{379} (acetyl-p53) were increased under osthole treatment. Our results also demonstrated that p53 was activated followed by generation of reactive oxygen species (ROS) and activation of c-Jun N-terminal kinase (JNK). Our study provides novel insights of p53-mediated responses under osthole treatment. Taken together, we concluded that osthole induces cancer cell death and inhibits migratory activity in a controlled manner and is a promising candidate for antitumor drug development.

1. Introduction

Colon cancer is one of the leading causes of cancer-related deaths worldwide. Surgery can be applied in the early stage, while chemotherapy and/or radiation therapy are used to treat malignant tumors. The metastatic dissemination of primary tumors is linked directly to patients' survival and accounts for about 90% of all colon cancer deaths. It should therefore be obvious that tools and methodologies that allow early cancer detection will affect the survival time and rate of patients. The development of colon cancer is increasing

in recent years; however, the knowledge of treatments is still limited.

The p53 tumor suppressor gene is one of the most commonly mutated genes found in human malignancies [1]. More than 50% of human cancer cells are associated with missense mutations or deletions of p53 [2], which results in chemoresistance of those cancer cells [3]. p53 protein is important in the transcription of growth inhibiting genes, apoptosis, cell cycle arrest, and DNA repair [4]. p53 is also a sequence-specific transcription factor that transactivates p21, which is involved in cell growth regulation [5]. The MAP

kinase family comprises extracellular-signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), and these signal pathways have been implicated in many physiologic processes, including cell growth and death through p53-dependent or p53-independent mechanisms [6]. Importantly, JNK signal transduction pathway is also activated by some anticancer drugs [7, 8]. JNK1 protein kinase is the major isoform in JNK family proteins that its activation requires phosphorylation on Thr^{183} and Tyr^{185} [9]. Dominant-negative mutation of JNK (DN-JNK) is a mutant at these sites (on Thr^{183} and Tyr^{185}), which effectively suppresses paclitaxel-induced cell apoptosis in cancer cells [10].

Frequent consumption of natural fruits and vegetables has been considered to reduce risk of developing cancers and mortality [11, 12]. Osthole (7-methoxy-8-(3-methyl-2-butenyl)coumarin) is an active constituent isolated from *Cnidium monnieri* (L.) *Cusson,* which has been shown to exert a wide variety of biological effects such as contractility-based motility of different cells and tissues [13]. Osthole has also been shown to have anti-inflammatory [14], antiosteoporosis [15], and antiseizure [16] effects. In recent years, accumulating evidence also suggests that osthole has antitumor activities that are thought to occur via the induction of apoptosis and inhibition of cancer cell growth and metastasis [17–19].

ROS plays a key role in regulation of biological functions including differentiation and immune responses [20]. ROS is generated through a number of environmental stimuli, and excessive production of ROS causes oxidative stress leading to adverse events like cell death [21, 22]. Previous reports showed that the protective effects of osthole were revealed by reducing ROS production in ischemia-reperfusion injury models [23, 24]. However, the effect of osthole in generation of ROS in cancer cells is still unknown, and the detailed mechanisms underlying the anticancer effects of osthole in human colon cancer remain unclear. This study investigates the effects and underlying mechanisms of osthole-induced cell death and migration in human colon cancer. Our study reports that osthole induces cell death and reduces cell migration through the induction of ROS production, JNK activation, and p53 activation.

2. Experimental Section

2.1. Reagents and Antibodies. Osthole was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, and Lipofectamine 2000 (LF2000) were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA, USA). Primary antibodies against Bax, Bcl2, procaspase-3, PARP, JNK, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against p-JNK Thr^{183}/Tyr^{185}, p-p53 Ser^{15}, and acetyl-p53 Lys^{379} were purchased from Cell Signaling and Neuroscience (Danvers, MA). The primary antibody against p53 was purchased from BD Pharmingen Transduction Laboratories (San Diego, CA). The dominant-negative (DN) mutant of JNK plasmid was a gift from Dr. W.-M. Fu (National Taiwan University, Taipei, Taiwan).

2.2. Cell Culture. Human colon carcinoma cell lines HCT116 and SW480 were obtained from the American Type Culture Collection (Manassas, VA, USA). The procedures of cell culturing were conducted according to previous reports [25, 26].

2.3. Reactive Oxygen Species (ROS) Assay. The procedures of ROS assay were conducted according to previous reports [27, 28]. ROS production was determined by the oxidation of specific probe, H_2DCFDA ($2',7'$-dichlorodihydrofluorescein diacetate), by using flow cytometry. Cells were incubated with H_2DCFDA ($10\,\mu M$) at $37°C$ for 30 min and then stimulated with osthole or hydrogen peroxide. Fluorescence intensities were determined with an excitation filter of 488 and 525 nm emission wavelengths.

2.4. Western Blot Analysis. Cells were lysed in a homogenization buffer on ice, and equal protein amounts of samples were loaded in an SDS-PAGE (polyacrylamide gel electrophoresis) [29]. The membrane was blocked with 5% nonfat milk and then probed with primary antibody. After several PBST washes, the membrane was incubated with a peroxidase-conjugated secondary antibody. The blots were visualized by enhanced chemiluminescence using Fuji medical X-ray film (Fujifilm, Tokyo, Japan). The blots were then stripped by incubation in stripping buffer [25] and reprobed a loading control. Quantitative data were obtained using a densitometer and Image J software (National Institute of Health, Bethesda, MA).

2.5. Migratory Activity Assay. *In vitro* migration assay was performed using Costar Transwell inserts (pore size, $8\,\mu m$) (Corning, Albany, NY) as described previously [30–32]. Cells in $200\,\mu L$ of media were seeded in the upper chamber, and $300\,\mu L$ of media was placed in the lower chamber (incubated at $37°C$ in 5% CO_2). After seeding cells in the upper chamber, cells were treated with osthole for 2 h. After a 24 h migration period, cells were stained with 0.05% crystal violet in 2% methanol. Nonmigratory cells on the upper surface of the filters were removed by wiping with a cotton swab. Cell number was counted in five random fields per well under a microscope at 200x magnification. Images of migratory cells were acquired using a digital camera and light microscope.

2.6. Reverse Transcriptase-PCR (RT-PCR). Total RNA was extracted from cells using TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using $2\,\mu g$ of total RNA, which was reverse transcribed into cDNA using the oligo(dT) primer and then amplified using oligonucleotide primers:

  p53: $5'$-AATTTGCGTGTGGAGTATTT-$3'$ and
  $5'$-GTGGAGTCTTCCAGTGTGAT-$3'$;
  p21: $5'$-AGGCACCGAGGCACTCAGAG-$3'$ and
  $5'$-AGTGA CAGGTCCACATGGTCTTCC-$3'$;
  GAPDH: $5'$-TGGGCTACACTGAGCACCAG-$3'$ and
  $5'$-GGGTGTCGCTGTTGAAGTCA-$3'$.

Each PCR cycle was carried out for 30 sec at $95°C$, 30 sec at $55°C$, and 1 min at $72°C$. PCR products were then separated

(a)

(b)

(c)
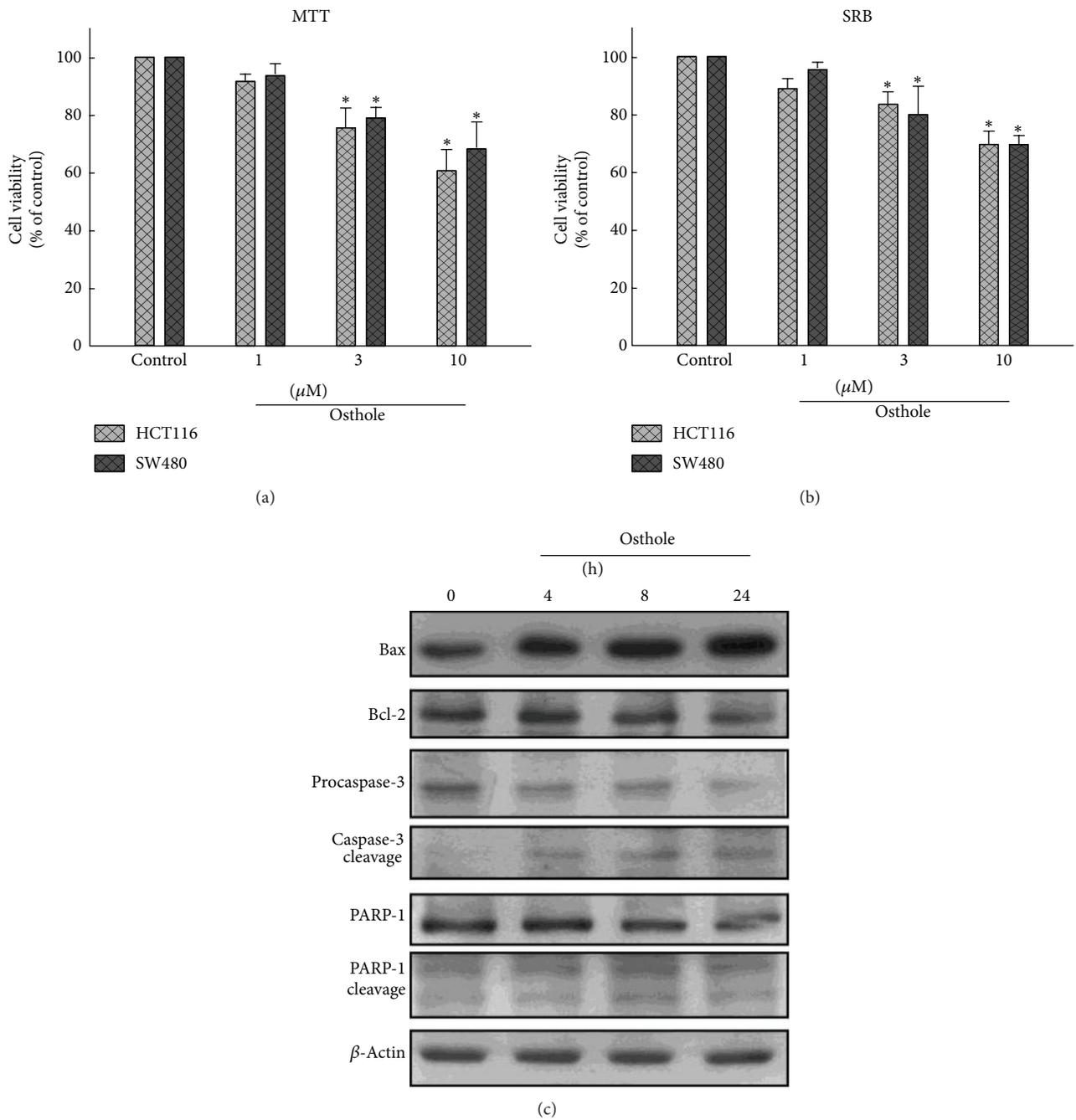
FIGURE 1: Osthole induces cell death in human colon cancer. Osthole-induced cell death of HCT116 and SW480 human colon cancer cells was shown. Cells were incubated with various concentrations of osthole (1, 3, or 10 μM) for 24 h, and the cell viability was examined by MTT (a) or SRB (b) assays. Results are expressed as the means ± S.E.M. from three independent experiments. $^*P < 0.05$, compared with the vehicle treatment group. (c) SW480 cells were incubated with osthole (10 μM) for indicated time periods, and the protein expressions of Bax, Bcl-2, procaspase-3, cleaved caspase-3, and PARP-1 were examined by western blot analysis. Results are the representative of three independent experiments.

electrophoretically in a 2% agarose gel and stained with Novel Juice (GeneDireX, Las Vegas, Nevada).

2.7. MTT and SRB (Sulforhodamine B) Assays. The procedures of MTT and SRB assays determining cell viability were conducted according to previous reports [33–35]. After treatment with various concentrations of osthole for 24 h, cell culture media were aspirated. In MTT assay, MTT (0.5 mg/mL) was added to each culture well and incubated for 2 h at 37°C. The MTT reagent was then removed and washed with PBS for several times. DMSO (200 μL per well)

(a)

(b)
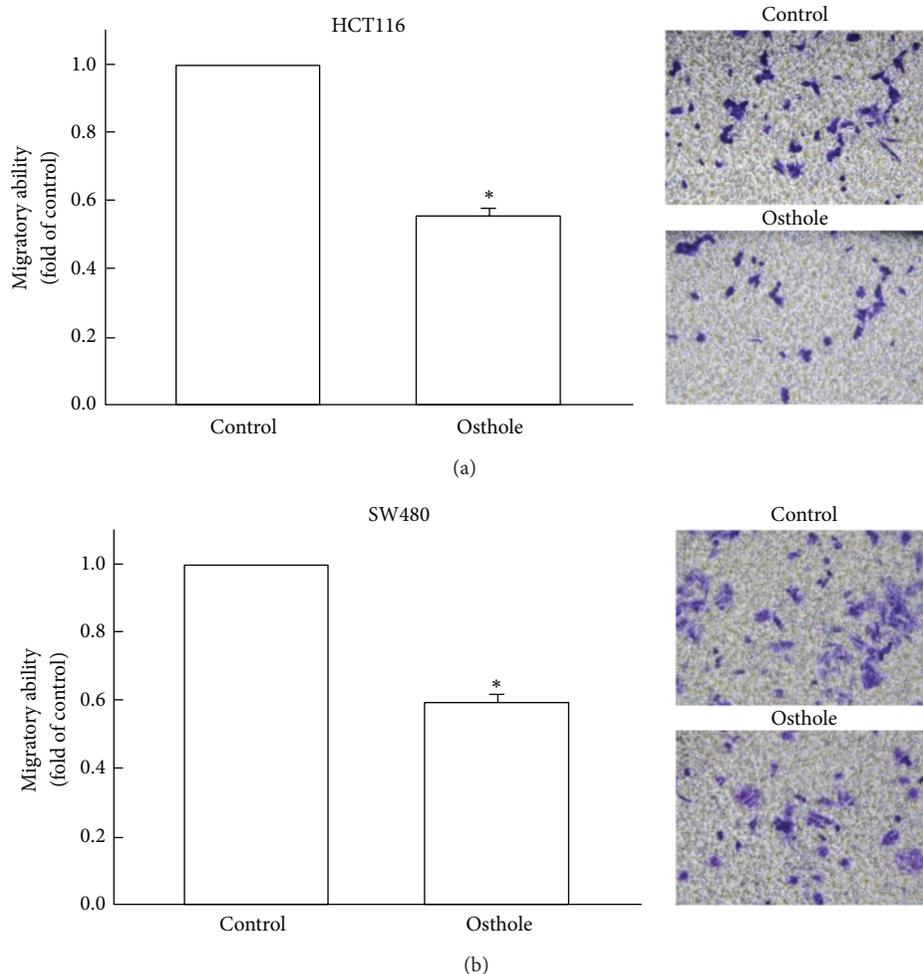
FIGURE 2: Osthole inhibits migration activity of human colon cancer. By using cell culture insert system, migratory activities of human colon cancer cells were examined. After incubating cells with osthole (10 μM) or vehicle control for 24 h, we found that osthole inhibited migration activity in both HCT116 (a) and SW480 (b) cells. Migrated cells were visualized by phase-contrast imaging (right panels). Results are expressed as means ± S.E.M. from three independent experiments. $^*P < 0.05$, compared with control group.

was added to dissolve formazan crystals. In SRB assay, cells were fixed *in situ* by gentle addition of 50 μL per well of 10% TCA, and culture plates were incubated for 1 h at 4°C. After discarding supernatant, cells were then washed with PBS and 50 μL of SRB solution was then added to each well for 10 min at room temperature. After staining, cells were washed with 1% acetic acid and dissolved in tris-base. The absorbance was determined at 550 nm (for MTT) or 515 nm (for SRB) using a microplate reader (Thermo Scientific, Vantaa, Finland).

2.8. Statistical Analyses. The values are reported as mean ± S.E.M. Statistical analyses between two groups were performed using Student's t-test. The difference was determined to be significant if the P value was <0.05.

3. Results

3.1. Osthole Induces Cell Death and Apoptosis in Human Colon Cancer. In order to investigate whether osthole affects cell viability, HCT116 and SW480 human colon cancer cells were incubated with various concentrations of osthole (1, 10, or 30 μM) for 24 h, and cell viability of both cell lines was determined by MTT and SRB assay. We observed that osthole induced cell death of human colon cancer in a concentration-dependent manner (Figures 1(a) and 1(b)). Treatment of SW480 cells with osthole also increased Bax protein expression and the cleavage of caspase-3 and PARP-1. Meanwhile, osthole decreased the protein expression of Bcl-2, procaspase-3, and PARP-1 as well (Figure 1(c)).

3.2. Osthole Inhibits Migratory Activity of Human Colon Cancer. Osthole-regulated human colon cancer migration was examined by using cell culture insert system. As shown in Figures 2(a) and 2(b), human colon cancer cells (HCT116 and SW480 cells) migrated from the upper to the lower chamber, and images of migrated cells were shown in right panels. Our results indicated that osthole effectively reduced human colon cancer migration in a concentration-dependent manner.

(a)

(b)

(c)
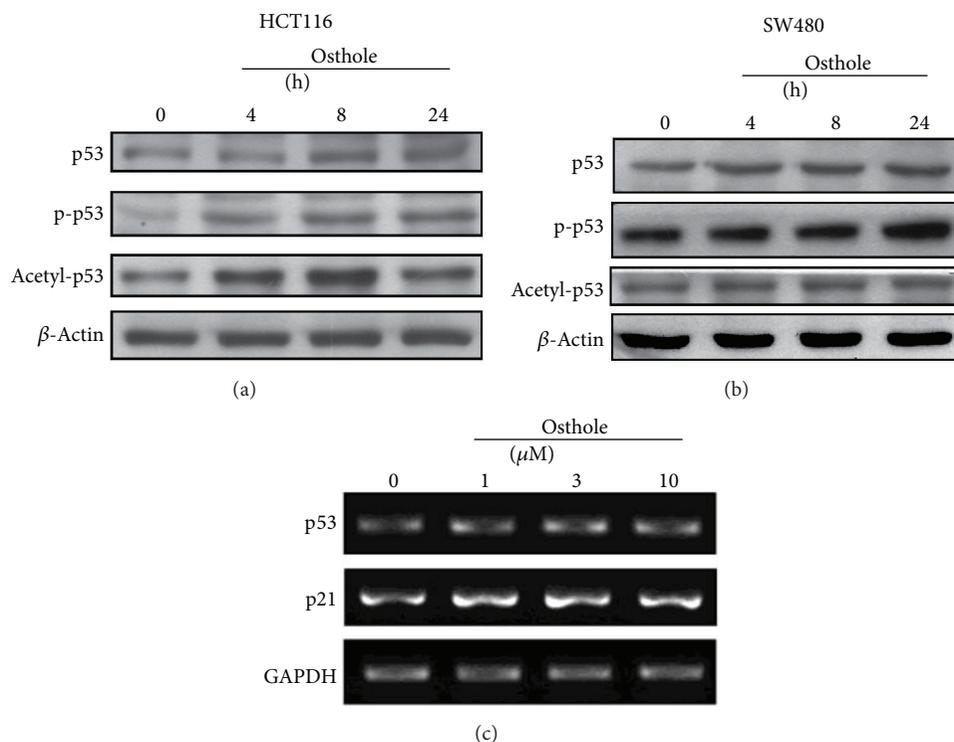
FIGURE 3: Osthole increases p53 protein activation in human colon cancer. HCT116 (a) and SW480 (b) were incubated with osthole (10 μM) for indicated time periods. p53, phosphorylated p53 (p-p53), and acetylated p53 (acetyl-p53) expressions were examined by western blot analysis. (c) SW480 cells were stimulated by osthole for 6 h, and p53 and p21 expressions were examined by RT-PCR analysis. Results are the representative of three independent experiments.

3.3. Osthole Induces p53 Protein Activation in Human Colon Cancer. As shown in Figures 3(a) and 3(b), HCT116 and SW480 cells were incubated with osthole before cell lysate extracts were collected. p53 protein levels, phosphorylation of p53 on Ser15 (p-p53), and acetylation of p53 on Lys^{379} (acetyl-p53) were all increased after osthole stimulation. The effects of osthole on gene expression of p53 and p21 were estimated in SW480 cells using RT-PCR analysis. As shown in Figure 3(c), p53 and p21 gene expression levels were mildly increased in osthole-treated group.

3.4. JNK Activation in Osthole Involves Protein p53-Induced Activation. After incubating the SW480 cells with various concentrations of osthole (1, 3, or 10 μM) for 24 h, we found that osthole increased the level of phosphorylated JNK (Figure 4(a)). Moreover, transfection with DN-JNK for 24 h reduced JNK phosphorylation (Figure 4(a)). Treatment with JNK inhibitor SP600125 reduced osthole-induced p53 protein activation (Figure 4(b)). Furthermore, transfection with DN-JNK also attenuated the effects of osthole-induced human colon cancer cell death (Figures 4(c) and 4(d)) and cell migratory effect (Figure 4(e)). Incubation with JNK inhibitor SP600125 also reversed osthole-inhibited cell migratory effect in SW480 human colon cancer cells (Figure 4(f)).

3.5. Osthole Induces p53 Protein Activation through Reactive Oxygen Species Production in SW480 Human Colon Cancer.

As determined by probe H_2DCF-DA which was analyzed by flow cytometry assay, osthole was found to increase intracellular ROS levels. Treatment with an ROS scavenger NAC (n-acetylcysteine) reduced hydrogen peroxide (H_2O_2) induced ROS production (Figures 5(a) and 5(b)). Moreover, incubation with osthole also dramatically increased ROS generation in human colon cancer cells (Figure 5(c)). Additionally, treatment with NAC antagonized osthole-induced ROS production in SW480 cells as well (Figure 5(d)). As shown in Figure 5(e), treatment with NAC along with osthole in human colon cancer also reduced osthole-enhanced p53 protein expression, p-p53, and acetyl-p53 activation.

4. Discussion

Several clinical uses of drugs were derived or modified from plant extracts and have been successfully applied to treat a variety of human cancers [36]. We have reported some natural products and chemical compounds exerting anticancer effects in human glioblastoma [27, 28] and colon cancer [25]. Recently, we also reported that osthole induces cell death and attenuates cell migration in brain tumor [37]. However, the detailed mechanisms underlying the anticancer effects of osthole remain unclear. Our present study reported that osthole increased phosphorylation of p53 on Ser^{15} (p-p53) and acetylation of p53 on Lys^{379} (acetyl-p53), which were regulated by ROS generation and JNK activation.

(a)

(b)

(c)

(d)

(e)

(f)
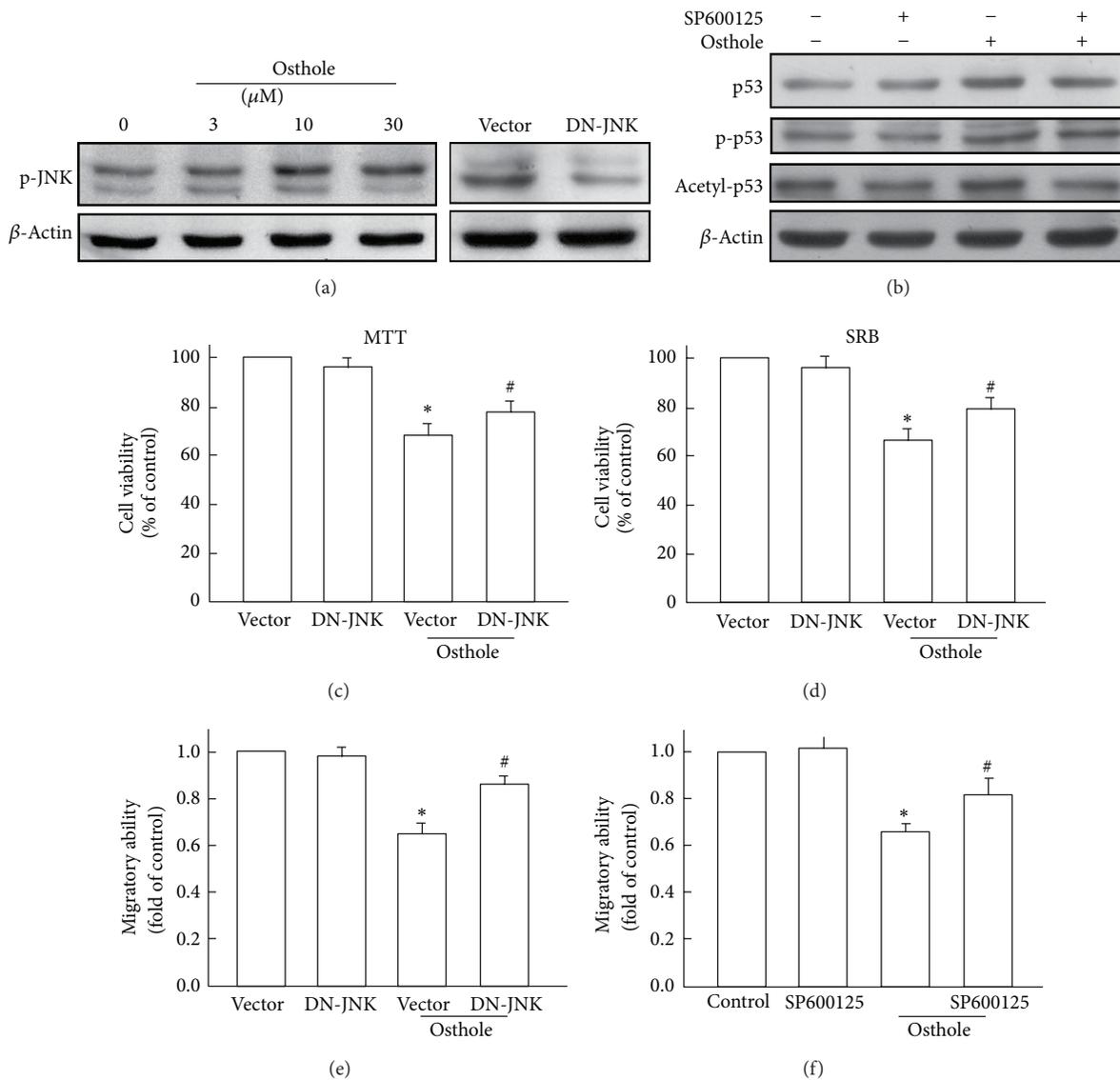
FIGURE 4: JNK activation contributes to osthole-induced p53 activation in SW480 human colon cancer. (a) Cells were incubated with various concentrations of osthole for 24 h (left panel). Cells were transfected with DN-JNK or empty vector for 24 h (right panel) before cell lysates were collected. Levels of phosphorylated JNK were determined by western blot. (b) Cells were pretreated with or without SP600125 (10 μM) followed by stimulation with osthole for another 24 h; the protein expressions of p53, p-p53, and acetyl-p53 were examined by western blot analysis. Results are the representative of three independent experiments. (c) Cells were transfected with DN-JNK for 24 h followed by stimulation with osthole for another 24 h, and cell viability was examined by MTT (c) or SRB (d) assays. Osthole-inhibited migratory activities were examined by cell culture insert system after transfecting cells with DN-JNK or empty vector for 24 h (e). (f) Cells were pretreated with SP600125 (10 μM) followed by stimulation with osthole for another 24 h, and migratory activities were also examined.

To our knowledge, this is the first report that shows the role of p53 in osthole-induced anticancer effects and identifies the molecular mechanisms through which ROS and JNK modulate p53 protein activation in human colon cancer cells.

Targeting p53, a tumor suppressor, is one of the promising strategies for anticancer therapy; therefore several compounds targeting p53 are currently being tested in clinical studies [38]. Numerous reports have supported this idea of pharmacological restoration of p53 activity for anticancer [39, 40]. Induction of p53 activation leads to cell growth arrest or cell death, but investigation of the detailed molecular mechanism regulating the cancer pathogenesis by p53 remains a key challenge in p53 biology of cancer cells [41]. Importantly, p53-mediated cell fate decisions also prevent cancer cells to be killed by chemotherapeutic drugs, thus leading to poor clinical outcomes. It is imperative to understand the mechanism of p53-mediated cell fate decisions for the efficient clinical application of drugs activating p53 [42]. Our current study reported the crucial role of ROS and JNK in p53-related proapoptotic function in human

(a)

(b)

(c)

(d)

(e)
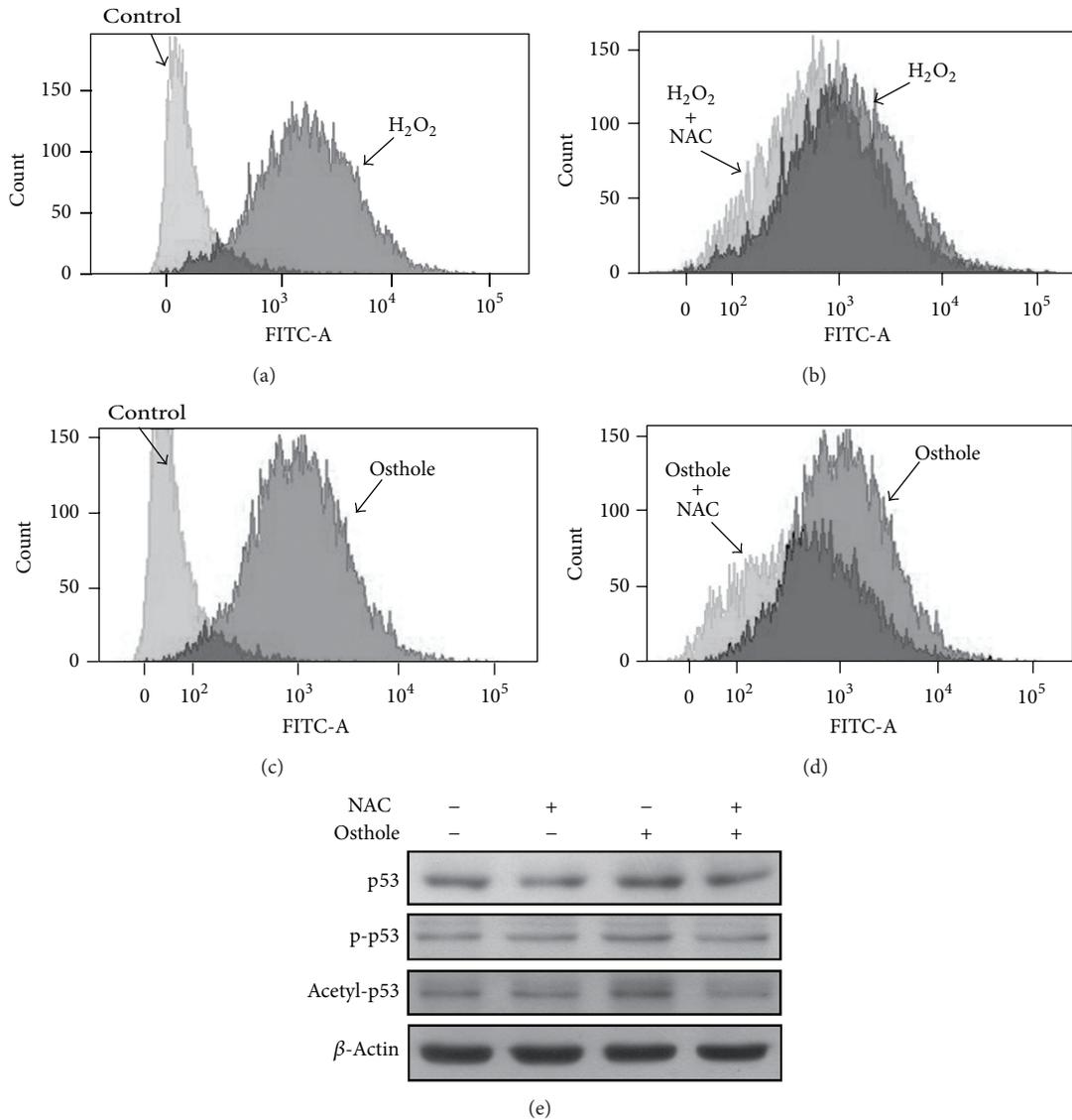
FIGURE 5: Reactive oxygen species involved in osthole-induced p53 activation. SW480 cells were incubated with H_2O_2 ($200\,\mu M$; (a)), H_2O_2 plus N-acetyl-L-cysteine (H_2O_2 + NAC; (b)), osthole ($10\,\mu M$; (c)), or osthole plus N-acetyl-L-cysteine (osthole + NAC; (d)) for 120 min. ROS generations were determined using flow cytometry with the fluorescence probe H_2DCFH-DA. (e) Cells were treated with NAC plus osthole for 24 h and the protein expressions of p53, p-p53, and acetyl-p53 were examined by western blot analysis. Results are the representative of three independent experiments.

colon cancer through activation of p53 by a natural product, osthole. Furthermore, our results also demonstrated that JNK-mediated mechanism played a key role in cell death and migratory ability. Interestingly, osthole induced significant cell death and inhibited migratory ability even in the p53-mutated colon cancer cell line SW480 cells.

Several lines of evidence demonstrate that the accumulation of ROS is correlated with the apoptotic response induced by several chemotherapeutic drugs [43]. ROS generation appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through the proapoptotic Bcl-2 proteins Bax and is further transformed sequentially into more toxic ROS, like hydrogen peroxide, which, consequently, induces cell death [44]. Our study showed that osthole elevated the intracellular ROS levels in human colon cancer cells. Furthermore, we observed that blocking ROS production with antioxidant NAC resulted in decreased intracellular ROS levels as well as osthole-induced p53 protein activation. These results indicated that ROS accumulation contributed to osthole-induced cell apoptosis in human colon cancer. Our results also reported that osthole-enhanced proapoptotic protein Bax expression and Bcl-2 degradation. Furthermore, treatment with osthole also increased caspase-3 activation and promoted PARP-1 cleavage. In conclusion, osthole-induced human colon cancer cell death may mediate by ROS generation, which subsequently induces Bax/Bcl-2 turnover and promotes caspase-3 activation and PARP-1 cleavage, resulting in cell apoptosis.

Moreover, our results in colon cancer are also in accordance with previous report that osthole effectively attenuates migratory ability in human cancer cells.

5. Conclusions

In the present study, osthole-induced p53 activation results from ROS generation and JNK activation, revealing a promising potential in the treatment of human colon cancer. The current study on a molecular basis provides valuable knowledge of osthole in effective antitumor therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors were involved in drafting this paper or revising it critically for important intellectual content, and all authors approved the final version to be published. Ssu-Ming Huang, Cheng-Fang Tsai, and Wei-Lan Yeh conceived and designed experiments. The acquisition, analysis, and interpretation of data were done by Ssu-Ming Huang and Wei-Lan Yeh. Ssu-Ming Huang, Min-Ying Wang, Dar-Ren Chen, and Wei-Lan Yeh drafted and revised the paper.

Acknowledgments

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Review Article

Evaluating the Cancer Therapeutic Potential of Cardiac Glycosides

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Cardiac glycosides, also known as cardiotonic steroids, are a group of natural products that share a steroid-like structure with an unsaturated lactone ring and the ability to induce cardiotonic effects mediated by a selective inhibition of the Na^+/K^+-ATPase. Cardiac glycosides have been used for many years in the treatment of cardiac congestion and some types of cardiac arrhythmias. Recent data suggest that cardiac glycosides may also be useful in the treatment of cancer. These compounds typically inhibit cancer cell proliferation at nanomolar concentrations, and recent high-throughput screenings of drug libraries have therefore identified cardiac glycosides as potent inhibitors of cancer cell growth. Cardiac glycosides can also block tumor growth in rodent models, which further supports the idea that they have potential for cancer therapy. Evidence also suggests, however, that cardiac glycosides may not inhibit cancer cell proliferation selectively and the potent inhibition of tumor growth induced by cardiac glycosides in mice xenografted with human cancer cells is probably an experimental artifact caused by their ability to selectively kill human cells versus rodent cells. This paper reviews such evidence and discusses experimental approaches that could be used to reveal the cancer therapeutic potential of cardiac glycosides in preclinical studies.

1. Introduction

Cardiac glycosides, also known as cardiotonic steroids, are natural products with a steroid-like structure and an unsaturated lactone ring. They usually contain sugar moieties in their structure and have cardiotonic activity. Cardiac glycosides containing the lactone 2-furanone are known as cardenolides and those containing the lactone 2-pyrone are known as bufadienolides (Figure 1). Most cardiac glycosides (e.g., digitoxin, digoxin, ouabain, and oleandrin) have been isolated from plants, including *Digitalis purpurea*, *Digitalis lanata*, *Strophanthus gratus*, and *Nerium oleander*. Some cardiac glycosides have also been found in amphibians and mammals, including digoxin, ouabain, bufalin, marinobufagenin, and telecinobufagin. Several cardiac glycosides are used in cardiology for the treatment of cardiac congestion and some types of cardiac arrhythmias. The mechanism by which these drugs affect cardiac contractility is thought to

be mediated by a highly specific inhibition of the Na^+/K^+-ATPase pump [1–3].

Over the years, several reports have suggested that cardiac glycosides may have an anticancer utilization (reviewed in [4–13]). *In vitro* and *ex vivo* experiments have revealed that some cardiac glycosides (e.g., digitoxin) induce potent and selective anticancer effects [4, 14, 15], which may occur at concentrations commonly found in the plasma of patients treated with these drugs [16]. Recent high-throughput screenings of drug libraries have identified several cardiac glycosides (e.g., digoxin, ouabain, and bufalin) as potent inhibitors of cancer cell growth [17–19]. These cardiac glycosides were also able to block tumor growth in mice xenotransplanted with human cancer cells, further supporting the idea that these compounds should be evaluated in cancer patients [17–19]. The cardiac drugs digitoxin and digoxin, the semisynthetic cardiac glycoside UNBS1450, and two extracts from the plant *Nerium oleander* have entered clinical trials for the treatment
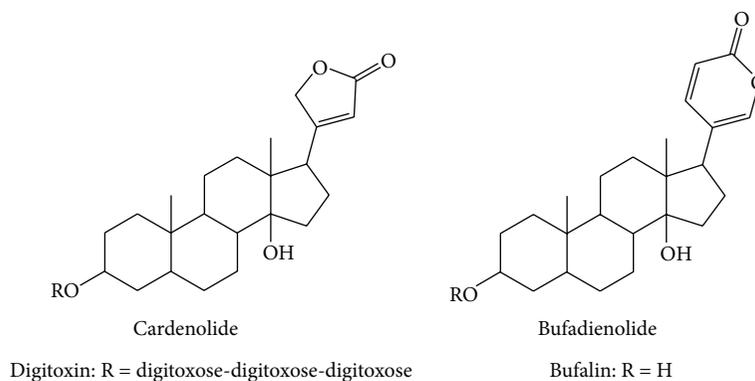
FIGURE 1: Chemical structure of cardiac glycosides. The basic skeletons of cardenolides and bufadienolides and the structures of the cardenolide digitoxin and the bufadienolide bufalin are shown.

of cancer (see http://clinicaltrials.gov/ and ref. [6, 7, 10, 20, 21]).

Research results also suggest, however, that cardiac glycosides may not inhibit cancer cell proliferation selectively in particular types of cancer [22–24] and the potent inhibition of tumor growth induced by cardiac glycosides in mice xenografted with human cancer cells is probably an experimental artifact caused by their ability to selectively kill human cells versus rodent cells rather than by their ability to selectively kill human cancer cells versus human normal cells [24–26]. After reviewing such evidence, this paper discusses experimental approaches that can be used to reveal the cancer therapeutic potential of cardiac glycosides in preclinical studies.

2. Possible Misinterpretation of Data from Preclinical Studies

Inhibition of cancer cell proliferation at low concentrations and inhibition of tumor growth in animal models are the most common parameters used by researchers to assess the therapeutic potential of drug candidates in preclinical studies. Based on this approach, researchers have proposed cardiac glycosides as candidates for evaluation in clinical trials. This section of the paper reviews evidence indicating that this approach may be inadequate to reveal the cancer therapeutic potential of cardiac glycosides.

2.1. Inhibition of Cancer Cell Proliferation at Low Concentrations Does Not Reliably Predict Therapeutic Potential. The key feature of an efficient anticancer drug candidate is its ability to kill (or to inhibit the proliferation of) human cancer cells at concentrations that do not significantly affect human nonmalignant cells. If the anticancer drug candidate does not have this feature, it does not really matter whether or not it can kill cancer cells at low concentrations. The reason is that the drug concentrations required to kill the tumor cells of cancer patients would also cause the death of their normal cells and, therefore, would be lethal to these patients. It is important to note that the therapeutic potential of a drug able to kill cancer cells at a concentration of 1 millimolar without

significantly affecting nonmalignant cells at a concentration of 10 millimolar is probably higher than that of a drug that kills both cancer and nonmalignant cells at a concentration of 1 nanomolar.

Cancer researchers do not commonly use human nonmalignant cells to assess the therapeutic potential drug candidates. Possible reasons are that they may consider that the inhibition of human cancer cell proliferation at low concentrations is an adequate parameter to predict therapeutic potential or they prefer using animal models instead. Researchers typically use mice xenotransplanted with human cancer cells to reveal whether their drug candidates inhibit cancer cell growth selectively. If their drugs inhibit tumor growth in these models without killing or significantly affecting the animals, they assume that their drugs also inhibit the proliferation of human cancer cells without significantly affecting that of human nonmalignant cells. Following this approach, researchers have proposed several cardiac glycosides as candidates for clinical testing in cancer patients [17–19, 27, 28].

Several research groups have evaluated the cancer therapeutic potential of cardiac glycosides by using human cancer cells and human nonmalignant cells. For instance, we recently observed that the cytotoxicity of digitoxin, digoxin, and ouabain in breast cancer cells (MCF-7) and melanoma cells (UACC-62) was similar than that in nonmalignant breast cells (MCF-10) and nonmalignant skin cells (VH-10) [24]. Clifford and Kaplan [23] have recently reported that human breast cancer cells were even more resistant to ouabain, digitoxin, and bufalin toxicity than human nonmalignant breast cells. Evidence has also shown, however, that digitoxin, digoxin, and ouabain were approximately 10 times more cytotoxic against human A549 lung cancer cells than against human MRC-5 nonmalignant lung cells (5–8 nM versus 29–75 nM) [24]. *Ex vivo* experiments, using cells from adult patients with B-precursor or T-acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL), as well as peripheral blood mononuclear cells from healthy donors, have also shown that digitoxin (but not ouabain) induced selective cytotoxicity (approximately 7-fold) in cells from patients with T- and B-precursor ALL [15]. In brief, although cardiac glycosides can inhibit

the proliferation of cancer cells at very low concentrations (nM), they usually inhibit the proliferation of human nonmalignant cells at similar concentrations; this strongly suggests that their potential for cancer therapy is low. In contrast, specific cardiac glycosides (e.g., digitoxin) can inhibit the proliferation of particular types of cancer cells (e.g., lung cancer and acute lymphoblastic leukemia) at concentrations that do not significantly affect human nonmalignant cells; these cardiac glycosides may have cancer therapeutic potential.

2.2. The Anticancer Activity of Cardiac Glycosides in Mice Xenografted with Human Cancer Cells Is Probably an Experimental Artifact. Several cardiac glycosides that are equally toxic to human cancer cells and human nonmalignant cells have shown potent anticancer effects in animal models. For instance, Clifford and Kaplan observed that human nonmalignant breast cells were more sensitive than human breast cancer cells (e.g., MDA-MB-231) to the cytotoxic effects of bufalin [23], and it has recently been reported that bufalin reduces tumor growth in mice xenotransplanted with human MDA-MB-231 breast cancer cells [19]. These apparent controversies can be explained by the ability of cardiac glycosides to kill human cells at concentrations much lower (approximately 100–1000 fold) than those required to kill rodent cells [24, 29].

Gupta and colleagues [29] evaluated some time ago the cytotoxicity of numerous cardiac glycosides (i.e., ouabain, digitoxin, digoxin, convallatoxin, SC4453, bufalin, gitaloxin, digoxigenin, actodigin, oleandrin, digitoxigenin, gitoxin, strophanthidin, gitoxigenin, lanatosides A, B, and C, alpha- and beta-acetyl digoxin, and alpha- and beta-methyl digoxin) against a number of independent cell lines established from human, monkey, mouse, Syrian hamster, and Chinese hamster. The authors observed that all cardiac glycosides exhibited greater than 100-fold higher toxicity towards the human and monkey cells in comparison to the rodent cells (mouse, Syrian hamster, and Chinese hamster). They also provided strong evidence that the species-related differences in sensitivity to cardiac glycosides were mediated by the Na^+/K^+-ATPase enzyme. They observed that the Na^+/K^+-ATPase enzyme of rodent cells was inhibited at much higher concentrations of cardiac glycosides than the Na^+/K^+-ATPase of human cells. They also observed a good correlation between these concentrations and those reported for inhibition of the Na^+/K^+-ATPase from isolated heart muscles of the same species [29]. More recent evidence suggests that the expression and cellular location of Na^+/K^+-ATPase alpha subunits in different types of cells may explain why they are more or less susceptible to the cytotoxic activity of cardiac glycosides [30–32].

Several years ago, a PNAS paper reported that digoxin blocked tumor growth in mice xenotransplanted with several types of human cancer cells [17]. The authors observed that digoxin prolonged tumor latency and inhibited tumor xenograft growth in mice when treatment was initiated before the implantation of P493-Myc, P493-Myc-Luc, PC3, and Hep3B cells. Digoxin also arrested tumor growth when treatment was initiated after the establishment of PC3 and

P493-Myc tumor xenografts [17]. Based on the observations of Gupta and colleagues [29] and on the plasma levels of digoxin in cardiac patients, we discussed the fact that the potent anticancer effects induced by digoxin in mice harboring human cancer cells [17] were not relevant to the treatment of human cancer and these anticancer effects were probably due to interspecies differences in sensitivity [25]. In other words, the marked reduction in tumor growth induced by digoxin in mice xenografted with human cancer cells was probably caused by the ability of cardiac glycosides to selectively kill human cells versus rodent cells rather than by their ability to selectively kill cancer cells versus normal cells. Perne et al. [22] later reported experimental data that further supported this idea. Despite these and other reports [24, 26], numerous publications containing this probable experimental artifact continue to appear in the scientific literature.

This section of the paper now reviews reports that have used mice xenotransplanted with human cancer cells to evaluate anticancer effects of cardiac glycosides (Table 1). The results of the following reports should probably be reinterpreted.

Digoxin. Svensson et al. [33] carried out *in vitro* and *in vivo* studies to evaluate the anticancer activity of the cardenolide digoxin. They studied the effect of digoxin on the growth of tumor cell lines and primary endothelial cells from different species. The most sensitive cell lines *in vitro* were the human SH-SY5Y and SK-N-AS neuroblastoma cell lines; the IC_{50} values were 34 and 22 ng/mL, respectively. They also reported that digoxin significantly reduced the growth of human SH-SY5Y neuroblastoma cells xenotransplanted in immunodeficient mice. The authors concluded that digoxin might be a specific neuroblastoma growth inhibitor. The authors also reported that the *in vitro* and *in vivo* anticancer effects of digoxin were dramatically reduced when the murine Neuro-2a neuroblastoma cell line was used instead of the human neuroblastoma cell lines [33]. Zavareh et al. [34] reported data suggesting that cardiac glycosides were inhibitors of N-glycan biosynthesis. Since aberrant N-linked glycans are known to contribute to cancer progression and metastasis, the authors studied whether digoxin could inhibit cellular migration and invasion. They used two mouse models of metastatic cancer in which human PPC-1 prostate cancer cells were injected into immunodeficient mice. They found that digoxin reduced distant tumor formation in both models and concluded that this cardiac glycoside could be a lead for the development of antimetastasis therapies. As discussed before, Zhang et al. [17] found that digoxin inhibited hypoxia-inducible factor 1 (HIF-1), a transcription factor highly involved in cancer development, and suggested that this effect might be observed in patients taking this drug. They also reported that digoxin blocked tumor growth in mice xenotransplanted with several types of human cancer cells. These data suggested that digoxin had anticancer potential [17]. Wong et al. [35] reported data suggesting that digoxin was a potential antimetastasis compound. They investigated whether digoxin could reduce metastases in human MDA-MB-435 tumor-bearing mice. Digoxin blocked metastatic

TABLE 1: The antitumor activity of cardiac glycosides in mice xenografted with human cancer cells is probably caused by their ability to selectively kill human cells versus rodent cells rather than by their ability to selectively kill human cancer cells versus human nonmalignant cells.

Cardiac glycoside	Antitumor activity in mice xenografted with human cancer cells	Selective cytotoxicity against human cells versus rodent cells	Selective cytotoxicity against human cancer cells versus human nonmalignant cells
Arenobufagin	Liver HepG2/ADM [28]	N.D.	N.D.
Bufalin	Breast MDA-MB-231 [19], osteosarcoma U2OS/MTX300 [50], and pancreatic Mia Paca-2 [49]	>1000-fold [29]	NO: breast cancer versus breast nonmalignant [23]; 10-fold: ovarian cancer versus endometrial nonmalignant [53]
Bufotalin	Liver R-HepG2 [51]	>100-fold [54]	N.D.
Digitoxin	N.D.	>1000-fold [29]; >700-fold [24]	10-fold: lung cancer versus lung nonmalignant [24]; 3-fold: lung cancer versus lung nonmalignant [55]; 7-fold: ALL versus PBMCs nonmalignant [15]; 4-fold: AML versus PBMCs nonmalignant [15]; 2-fold: CLL versus PBMCs nonmalignant [15]; 2-fold: breast cancer versus breast nonmalignant [24]; NO: breast cancer versus breast nonmalignant [23]; NO: skin cancer versus skin nonmalignant [24]
Digoxin	Brain SH-SY5Y [33], brain SK-N-AS [33], breast MDA-MB-231 [36, 37], breast MDA-MB-435 [35], liver Hep3B [17], prostate PC3 [17], prostate PPC-1 [34], and transformed human B-lymphocytes P493-Myc [17]	>1000-fold [24, 29]	NO: breast cancer versus breast nonmalignant [24]; 8-fold: lung cancer versus lung nonmalignant [24]; NO: skin cancer versus skin nonmalignant [24]; 8-fold: brain cancer versus umbilical vein endothelial nonmalignant [33]; NO: breast cancer versus umbilical vein endothelial nonmalignant [33]; NO: colorectal cancer versus umbilical vein endothelial nonmalignant [33]
Lanatoside C	Brain U87 [48]	>100-fold [29]	N.D.
Ouabain	Brain SH-SY5Y [27], ocular Y79LUC [39], pancreatic BON1 [40], promyelocytic leukemia HL-60 [18], and prostate PPC-1 [40]	>1000-fold [24, 29, 44]	NO: breast cancer versus breast nonmalignant [23, 24]; 5-fold: lung cancer versus lung nonmalignant [24]; NO: skin cancer versus skin nonmalignant [24]; 2-fold: ALL versus PBMCs nonmalignant [15]; 2-fold: AML versus PBMCs nonmalignant [15]; NO: CLL versus PBMCs nonmalignant [15]
Periplocin	Liver Huh-7 [47] and lung A549 [46]	>1000-fold [47]*; NO: [46]	>1000-fold: liver cancer versus PBMCs nonmalignant [47]*
UNBS1450	Brain U373-MG [44], lung A549 [41, 42], lung NCI-H727 [41, 42], prostate PC-3 [43], and skin VM-48 [45]	>100-fold [44]	10-fold: brain cancer versus lung and skin nonmalignant [44]; 100-fold: prostate cancer versus lung and skin nonmalignant [43]

N.D.: not determined; NO: no selective cytotoxicity; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; PBMCs: peripheral blood mononuclear cells; *not specified if the PBMCs were human cells or rodent cells (we contacted the authors without success).

niche formation and breast cancer metastasis in the lungs, and the authors discussed the fact that this effect was probably due to inhibition of HIF-1. The most relevant conclusion of this work was that digoxin might be useful to treat patients with HIF-1-overexpressing breast cancers [35]. Zhang et al. [36] observed that digoxin reduced tumor growth and inhibited the metastasis of human MDA-MB-231 breast cancer cells to the lungs in mice xenografted with these cells, without causing any sign of toxicity in the animals. They concluded that clinical trials were warranted to investigate whether the concentrations of digoxin achievable in patients are sufficient to inhibit tumor growth and metastases [36]. Schito et al. [37] reported that HIF-1 promoted lymphatic metastases of breast cancer and the use of the HIF-1 inhibitor digoxin strongly

decreased tumor growth and blocked lymphangiogenesis and lymphatic metastasis in mice bearing human breast cancer cells. The authors suggested that digoxin might be useful to treat patients with high risk of lymphatic metastases [37]. Gayed et al. [38] observed that specific concentrations of digoxin inhibited blood vessel formation but not tumor growth in mice injected with the human C4-2 prostate cancer cell line.

Ouabain. Several cardiac glycosides were identified by Antczak et al. [39] as potent antiretinoblastoma agents *in vitro.* One of them, the cardenolide ouabain, induced a drastic tumor regression in immunodeficient mice injected with human Y79LUC retinoblastoma cells, without inducing any

significant toxicity on the host. In light of the results of their study, the authors proposed that digoxin, which is widely used in patients with cardiac disease, could be repositioned for the treatment of retinoblastoma [39]. Simpson et al. [40] identified the cardiac glycosides ouabain, peruvoside, digoxin, digitoxin, and strophanthidin as anoikis sensitizers. Because resistance to anoikis permits cancer cells to survive in the circulation and improves their metastatic potential, the authors evaluated in mouse models of metastasis whether ouabain could block distant tumor formation. They observed that ouabain reduced the number of tumors in human PPC-1 prostate cancer cells bearing mice. They also reported that systemic administration of ouabain decreased the survival and growth of human PPC-1 prostate cancer cells and human BON1 pancreatic cancer cells xenografted into nude mice [40]. Hiyoshi et al. [27] reported that ouabain induced quiescence in neuroblastoma cells *in vitro* and a marked reduction in tumor growth when human neuroblastoma cells were xenografted into immune-deficient mice. Based on these findings, the authors concluded that ouabain could be used in chemotherapies to suppress tumor growth and/or arrest cells to increase the therapeutic index in combination therapies. Tailler et al. [18] identified the cardiac glycoside ouabain as a potential antileukemic compound. They observed that ouabain was highly efficient in inhibiting the growth of human acute myeloid leukemia cells xenotransplanted in immunodeficient mice, without exerting significant toxicity on the host. The authors concluded that ouabain was a promising antileukemic agent whose activity should be evaluated in prospective clinical studies [18].

UNBS1450. Mijatovic et al. [41] investigated the *in vitro* and *in vivo* anticancer activity of UNBS1450, a semisynthetic derivative of the natural cardenolide UNBS1244 (isolated from the African plant *Calotropis procera*). They observed that UNBS1450 was able to inhibit cell growth of four different non-small cell lung cancer cells (A549, NCI-H727, A427, and CAL-12T) at nanomolar concentrations. This cardenolide also significantly decreased tumor growth in nude mice xenografted with human NCI-H727 cancer cells and increased the survival rates in mice xenografted with human A549 cancer cells. The authors observed in another study [42] that the cytotoxic potency of UNBS1450 in A549 lung cancer cells was similar than that of the anticancer drugs paclitaxel and SN38 (the active metabolite of irinotecan) and much higher than that of cisplatin, carboplatin, and oxaliplatin. UNBS1450 also decreased tumor growth in mice xenotransplanted with A549 lung cancer cells and human NCI-H727 lung cancer cells [42]. Another study revealed that UNBS1450 inhibited the proliferation of human prostate cancer cells (LNCaP, PC-3, and DU145) and increased the survival of mice transplanted with human PC-3 prostate cancer cells [43]. Lefranc et al. [44] reported that UNBS1450 was more cytotoxic on human glioblastoma cells (U373-MG and T98G) than on human normal fibroblasts (WI-38 and WSI) at nanomolar concentrations. This compound also inhibited the proliferation of rat C6 glioblastoma cells at micromolar concentrations. UNBS1450 increased the survival of mice grafted with human U373-MG glioblastomas cells, without

observable toxic effects on the animals. Mathieu et al. [45] reported that UNBS1450 blocked cell proliferation in several human melanoma cell lines *in vitro* (IC_{50} values between 5 and 45 nM) and improved the survival of immunodeficient mice grafted with human VM-48 melanoma brain metastasis cells.

Periplocin. Lu et al. [46] reported that the natural cardenolide periplocin induced similar cytotoxicity against a panel of human lung cancer cell lines than against a rodent lung cancer cell line (LL/2). They also observed antitumor activity in mice transplanted with both the human A549 lung cancer cell line and the murine LL/2 Lewis lung cancer cell line. Cheng et al. [47] have recently reported that periplocin displayed a potent cancer cell growth inhibitory activity *in vitro* and *in vivo*. Periplocin inhibited cell growth of human HA22T/VGH hepatocellular carcinoma with an IC_{50} of 27 nM and was less toxic to normal peripheral blood mononucleated cells. The authors also observed that periplocin showed an inhibition of tumor growth when human Huh-7 hepatoma cells were injected into immunodeficient mice, without observing clear side effects on the host.

Lanatoside C. Badr et al. [48] identified the cardenolide lanatoside C as a sensitizer of glioblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cell death. They observed that lanatoside C, alone or in combination with TRAIL, reduced tumor growth in nude mice harboring human U87 glioblastoma cells.

Bufalin. Chen et al. [49] identified the bufadienolide bufalin as a potential agent for the treatment of pancreatic cancer in combination with the standard anticancer drug gemcitabine. They found that bufalin inhibited the growth on three pancreatic cancer cell lines (Bxpc-3, Mia PaCa-2, and Panc-1) and it synergistically increased gemcitabine-induced cancer cell growth inhibition and apoptosis. The combination of bufalin with gemcitabine was also found to significantly reduce tumor growth in mice bearing human Mia Paca-2 pancreatic cancer cells. Xie et al. [50] investigated the *in vitro* and *in vivo* antiosteosarcoma activity of bufalin. They observed that bufalin strongly inhibited the cell growth of different human osteosarcoma cell lines, including the methotrexate-resistant U2OS/MTX300 cell line. They also found that the treatment with bufalin induced significant tumor growth inhibition in mice xenotransplanted with the human U2OS/MTX300 osteosarcoma cell line, without decreasing the body weight of the animals. The authors concluded that bufalin might be an alternative chemotherapeutic agent to treat osteosarcoma, particularly in methotrexate-resistant cancers [50]. Wang et al. [19] have recently reported that bufalin was a potent inhibitor of the steroid receptor coactivators SRC-3 and SRC-1. Because these coactivators have been implicated in cancer progression, the authors investigated whether bufalin could also block cancer cell growth in cell culture and animal models. They observed that bufalin inhibited the growth of human MCF-7 breast cancer cells and human A549 lung cancer cells at nanomolar concentrations (3–5 nM); these concentrations also resulted in inhibition of the steroid

receptor coactivator SRC-3 and were below those reported to be tolerated by humans (8.75 nM). They also found that bufalin inhibited tumor growth in mice xenotransplanted with human MDA-MB-231 breast cancer cells.

Arenobufagin. Zhang et al. [28] recently observed that the bufadienolide arenobufagin induced a potent cell growth inhibitory activity on cancer cells both *in vitro* and *in vivo*. They tested its anticancer activity on several human cancer cell lines (hepatoma, breast adenocarcinoma, cervix adenocarcinoma, lung cancer, colon cancer, leukemia, and gastric adenocarcinoma). Arenobufagin inhibited the growth of all cancer cell lines at nanomolar concentrations, including multidrug-resistant cancer cell lines. Arenobufagin also inhibited the growth of human HepG2/ADM hepatocellular carcinoma cells xenografted into immunodeficient mice, without causing side effects on the hosts. The authors concluded that their results may provide a rationale for future clinical application using arenobufagin as a chemotherapeutic agent for the treatment of patients with hepatocarcinoma [28].

Bufotalin. Zhang et al. [51] observed that four bufadienolides from Venenum Bufonis, a traditional Chinese medicine, displayed inhibitory effects on the growth of human HepG2 hepatocarcinoma cells and human R-HepG2 multidrug hepatocarcinoma cells. One of them, bufotalin, was also able to significantly inhibit the growth of human R-HepG2 cells xenografted into immunodeficient mice, without observing any life-threatening toxicity in the animals. The authors discussed the fact that their study supports the possible development of bufotalin as a potential agent in the treatment of multidrug resistant hepatocellular carcinoma [51].

Data from preclinical studies reporting antitumor effects in rodent xenografts of plant extracts containing cardiac glycosides may also need reinterpretation. For instance, Han et al. [52] reported that an extract from the plant *Streptocaulon juventas* induced a strong inhibitory effect on the proliferation of human lung A549 adenocarcinoma cells. A bioassay-guided fractionation revealed that the most cytotoxic fraction *in vitro* also induced antitumor effects in athymic nude mice transplanted with human A549 cancer cells without exerting side effects on the mice. Following HPLC and NMR spectrometry, the main components of this active fraction were identified as the cardiac glycosides digitoxigenin, periplogenin, and periplogenin glucoside [52].

3. Possible Approaches to Reveal the Cancer Therapeutic Potential of Cardiac Glycosides in Preclinical Studies

As discussed before, the key feature of an efficient anticancer drug candidate is its ability to kill (or to inhibit the proliferation of) human cancer cells at concentrations that do not significantly affect human nonmalignant cells. Ideally, the drug candidate should kill all the cancer cells of the patients without significantly affecting their normal cells. Because this is difficult to achieve, one can settle for less. A drug that

improves the ability of our current anticancer drugs to kill cancer cells at concentrations that do not significantly affect nonmalignant cells could be therapeutically useful.

In vitro, one can evaluate whether the drug candidate improves the selective cytotoxicity of the standard anticancer drugs towards cancer cells by using the following approach. The first step in this approach is the selection of a panel of human cancer cell lines and human nonmalignant cell lines (or primary cells). Because the cytotoxicity of some drugs depends on the nature of the tissue from which they originate, one should select nonmalignant cell lines of the same tissue origin than that of the selected cancer cell lines. A small number of cancer cell lines may be sufficient to reveal the therapeutic potential of a drug for a particular type of cancer. However, the selection of a low number of nonmalignant cell lines reduces the chances of finding toxicity on a specific tissue that would limit the possible therapeutic use of the drug. The next step is to treat the selected cell lines with several concentrations of the drug candidate and of the anticancer drugs most commonly used in the treatment of the selected cancers. Then, cell viability or cell death is estimated with a cytotoxicity test (e.g., SRB assay and MTT assay), and cytotoxic parameters (e.g., IC_{50} values) are calculated. The following step is to calculate one or several selectivity indexes for the drug candidate and for the anticancer drug. These selectivity indexes can be calculated by dividing the IC_{50} values in the nonmalignant cell lines by the IC_{50} values in the cancer cell lines. For instance, if the mean IC_{50} value of a drug in a variety of nonmalignant cells originated from several tissues is 100 μM and the mean IC_{50} value of the drug in several cell lines derived from a specific cancer is 20 μM, the selectivity index for this particular cancer would be 5. Finally, the following question must be answered: is the selectivity index of the drug candidate higher (or at least similar) than that of the standard anticancer drug? If the answer is no, the drug candidate does not have therapeutic potential and should not be tested in animal models. If the answer is yes, the drug candidate has chemotherapeutic potential, which should be confirmed by using *in vivo* experiments.

Rodent xenograft models are the most common animal models used by researchers to evaluate the therapeutic potential of anticancer drug candidates *in vivo*. However, as discussed before, these models may be inadequate to evaluate the therapeutic potential of cardiac glycosides. To the authors' knowledge, all cardiac glycosides tested in human cells and rodent nonmalignant cells have shown greater than 100-fold higher toxicity towards the human cells in comparison to the rodent cells. This does not mean, however, that all compounds having the basic chemical structure of cardiac glycosides (a steroid skeleton with an unsaturated lactone ring) will be more toxic against human cells than against rodent cells. One can test the suitability of using tumor xenografts to evaluate the *in vivo* therapeutic potential of a particular cardiac glycoside by testing if the cytotoxicity of the cardiac glycoside against a panel of human nonmalignant cells is similar than that against a panel of rodent nonmalignant cells. If the compound behaves similarly in both types of cell lines, its *in vivo* anticancer activity can be evaluated in mice xenografted with human cancer cells. If the rodent

cell lines are more resistant than the human cell lines to the cytotoxicity of the cardiac glycoside, these models are probably inadequate to evaluate its anticancer effects *in vivo*. Animal models using mice transplanted with mouse cancer cells may also be inadequate when human cells are more sensitive than rodent cells to the cytotoxicity of the cardiac glycoside. The reason is that the therapeutic target responsible for the death of the human cells may be different than that responsible for the death of the rodent cells and, therefore, results obtained in mice may not be extrapolated to humans.

The anticancer activity of cardiac glycosides displaying a similar cytotoxic profile in nonmalignant cells originated from both human and mouse tissues can be assessed by using tumor xenografts or other rodent models. It is important to remember that most cancer patients requiring therapy with anticancer drugs have metastatic disease and patient survival is the parameter used by oncologists as an endpoint of clinical interventions designed to assess drug efficacy in patients with cancer (other parameters used by many preclinical researchers as an endpoint for their experiments, such as measurements of tumor volumes, do not necessarily predict survival). It is essential, therefore, to select animal models of metastasis and to assess animal survival as an endpoint for the experiments. In our opinion, animals with metastasis should be treated with equitoxic concentrations of the cardiac glycoside and of the standard anticancer drug used in the type of cancer under study. Then, one should evaluate whether the cardiac glycoside improves the survival rates induced by the standard anticancer drug. If the cardiac glycoside improves (or at least matches) the selectivity index (*in vitro*) and the survival rates (*in vivo*) of the standard anticancer drugs, it should be considered for clinical trials testing.

Rodent models are inappropriate for testing the anticancer activity of cardiac glycosides that kill human nonmalignant cells at lower concentrations than those required to kill rodent nonmalignant cells. These models, however, could provide information on the pharmacokinetics of the cardiac glycoside. These models may also help detect possible toxicity not detected by using a panel of human nonmalignant cell lines; they could help detect toxicity not mediated by inhibition of the Na^+/K^+-ATPase (which seems to be the main determinant for the species differences in sensitivity to cardiac glycosides). In our opinion, a cardiac glycoside that kills human nonmalignant cells at lower concentrations than rodent nonmalignant cells should pass the following tests before being considered for evaluation in clinical trials. First, it should match or improve the selectivity indexes of the standard anticancer drugs when they are evaluated in a panel of human cancer cell lines derived from a particular type of cancer versus a variety of human nonmalignant cell lines and primary cells derived from a variety of human tissues. Second, *in vivo* experiments (e.g., rodent models) should exclude pharmacokinetic and toxicological limitations that may compromise the *in vivo* anticancer activity of the cardiac glycoside. Finally, if the cardiac glycoside is in clinical use for the management of other diseases or if clinical data already exist on its plasma and tissue concentrations, one should also consider whether the anticancer effects observed in preclinical studies may occur at concentrations within or below the concentration range tolerated by humans.

4. Conclusion

Preclinical research has shown that cardiac glycosides can both inhibit cancer cell proliferation at very low concentrations and induce potent anticancer effects in mice transplanted with human cancer cells. Based on these observations, cardiac glycosides have been considered as potential anticancer drug candidates that should be evaluated in clinical studies. This paper has reviewed evidence indicating that cardiac glycosides may not selectively inhibit the proliferation of human cancer cells and these compounds have the ability of killing human cells at concentrations much lower than those required to kill rodent cells (approximately 100–1000 fold). This strongly suggests that the potent anticancer effects induced by cardiac glycosides in mice transplanted with human cancer cells may be an experimental artifact caused by their ability to selectively kill human cells versus rodent cells rather than by their ability to kill human cancer cells versus human nonmalignant cells. It has also been discussed that inhibition of cancer cell proliferation at low concentrations is not an adequate parameter to predict the therapeutic potential of a drug candidate. The key feature of an efficient anticancer drug is its ability to kill (or inhibit the proliferation of) human cancer cells at concentrations that do not significantly affect human nonmalignant cells. Based on this principle, an approach to evaluate the therapeutic potential of cardiac glycosides in preclinical *in vitro* studies has been proposed. This approach is also based on the idea that only drug candidates that match or improve the ability of the approved anticancer drugs to kill human cancer cells at concentrations that do not significantly affect human nonmalignant cells have a chance to be ultimately used in cancer therapy. A test for revealing the suitability of using rodent models for the evaluation of the anticancer activities of cardiac glycosides *in vivo* has also been proposed. If the cardiac glycoside passes this test, several recommendations have been made for the evaluation of its cancer therapeutic potential in these models. If the cardiac glycoside fails to pass this test, an alternative approach for revealing its possible therapeutic potential has been discussed. It is the hope of the authors that this paper may help researchers evaluate the therapeutic potential of cardiac glycosides in preclinical studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Anti-Proliferative Effect and Phytochemical Analysis of *Cymbopogon citratus* Extract

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The antiproliferative and antioxidant potential of *Cymbopogon citratus* (Lemon grass) extracts were investigated. The extracts were isolated by solvent maceration method and thereafter subjected to antiproliferative activity test on five different cancer cells: human colon carcinoma (HCT-116), breast carcinoma (MCF-7 and MDA-MB 231), ovarian carcinoma (SKOV-3 and COAV), and a normal liver cell line (WRL 68). The cell viability was determined using MTT assay. The DPPH radical scavenging assay revealed a concentration dependent trend. A maximum percentage inhibition of 45% and an IC50 of 278 μg/mL were observed when aqueous extract was evaluated. In contrast, 48.3% and IC50 of 258.9 μg/mL were observed when 50% ethanolic extract was evaluated. Both extracts at concentration of 50 to 800 μg/mL showed appreciative metal chelating activity with IC50 value of 172.2 ± 31 μg/mL to 456.5 ± 30 μg/mL. Depending on extraction solvent content, extract obtained from 50% ethanolic solvent proved to be more potent on breast cancer MCF-7 cell line (IC50 = 68 μg/mL). On the other hand, 90% ethanolic extract showed a moderate potency on the ovarian cancer (COAV) and MCF-7 cells having an IC50 of 104.6 μg/mL each. These results suggested antiproliferative efficacy of *C. citratus* ethanolic extract against human cancer cell lines.

1. Introduction

Cancer is among the leading causes of mortality among human population of all ages. In fact, it is responsible for 7.6 million deaths in 2008 [1]. It has been projected that the cancer mortality rate will extend to about 30.1 million by 2030 [1]. Current therapeutic interventions mostly involve malign surgery, radiotherapy, and chemotherapy, and at times the therapeutic efficiency is very low. This incurs the current increase in research on mild alternative cancer therapy. Bioactive phytochemicals exhibiting the ability to inhibit cancer cytogenesis by suppressing the tumor initiation, promotion, and progression are being considered as potential biocompatible anticancer agents. In this regard, the antiproliferative activity of several phytochemical extracts was reported [2–4].

Among the medicinal plants used, *Cymbopogon citratus* (lemon grass) is prominent and commonly explored in folk alternative medicine for the treatment of diverse ailments. Although, several bioactive compounds were reported to be isolated from *C. citratus*; among them is the acyclic monoterpene aldehydes described as citral that comprises of isomeric geranial and neral. Citral was reported to be the major bioactive component that incurs most of the plant's bio efficacy [5]. Popularly, the aqueous infusion of this plant is called "abafado" by Portuguese, and was said to have bioactive efficacy against nervous and gastrointestinal disturbances when administered orally [6]. In addition, it is also reported to be a potent free radical scavenger of reactive oxygen species [7]. Furthermore, citral was shown to possess activities like anti mutagenicity [8], antiproliferative effect against *Trypanosoma cruzi* [9], and antinociceptive and[10], antiparasitic effects against leishmaniasis [11, 12].

The efficient potency of *C. citratus* on free radical scavenging and antioxidation ability led us to evaluate the effect of its

aqueous ethanolic extracts on proliferation and cell growth of several human cancer cell lines such as those of breast cancer [MDA-MB 231 and MCF-7], ovarian cancer [SKOV-3 and COAV], and colon cancer [HCT-116]. In addition, the phytochemical content analysis of the extracts is also reported.

2. Materials and Methods

2.1. Plant Materials, Phytochemicals Extraction and GCMS Analysis. C. citratus leaves were identified and obtained by MABLEAJAZ chair for Scientific Research in Prophetic Medicine, Faculty of Medicine, Taibah University, Saudi Arabia (Specimen Voucher Number: TU/JX03/SP2765). The leaves were cleaned, dried under shade for 7 days then grounded, weighed, homogenized in water (Ew), 50% ethanol (E50), and 90% ethanol (E90) at a ratio of 1 : 10 of plant powder to solvent, and left to macerate for 5 days at ambient temperature ($25 \pm 1^\circ$C) with occasional shaking and stirring. The mixture was then filtered and the resulting liquid was concentrated under reduced pressure at 40°C in an EYELA rotary evaporator yielding a dark brown to green extracts. The concentrated extracts were then kept in *vacuo* at 45°C for 3 days to evaporate the residual solvents resulting in the respective dried crude extract of either Ew, E50, or E90, respectively. Extracts were then dissolved in 0.5% DMSO before being used in the cell cultures at concentrations of 3, 6, 12, 25, 50, and 200μg/mL.

Another portion of the extract (1 mg) was dissolved in 1 mL methylene chloride in a screw capped test tube. To this mixture, 1 mL of acidified methanol (methanol containing 15% H_2SO_4) was added, then tightly capped and incubated at 100°C for 2 hours. At the end of heating, the reaction mixture was allowed to cool down to room temperature, followed by addition of 1 mL of deionized water and vortex to induce phase separation, and to stand for a minute. Using Pasteur pipette, about 1 mL of the organic phase was carefully withdrawn into GC vials for GCMS phytochemical content analysis.

The GCMS analysis was conducted on Agilent 7000B triple quadrupole GCMSMS machine carrying triple axis detector and Agilent HP-5 MS separation column that has been impregnated with 5% phenyl methyl silox (30 m long $\times$ 0.25 mm internal diameter $\times$ 0.25 μm film thickness). A sample (1 μL) was automatically injected into the machine at a split ratio of 1 : 20. The injection temperature was set at 280°C. The oven ramping temperature profile was as follows: 40°C for 2 min then increased to 140°C at 3°C min^{-1}, held at 140°C for 2 min then increased to 250°C at 10°C min^{-1}, and then held at 250°C for 5 min. Helium was used as the carrier gas at a flow rate of 14 mL min^{-1}. Mass spectra were acquired at 1250 scan speed using electron impact energy of 70 eV at 230°C ion-source temperature and 250°C interface temperature.Corresponding spectrum for each chromatogram peak was compared with deposited spectra in NIST database for compound identification.

2.2. DPPH Assay. Radical scavenging activities of the extracts were determined by a spectrophotometric assay using alcoholic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as

reported somewhere else [7]. Briefly, different concentrations of extracts-DMSO solutions (15.6–250 μg/mL) are added to a solution of DPPH (200 mM) in absolute ethanol and incubated in dark for 30 min at room temperature ($25 \pm 1^\circ$C). The change in chrometric status of DPPH from purple to yellow upon reduction was measured spectrophotometrically at 517 nm for each sample after incubation against a control solution of DPPH and DMSO alone. Ascorbic acid was used as standard control. The free radical scavenging activities of the extracts were calculated as a percentage of radical reduction in (1). All experiments were performed in triplicates, and the IC_{50} values were determined from a calibration curve for each extract:

Radical scavenging activity %

$$= \left(1 - \left(\frac{\text{Abs}_{517} \text{ control}}{\text{Abs}_{517} \text{ sample or standared control}}\right)\right) \times 100.$$

(1)

2.3. Metal Chelating Assay. The metal chelating abilities of the extracts were determined according to the method of Wang et al. [35] with minor modifications. The stock solutions of the extracts (100 μL of 5 mg/mL) were mixed with 135 μL of distilled water and 5 μL of 2 mM $FeCl_2$ in a microplate. The reaction was initiated by the addition of 10 μL of 5 mM ferrozine. Thereafter solutions were mixed and allowed to stand for 10 min at room temperature ($25 \pm 1^\circ$C). After incubation, the absorbance was measured at 562 nm with a microplate reader (GF-M3000, UNICOM-OPTICS). Distilled water (100 μL) instead of sample solution was used as a control. Distilled water (10 μL) in place of ferrozine solution was used as a blank, which is used for error correction because of unequal colour of the sample solutions. EDTA-Na_2 was used as reference standard. All measurements were performed in triplicate, and the ferrous ion-chelating ability was calculated according to (2):

Metal chelating ability%

$$= \left(\frac{[(\text{Abs}_{562} \text{ control} - (\text{Abs}_{562} \text{ sample} - \text{Abs}_{562} \text{ blank}))]}{\text{Abs}_{562} \text{ control}}\right)$$

$$\times 100.$$

(2)

2.4. FRAP Assay. The ferric reducing antioxidant powers (FRAP) of the extracts were assayed according to the previously described method [36] with slight modification. In brief, the FRAP reagent was prepared by adding 300 mM acetate buffer (3.1 mg sodium acetate/mL, pH 3.6) to 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution and 20 mM $FeCl_3 \cdot H_2O$ (5.4 mg/mL). A portion of TPTZ reagent (290 μL) was added to each well of 96-well titre plate in triplicate, and aliquot sample (10 μL) of 1 mg/mL of prepared C. citratus extracts was used to read the absorbance at 593 nm in ELISA reader (Shimadzu, Japan) after every 4 min for 2 h.

2.5. Nitric Oxide Assay.

Nitric oxide (NO) scavenging activity of the extracts was determined using Griess reaction. According to this reaction, when sodium nitro-prusside is used in aqueous solution at physiological pH, it generated NO$^\bullet$ radicals that react with oxygen to produce nitrite ions. The produced ions are then quantified via spectrophotometric analysis as reported by Nagmoti et. al. [37]. In brief, $1\,\mu$L of 10 mM sodium nitro-prusside was mixed with 1 mL of test extracts or curcumin (as a reference control) at various concentrations (400–1600 μg/mL) dissolved in methanol and a control without test extracts, but only with an equivalent amount of methanol. The mixture was then incubated for 30 min at room temperature (25°C). After 30 min of incubation, 1 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethylenediamine dihydrochloride) was added to 1 mL of the incubated solution and vortex. The absorbance of the pink coloration during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm. All the tests were performed in triplicate. Percentage inhibition was calculated using (3):

$$\text{NO scavenging \%} = \left(\frac{[\text{Abs}_{546} \text{ control } - \text{ Abs}_{546} \text{ sample}]}{\text{Abs}_{546} \text{ control}} \right) \times 100. \quad (3)$$

2.6. Evaluation of C. citratus Cytotoxicity by MTT Assay.

Three different extracts were prepared for studying the antiproliferative effect of *C. citratus* extracts against the human cancer cell lines in reference to normal liver cell line WRL 68, these were Ew, E50, and E90 representing aqueous and 50% and 90% ethanol, respectively. Both the normal cell line (WRL 68) and the human cancer cell lines were used (breast cancer [MDA-MB 231 and MCF-7], ovarian cancer [SKOV-3 and COAV], and colon cancer cell lines [HCT-116]) were obtained from American Type Culture Collection and supplied by Department of Molecular Medicine, University of Malaya. Extract's antiproliferative effect by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was evaluated according to modified protocol [38]. Briefly, cell lines were cultured in RPMI-1640 growth medium and supplemented with *C. citratus* extracts at different concentrations (3–200 μg/ml), 10% (v/v) sterile fetal bovine serum (FBS, PAA Lab, Austria), 100 mg/mL streptomycin, 100 U/mL penicillin (PAA Lab, Austria) and 50 mg/mL fungizone (Sigma Aldrich). Cultures were incubated in 5% CO_2 incubator at 37°C in a humidified atmosphere. The cells were harvested by detaching the cells from the culture flask using trypsin after the flask get confluent enough with the cells. The harvested cells were then aseptically introduced into 50 mL sterile falcon tube and washed with physiological buffer (pH 7.2) under spinning at 1200 rpm for 10 minutes. The supernatant was discarded, and the cells pellets were mixed with 1 mL of sterile media to form a cell suspension. The viable cells count was determined using trypan blue assay. About 10 μL of the cell suspension was mixed with 10 μL of trypan blue, and aliquot sample (10 μL) of this mixture was used to count the cells in Neo Bar chambers.

The Harvested cells were then seeded into 96-well culture plates at 5000 cells/well and allowed to adhere overnight. Both the water and alcoholic extracts of the *C. citratus* were dissolved in 5% dimethyl sulphoxide (DMSO) with final concentration of 0.5% and diluted to different concentration spanning from 3–200 μg/mL. Blank 5% DMSO was used as a control. Cells were incubated with the samples (three wells on a plate for each concentration) from 48 to 72 h. Thereafter, 10 μL of MTT (5 mg/mL) (Sigma) was added to each well and the plates were incubated at 37°C for 4 h. The media was then gently aspirated, and about 200 mL of DMSO was added to dissolve the formazan crystals. The amount of formazan product was measured spectrophotometrically at 570 nm using a microplate reader (GF-M3000). The percentage cell viability was calculated according to (4):

$$\text{Cell viability \%} = \left(\frac{\text{Abs}_{570} \text{ treated}}{\text{Abs}_{570} \text{ untreated}} \right) \times 100. \quad (4)$$

2.7. Statistical Analysis.

The obtained experimental data were evaluated statistically as mean ± S.E.M. (standard error mean). The statistical differences between the groups were determined based on 95% confidence intervals using one-way ANOVA in SPSS program (SPSS Inc., USA). All obtained data were analyzed using Analysis of Variance (ANOVA). A probability value of $P < 0.05$ was considered as significant between the measurements of the two compared groups.

3. Results and Discussion

3.1. Analyses of Phytochemical Extracts, Radical Scavenging, and Antioxidant Efficacy.

In this research, maceration using aqueous-solvent extraction of lemon grass yielded 5.4 g/100 g on dry weight basis. Based on GCMS qualitative analysis, eighteen (18) phytochemicals compounds from the extracts were identified (Table 1). The analysis revealed that the constituents of lemon grass extracts mostly belong to monoterpene, sesquiterpene, and phenolic acids. This phytochemical composition analysis was found to be in accord with previously reported studies [39, 40]. The antioxidants potential of *C. citratus* leaves extracts on the DPPH radical scavenging were determined by their hydrogen donating ability. Evaluating the DPPH radical scavenging activity of both Ew and E50 extracts against ascorbic acid as a standard control (Figure 1(a)), it is observed that at lower concentrations (100–200 μg/mL) both extracts revealed almost similar radical scavenging activity (Figure 1(a)). However, as the concentration increases, the Ew extract seems to have a logarithmic increase in percentage inhibition over the concentration range, achieving a maximum inhibition of about 45% and an IC$_{50}$ value of about 278 as shown in Figure 1(a). On the other hand, increasing the concentration of E50 extract to 750 μg/mL revealed an increase in percentage inhibition. Beyond this value, the percentage inhibition appeared to approach plateau with the increment in the extract's concentration attaining a maximum percent inhibition of 48.3% with corresponding IC$_{50}$ of 258.90 (Figure 1(a)). This

TABLE 1: Phytochemical composition of aqueous extract of *Cymbopogon citratus*.

Number	Compound	Extract		Reported application	Reference
		Ew %	E50 %		
1	*Hydroquinone*	0.8	ND	Treatment of melasma	[13]
2	*Nerolidol*	24	ND	Anticancer	[14]
3	*β-Elemene*	42	ND	Anticancer	[15]
4	*β-Eudesmol*	12	ND	Anticancer	[16]
5	*Myrtenal*	4	0.6	(i) Inhibits Alzheimer's acetylcholinesterase (ii) Anticancer (iii) Insect repellent	[17] [18] [19]
6	*Piperitone*	2.1	4.5	Antimicrobial	[20] [21]
7	*Nonadiyne*	ND	0.6	Inhibits the release of endogenous nitric oxide	[22]
8	*α-Cubebene*	ND	0.5	Cytotoxic and antimicrobial	[23]
9	*α-Copaene*	ND	0.3	Antidiabetic	[24]
10	*L-calamenene*	ND	0.2	Anticancer	[25]
11	*Elemol*	ND	41	Antimosquitoes	[26]
12	*Humulene*	ND	4	Anti-inflammatory and inhibits the generation of tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL1β)	[27]
13	*Caryophyllene*	ND	0.9	Antiulcerogenic and anti-inflammatory	[28]
14	*Cubenol*	ND	2	Cytotoxic effect	[29]
15	*Cubebol*	ND	4.7	Mosquito larvicidal activity	[30]
16	*Carvone*	0.7	2	Sprout suppression and antifungal activity in potato	[31]
17	*β-Eudesmol*	ND	45	Antimutagenic Antiangiogenic	[32] [33]
18	*Nonanenitrile*	ND	0.9	Antipancreatitis and antiulcerogenic	[34]

*ND: not detected.

could be due to the difference in the extracted terpenes content. When compared to the ascorbic acid, it takes about 1500 μg/mL of the extract to achieve the percentage inhibition of the standard control at a concentration about 20 μg/mL with corresponding IC$_{50}$ of 6.21 μg/mL. These observations were found to be in agreement with similar observations on a higher IC$_{50}$ values for *C. citratus* extracts [41–43].

The antioxidant results presented so far indicated that the phytochemical extracts of *C. citratus* leaves had both proton and electron donating abilities of primary antioxidant efficacy. However, it has been reported that secondary antioxidants serve as effective ligands in chelating metal ions, thereby suppressing the formation of hydroxyl radicals by Fenton's reaction [44]. Interestingly, in this assay, both the Ew and E50 extracts at concentrations of 50 to 800 μg/mL showed appreciative metal chelating activity (Figure 1(b)). Among the extracts studied, the highest activity was observed in Ew with corresponding metal chelating inhibition of 71.2% and an IC$_{50}$ value of 172.2 $\pm$ 31 μg/mL, while the E50 extract showed the chelating inhibition of 40% and a corresponding IC$_{50}$ value of 456.5 $\pm$ 30 μg/mL. In contrast to the standard control (EDTA-Na), the observed results were found to equal the standard's chelating performance of about 40 μg/mL (Figure 1(b)).

The ferric ion reducing capacity (FRAP) showed both the extracts to possess almost similar activity. The assay activity trend revealed an increase in value from 0.21 Fe^{2+}/g in the Ew extract to 0.3 Fe^{2+}/g in the E50 extract, signifying the effectiveness of the E50 extract as compared to the Ew extract. Runnie et al. [45] reported similar observation on the FRAP activity of *C. citratus* leaf extract. Within the animal cells, it is a fact that most inflammatory responses were associated with nitric oxide [46]. In this study, both the Ew and E50 extracts were checked for their inhibitory effect against nitric oxide production (Figure 1(c)). Among the analyzed samples and reference to the standard curcumin (IC$_{50}$ = 10.8 $\pm$ 1.2 μg/mL), the E50 extract showed the highest nitric oxide inhibitory activity of 49.3% (IC$_{50}$ 462.9 $\pm$ 29 μg/mL) as compared to the Ew extract of 40.1% (432.4 $\pm$ 24 μg/mL). In fact, the extracts were found to have a nitric oxide scavenging activity equivalent to about 40 μg/mL of the standard curcumin (Figure 1(c)).

3.2. Antiproliferative Effect of C. citratus Extract on Different Cancer Cell Lines. The effects of extracts concentrations on the viability and growth of tumor cell lines in reference to the normal cell line have been observed (Figures 2(a)–2(f)). Generally, when the extracts treatments in cancer cell lines
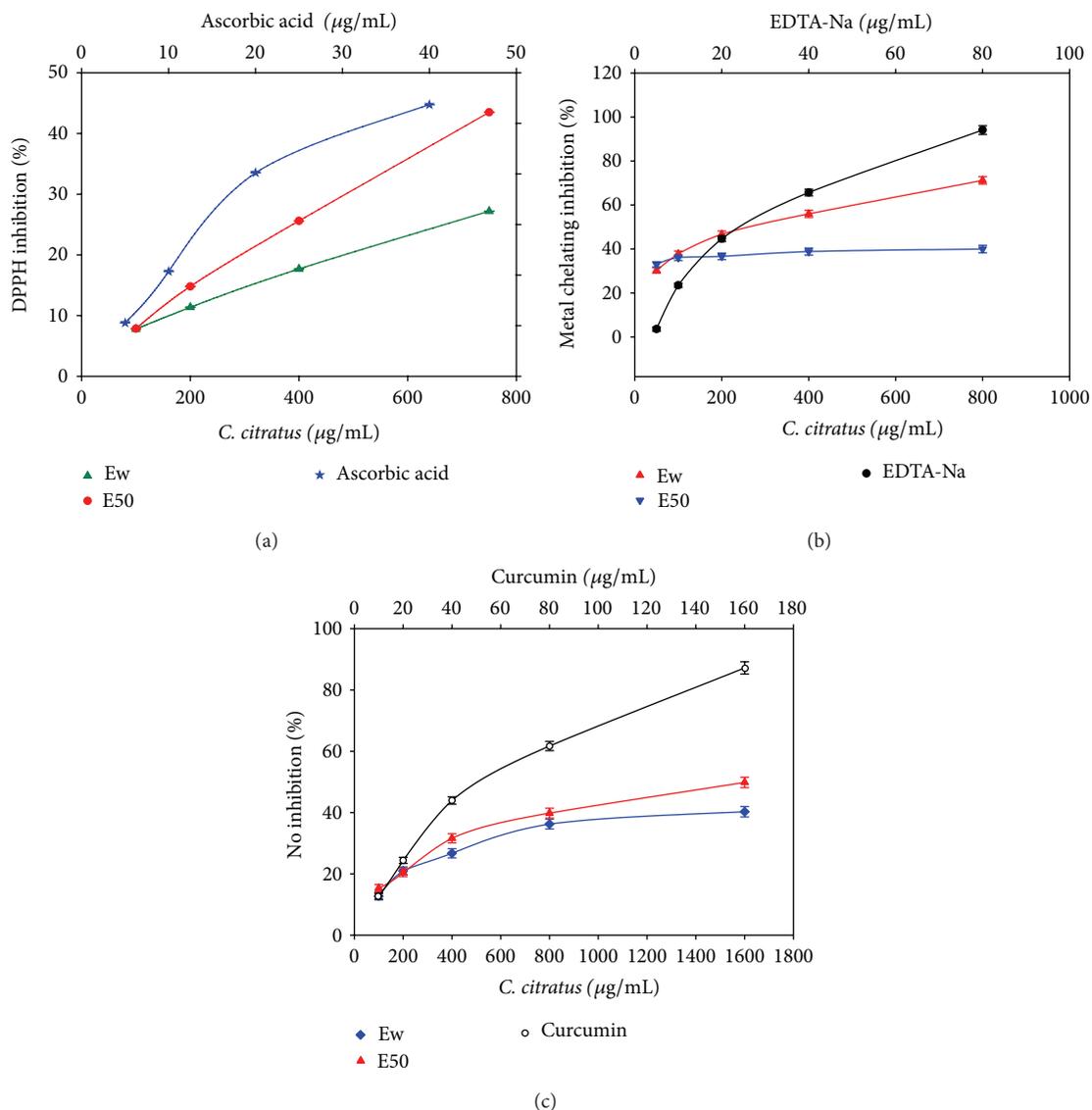
Figure 1: Antioxidant and radical scavenging property of *C. citratus* aqueous (Ew) and 50% ethanolic (E50) extracts. (a) DPPH radical scavenging activity. (b) Metal chelating capacity. (c) NO inhibition activity. All values are expressed as the means ± S.E.M. Statistical difference is significant at the $P < 0.05$ level.

(Figures 2(a)–2(e)) were compared with that of the normal cell line WRL 68 (Figure 2(f)), the antiproliferative effect of the extract could be observed to be exerted more on the cancer cell lines, the extract was observed to cause an inhibition of less than 50% even at higher dosage (200 μg/mL). On the other hand, using the extract even at lower concentrations <50 μg/mL caused marked inhibition in the cell growth. At the lower concentrations (i.e. <50 μg/mL), it is observed that E50 extract has the highest inhibition activity. However, as the treatment concentration increases beyond 50 μg/mL, E90 showed the highest efficacy (Figures 2(a)–2(e)). Among the breast cancer cell lines tested, E90 showed lower inhibition in MDA-MB 231 cell line with corresponding 40% inhibition (Figure 2(a)) and IC$_{50}$ >200 μg/mL as compared to observed inhibition activity on MCF-7 cell line (Figure 2(b)), which showed percent inhibition of 82.9% and IC$_{50}$ of 104.6 μg/mL.

In comparison to E90 extract, E50 was observed to be more potent on MCF-7 cell line (IC$_{50}$ = 68 μg/mL) than on MDA-MB 231 cell line (IC$_{50}$ $\geq$ 200 μg/mL). It has been reported that the antiproliferative activities of phytochemical extracts could be due to their influence on incurring increase expression of kinase and their activities of positive G1/S and G2/M regulators with simultaneous expression of p21 in presence of high level of p27 and p53 [4]. These effects caused a blockade in cell cycle, thus inducing the apoptosis in MCF-7 cells.

Evaluating the extract's antiproliferative effect on SCOV-3 (Figure 2(c)) and COAV (Figure 2(d)) cell lines, increasing the E90 concentration to 100 μg/mL resulted in a logarithmic increase in percentage inhibition with an observed IC$_{50}$ of 200 and 104.6 μg/mL in SCOV-3 and COAV, respectively. Thereafter, increasing the concentration above 100 μg/mL resulted in minimal increase in the percentage inhibition

(a)

(b)

(c)

(d)

(e)

(f)
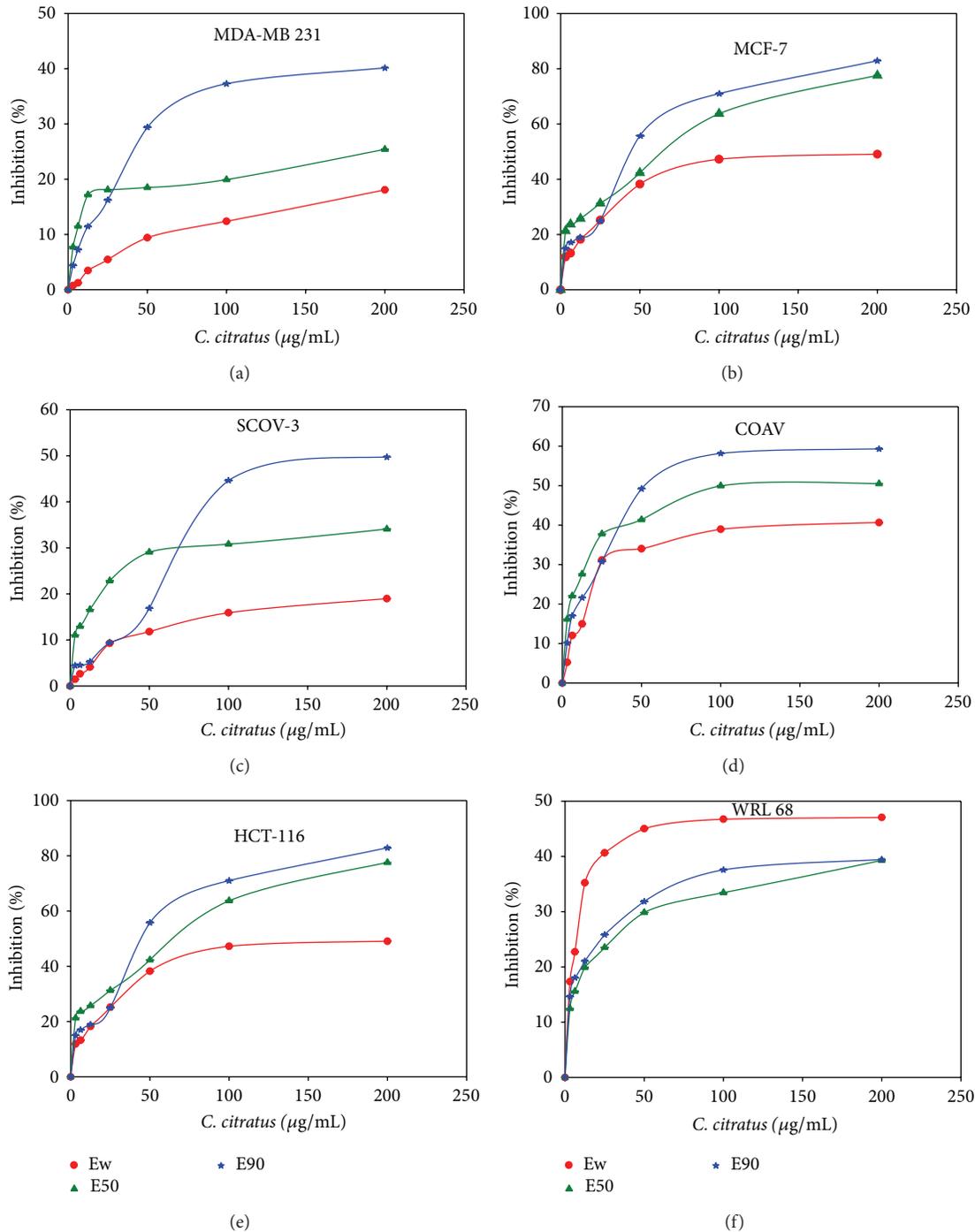
FIGURE 2: Antiproliferative efficacies of *C. citratus* extracts by MTT assay on (a) MDA-MB 231 (b) MCF-7, (c) SCOV-3, (d) COAV, (e) HCT-116, and (f) WRL 68 cell lines. Ew, E50, and E90 represent the aqueous, 50% and 90% ethanolic extracts, respectively. All values are expressed as the means ± S.E.M. Statistical difference is significant at the $P < 0.05$ level.

achieving a maximum inhibition of about 50% and 59% in both SCOV-3 and COAV, respectively. This stabilization in the percentage inhibition at higher concentration could be attributed to the major death of the cell population. Similar observation has been reported by Konrad et al. [47], who observed a reduction in growth inhibition with increasing *Urtica dioica* root extract concentration on human prostate

cancer (LNCaP) cell line. Analyzing the extract's efficacy on colon cancer cell line HCT-116 (Figure 2(e)), revealed a different trend, since the percentage inhibition appeared to continue to increase with increasing concentration up to 200 μg/mL resulting in maximum inhibition of about 83% and $IC_{50} > 200$ μg/mL. According to Dudai et al. [48] the mechanism of the inhibition is mostly accompanied by DNA

fragmentation and caspase-3 catalytic activity induction. Although at higher ethanolic content extraction there is an appreciative inhibitory activity, the extract performance was found to be lower than that of aqueous extracts. In this extract all samples tested were found to have an $IC_{50} > 200 \, \mu g/mL$. In general, the results presented here on cell growth inhibition by *C. citratus* extract are further supported in the light of the antiproliferative effects of the plant's phytochemical constituents that have been published before as shown in Table 1.

4. Conclusions

The oxidative radical scavenging and chemo-preventive efficacy of *C. citratus* extracts were evaluated. Ew and E50 extracts have shown DPPH antioxidant activities. In addition, the extracts were found to show an appreciative iron chelating and nitric oxide scavenging activities. On evaluating the antiproliferative effect, five (5) human cancer cell lines were compared in this analysis. The E50 extract proved to be more potent on breast cancer MCF-7 cell line. On the other hand, E90 extract showed a moderate potency on the ovarian cancer (COAV) and MCF-7 cell lines. Compared to extracts from higher ethanolic content, aqueous extracts were observed to show lower inhibitory activity with a generalized $IC_{50} > 200 \, \mu g/mL$ in all samples tested. In general, the observed efficacy could probably be due to the phytochemical constituents of this plant.

Conflict of Interests

The authors declare that there is no conflict of interests in this paper.

Acknowledgment

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