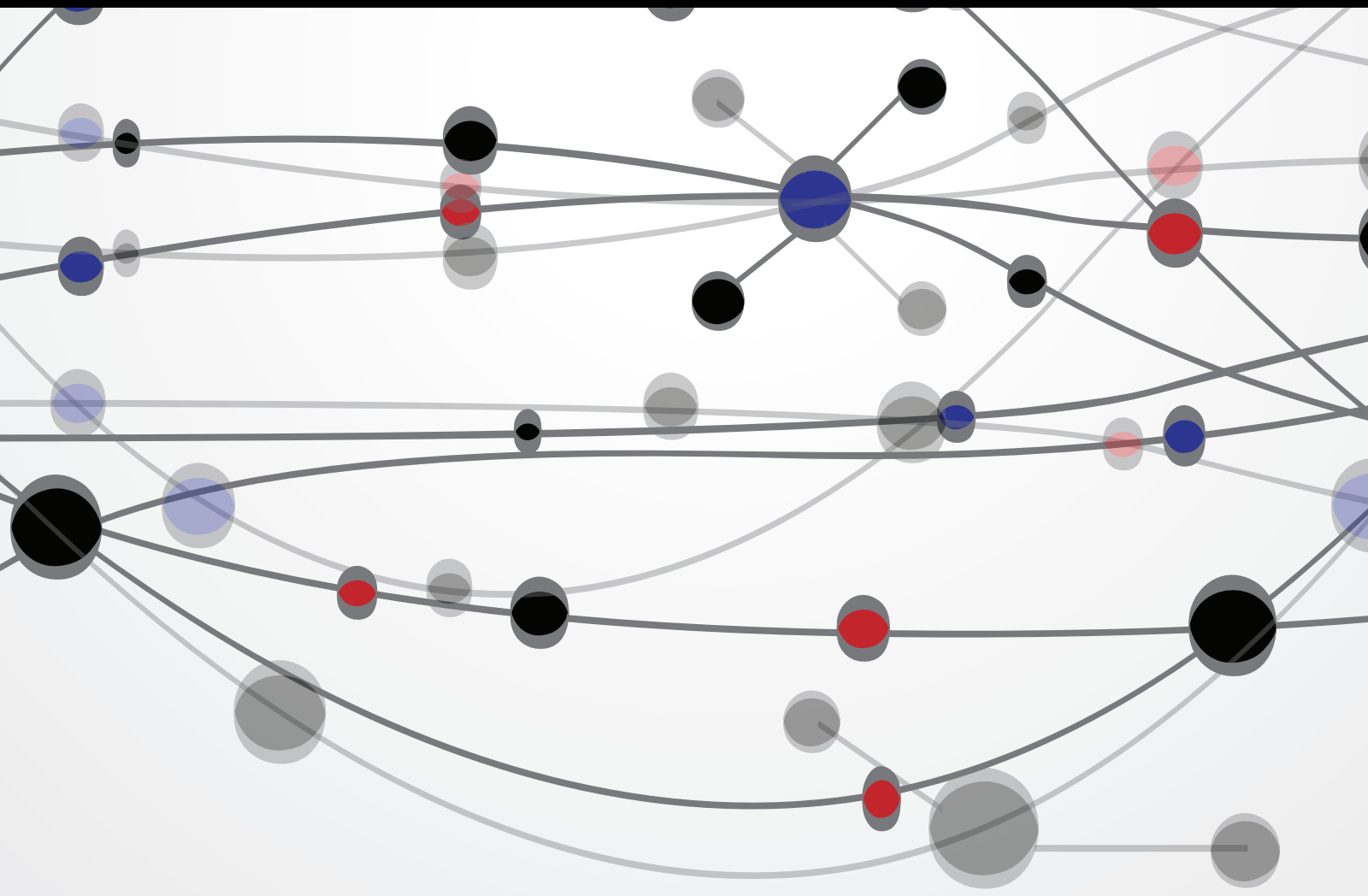
The Scientific World Journal

Natural Products: Bioactivity, Biochemistry, and Biological Effects in Cancer and Disease Therapy

Guest Editors: Hsueh-Wei Chang, Li-Yeh Chuang, Sanjay Guleria, and Sammia Yasmin



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Editorial

Natural Products: Bioactivity, Biochemistry, and Biological Effects in Cancer and Disease Therapy

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The drug discovery for chemoprevention and chemotherapy remains a challenge. Natural products-derived extracts and compounds are frequently reported to discover therapeutic agents for disease and cancer.

The overall scenario of this special issue of The Scientific World Journal presents the recent advances in biological function of selected natural products for cancer and disease therapy in terms of crude extracts and components. Some studies describe the bioinformatics tool to help to investigate the field of natural products.

The papers by S. Guleria et al. and C.-C. Lee et al. provide the essential oil and/or extracts of herb *Zanthoxylum alatum* and *Zingiber officinale* for its antioxidant and antimicrobial properties, respectively. O. O. Igbinosa et al. and C.-C. Lee et al. provide the animal experiments using extractsfrom *Jatropha curcas* (Linn) leaf and from supercritical carbon dioxide extracted ginger, respectively. Three studies (F. M. Al-Jasass and M. S. Al-Jasser, X.-W. Chen et al., and C.-Y. Lo et al.) focus on biological functions of the compounds from Saudi Arabia herbs, Chinese herb Huang Lian (*Rhizoma coptidis*), and from *Alpinia galangal*, respectively. Further, C.-Y. Yen et al. provide the toxicological study for cardiotoxin III in growth inhibition of oral cancer. C.-Y. Lin et al. provide a review article for the chemoprevention of cytochrome P450 in oral potentially malignant disorders (OPMDs) patients in terms of betel quid metabolism. S.-S. Liang et al. introduce the novel technique for online monitoring oxidative products and metabolites of nicotine based on tandem mass spectrometry.

Some papers introduce the bioinformatics methods or resources to study or review the natural products-related studies. L. Wang et al. introduce the gene ontology (GO) network for the systems-theoretical analysis of human hepatocellular carcinoma. Y.-C. Lin et al. provide the database (TIPdb) for anticancer, antiplatelet, and antituberculosis phytochemicals from indigenous plants in Taiwan. Three papers (W.-H. Huang et al. and J.-Y. Tang et al.) provide the drug discovery for cancer and disease therapy in terms of RNA editing, alternative splicing, and long noncoding RNAs as well as the summary for their bioinformatics resources.

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Research Article

Antimelanoma and Antityrosinase from *Alpinia galangal* Constituents

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Two compounds, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (BHPHTO) and bisdemethoxycurcumin (BDMC) they have been isolated from the rhizomes of *Alpinia galangal*, and the structures of both pure constituents were determined using spectroscopic analyses. The study examined the bioeffectivenesses of the two compounds on the human melanoma A2058 and showed that significantly inhibited the proliferation of melanoma cells in the cell viability assay. This research was also taken on the tests to B16-F10 cell line and showed minor inhibitory consequences of cellular tyrosinase activities and melanin contents. Our results revealed the anticancer effects of *A. galangal* compounds, and therefore, the target compounds could be potentially applied in the therapeutic application and the food industry.

1. Introduction

Alpinia galangal belongs to the Zingiberaceae family, and the herb grows mainly in South East Asia. It is now cultivated throughout tropical and subtropical Asia, such as India, Egypt, Thailand, Malaysia, Indonesia, and China. The herbs are usually not only used for seasoning but also for traditional medicine. The rhizomes contain essential oil. Many essential oils were obtained by hydrodistillation. The yield of essential oils ranged from 1.32 to 0.143% [1–3]. The wide range of major volatile compounds was identified and characterized by GC and GC/MS as *endo*-fenchyl acetate, zerumbone, 1,8-cineole and myrcene. For *A. galangal* rhizome chemical studies, a group of related phenylpropanoids was identified [4, 5]. Among these phenylpropanoids, 1′S-1′-acetoxychavicol acetate (galangal acetate) was the most studied and reported to possess various activities, such as antioxidative, antifungal, antitumor, and anti-inflammatory activities. However, only some studies for *A. galangal* grown in Taiwan were published [6]. Human skin is normally contacted with damage stress, which is produced by external and intrinsic sources, such as ultraviolet (UV) radiation, free radicals, and reactive oxygen species [7]. There are many studies about the skin exposed to oxidative stress or UV radiation and are responsible for aging or tumorigenesis [8]. Melanoma, a malignant tumor of epidermal melanocytes, is one of the most deadly skin cancers. Within the past several decades, the occurrences of cutaneous malignant melanoma have increased because it has a strong propensity to metastasize and, therefore, is one of the most aggressive skin cancers. Unlike other cancers, malignant melanoma is not easy to treat with surgery, radiotherapy, or chemotherapy. A good chemotherapeutic agent will be a naturally occurring agent and can induce cytotoxicity in cancer cells.

In mammals, skin, hair, and eyes, darkening is determined by the synthesis and distribution of melanin. In skin, it is a mixture of pigmented biopolymers that is synthesized in a unique organelle, the melanosome of melanocytes. Excessive biosynthesis of melanin induces various related pigment disorders, such as senile lentigo, melasma, freckles, and pigmented acne scars, that are of particular concern to women as well as men. Their treatment usually involves the use of medicines or medicinal cosmetics containing depigmenting or skin-whitening components. Safe and effective regulators that act to minimize skin pigmentation abnormalities include natural and synthetic depigmenting agents. However, only a few are used as therapeutic agents, primarily because of various safety concerns and low whitening bioactivity. In melanogenesis, L-tyrosine is hydroxylated to dihydroxyphenylalanine (L-DOPA), and then L-DOPA is oxidized to DOPA-quinone with two initial steps [9]. Pigment coloring hair, skin, and eyes because of the key protein, tyrosinase, is recognized to be the first two and rate-limiting enzyme in the biosynthesis of melanins [10]. Recently, much attention has been drawn to the application of tyrosinase inhibitors to medical treatments and cosmetic businesses. Therefore, in clinical usage, tyrosinase inhibitors are being taken for dermatological disorder treatments related to melanin hyperaccumulation and are thus fundamental in cosmetics for depigmentation [11].

2. Materials and Methods

2.1. Reagents and Materials. All solvents were at analytical grade. Lipophilic Sephadex (LH-20) resin was purchased from Sigma-Aldrich Inc., (St. Louis, MO, USA). Reverse phase (C18; 25–40 μm) sorbent was purchased from Merck Chemical Co. (Darmstadt, Germany).

2.2. Extraction and Purification of BHPHTO and BDMC. The rhizomes of *A. galangal* were collected from the Native Plant Ecological Garden in National Chiayi University. The air-dried rhizomes (300 g) of *A. galangal* were ground into a fine powder and extracted successively with 95% ethanol at room temperature. The crude ethanol extract (CEE) was filtered and evaporated to slurry using a rotary evaporator. The slurry was then suspended with water and partitioned successively three times with hexane (×3) and ethyl acetate (×3). The hexane and ethyl acetate extracts were separately dried using a rotary evaporator at 40°C, followed by freeze-drying for 48 h to give dried hexane and ethyl acetate extract (AG-EtOAC), respectively. The AG-EtOAC fraction was first subjected to passage over LH-20, using 95% ethanol. The eluted solution was collected by test tubes. Only the tubes that contained major components were concentrated and followed by eluting isocratically in the self-pack C18 with 80% methanol solution to obtain BHPHTO and BDMC. The identities of 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (BHPHTO) and bisdemethoxycurcumin (BDMC) (Figure 1) were confirmed by comparison of their NMR and MS spectral data with those available in the literature [12].

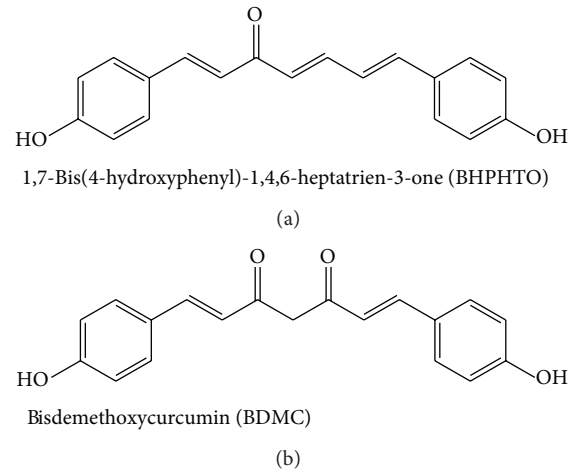

Figure 1: The structure of 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (BHPHTO) and bisdemethoxycurcumin (BDMC).

2.3. Human Melanoma A2058 Cell and Mouse B16-F10 Melanoma Cell Cultures. The human skin cancer A2058 cell lines were derived and purchased from the Bioresource Collection and Research Center (BCRC number: 60240, Hsinchu, Taiwan, ROC) the American Type Culture Collection (ATCC number: CRL-11147, Manassas, VA, USA). The human metastatic melanoma A2058 cell line is derived from 43-year-old male [13].

And the melanoma B16-F10 cells (BCRC 60031) were obtained from ATCC (Manassas, VA, USA), cultured in DMEM (13.4 mg/mL Dulbecco's modified Eagle's medium, 10 mM HEPES, 143 U/mL benzylpenicillin potassium, 100 mg/mL streptomycin sulfate, and 24 mM $NaHCO_3$, pH 7.1) containing 10% FBS, 1% P/S, and incubated at 37°C under 5% CO_2 atmosphere [14].

2.4. Cell Proliferation Assay. The effects of compounds on cell growths were according to the MTT assay procedures [15]. The method is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form impermeable crystals of a dark-blue formazan, thus resulting in accumulation within healthy cells. Briefly, cells were seeded in 96-well plates at a density of 8×10^3 cells/well. The medium was then changed, and cells were maintained in either solvent alone (control cells) or in the presence of the indicated each sample in a final volume of 100 μL in 10% FBS culture medium. Each sample was added to a microplate and incubated under the same conditions as above for 24 h. After 24 h of incubation, the medium was replaced with 100 μL of fresh medium including 0.5 mg/mL MTT. The plate was cultured in a 37°C incubator filled with 5% CO_2 for 2 h. Each precipitate in a specific dish was dissolved in 100 μL of DMSO to dissolve the purple formazan crystals. After the dishes were gently shaken for 10 min in the dark to ensure maximal dissolution of formazan crystals, the absorbance (A) values of the supernatant were

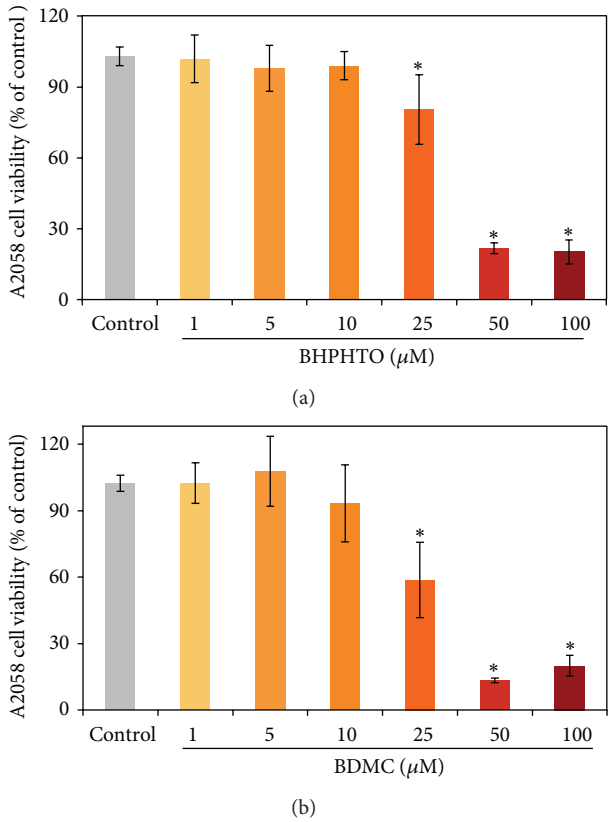

Figure 2: The effects of ginger extracts (BHPHTO and BDMC) on A2058 cell growths measured by MTT assay. The data represented the mean value ± SD of triplicate values for three independent experiments. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

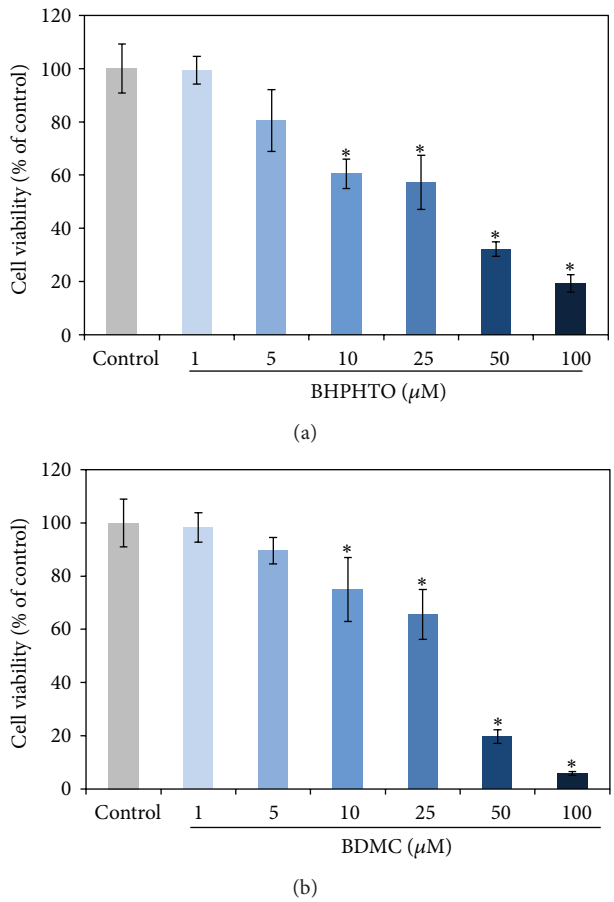

Figure 3: The impact of BHPHTO and BDMC to B16-F10 melanoma cell viabilities. The data represented the mean value ± SD of triplicate values for three independent experiments. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

measured at 595 nm (UV_vis, BioTek, Winooski, VT). Cell growth was calculated as

$$\text{Cell viability}\,(\%) = \frac{\left(A_{\text{sample}} - A_{\text{blank}}\right)}{\left(A_{\text{control}} - A_{\text{blank}}\right)} \times 100\%. \quad (1)$$

2.5. Measurement of B16-F10 Cellular Tyrosinase Activity. Testing cell tyrosinase activity *in vitro*, B16-F10 melanoma cells (10^5 cells per well) were placed in 24-well plates in 300 μL of medium containing various concentrations of testing samples and incubated for 2 days [16]. The sample-treated cells were washed with phosphate-buffered saline (PBS) and lysed with 1% Triton X-100/PBS. The enzyme extract of cellular lysate was added to 50 μL of 2 mM L-tyrosine. This reaction was then incubated at 37°C for 3 h in a dark environment, and the absorbance at 490 nm was measured on a spectrophotometer.

2.6. Determination of B16-F10 Cellular Melanin Contents. Briefly, we followed the previous method with minor modifications [17]. Cell pellets were dissolved in 2.0 N NaOH containing 10% DMSO and heated at 90°C for 1 h, and suspensions were clarified by centrifugation for 10 min at 10,000 ×g. The amount of melanin was determined spectrophotometrically based on the absorbance at 475 nm [18].

2.7. Statistical Analysis. The data were expressed as the mean value obtained in three experiments. Statistical comparisons were performed by Student's t-test for paired values.

3. Results and Discussion

3.1. Cellular Proliferative Depressing Effects of BHPHTO and BDMC on Melanoma Cells. The main reason of the death of a patient is due to the tumor proliferation and metastasis [12]. Therefore, it is urgent to find valuable and significant novel biomedicinal components for anticancer therapies. MTT assay illustrated the anticell proliferation of two kinds of human skin melanoma cells, A2058 cells for 24 h treatments. In Figure 2, to evaluate the effects of several ginger (BHPHTO and BDMC) extracts on cancer cytotoxicity, cells were treated with various concentrations from 0 to 100 μM. We demonstrated the cytotoxic abilities on human skin melanoma A2058 cells of the extracts, and cellular proliferations were inhibited in dose-dependent manner when cells

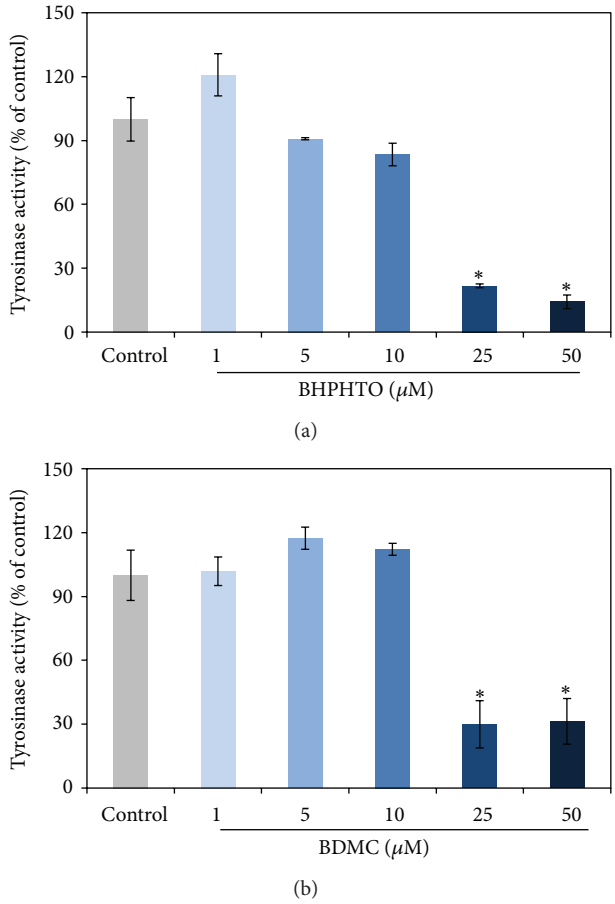

Figure 4: The tyrosinase activity of B16-F10 cells treated with various concentrations of BHPHTO and BDMC. The data represented the mean value ± SD of triplicate values for three independent experiments. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

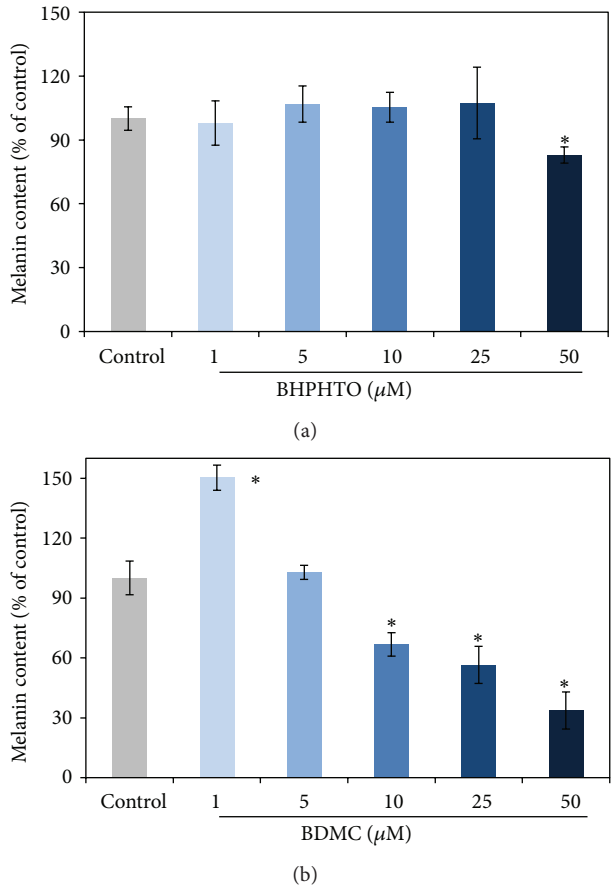

Figure 5: The melanin content of B16-F10 cells treated with various concentrations of BHPHTO and BDMC. The data represented the mean value ± SD of triplicate values for three independent experiments. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

were exposed to a high dose of all extracts, BHPHTO and BDMC (50 μM), and cell viabilities exhibited less than 25% after 24 h of treatment.

3.2. Cytotoxicity of BHPHTO and BDMC in B16-F10 Cells. 3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was investigated to study the cytotoxicities of BHPHTO and BDMC in B16F10 cells. The samples were treated with various concentrations, from 1 μM to 100 μM, and the vehicle control group had no testing agents with 0.5% DMSO. As shown in Figure 3. With 25 μM BHPHTO, about 55% B16-F10 cells remained, and with 25 μM BDMC, still 65% B16-F10 cells remained.

3.3. B16-F10 Cellular Tyrosinase Activity and Melanin Content of BHPHTO and BDMC. Melanin is a vitally important factor in determining the skin color of human. The melanogenesis pathway consists of the enzymatic L-tyrosine hydroxylation and the oxidation of L-dopa to its corresponding dopaquinone [9]. After the two tyrosinase-catalyzed steps, additional multiple biosynthesis steps followed and yielded melanin [10]. We tried to investigate if BHPHTO and BDMC have mouse B16F10 cellular tyrosinase-inhibiting abilities (Figure 4) and melanin content (Figure 5) decreasing power [11]. Although, the tyrosinase activity of B16-F10 results showed that with 5 or 10 μM of BHPHTO and BDMC there is no obvious variation and with concentrations higher than 25 μM tyrosinase activity decreased for low cell viability. But interestingly, even the cell viability is low, the tyrosinase activity is completely high. Melanin content, however, did not show evident reduction and on the contrary, with highest concentrations of BHPHTO and BDMC at 25 μM, the melanin content decreased for low cell viability. As the tendency of tyrosinase activity, melanin content is completely higher than cell viability.

Conflict of Interests

No contributing author has a conflict of interests in the publication of this study.

Authors' Contribution

Chih-Yu Lo and Po-Len Liu contributed equally to this work.

Acknowledgment

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Review Article

Cytochrome P450 Metabolism of Betel Quid-Derived Compounds: Implications for the Development of Prevention Strategies for Oral and Pharyngeal Cancers

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Betel quid (BQ) products, with or without tobacco, have been classified by the International Agency for Research on Cancer (IARC) as group I human carcinogens that are associated with an elevated risk of oral potentially malignant disorders (OPMDs) and cancers of the oral cavity and pharynx. There are estimated 600 million BQ users worldwide. In Taiwan alone there are 2 million habitual users (approximately 10% of the population). Oral and pharyngeal cancers result from interactions between genes and environmental factors (BQ exposure). Cytochrome p450 (*CYP*) families are implicated in the metabolic activation of BQ- and areca nut-specific nitrosamines. In this review, we summarize the current knowledge base regarding *CYP* genetic variants and related oral disorders. In clinical applications, we focus on cancers of the oral cavity and pharynx and OPMDs associated with *CYP* gene polymorphisms, including *CYP1A1*, *CYP2A6*, *CYP2E1*, and *CYP26B1*. Our discussion of *CYP* polymorphisms provides insight into the importance of screening tests in OPMDs patients for the prevention of oral and pharyngeal cancers. Future studies will establish a strong foundation for the development of chemoprevention strategies, polymorphism-based clinical diagnostic tools (e.g., specific single-nucleotide polymorphism (SNP) "barcodes"), and effective treatments for BQ-related oral disorders.

1. Introduction

Oral and pharyngeal cancers are some of the most common cancers worldwide [1]. Taiwan is a hyperendemic area for oral and pharyngeal cancers [2]. In 2010, the age-standardized incidence rate adjusted by 2000 years world population (ASRW) of oral and pharyngeal cancers was 40.56 per 100 000 Taiwanese males and was ranked the fourth most prevalent cancer in Taiwan [3]. The ASRW of oral and pharyngeal cancers among Taiwanese males was also ranked one of the highest worldwide [2].

Oral submucous fibrosis (OSF), leukoplakia, erythroplakia, and lichen planus are a group of oral potentially malignant disorders (OPMDs) thought to be linked to the development of oral and pharyngeal cancers [4, 5]. Cases of oral and pharyngeal cancers are concentrated in Southeast Asia, where betel quid (BQ) chewing is prevalent [2]. Habitual BQ chewing is associated with an increased risk of oral and pharyngeal cancers and OPMDs [5–9]. Ko et al. demonstrated a significant association between BQ chewing without tobacco and the incidence of oral cancer [6]. In addition, BQ chewing is a major risk factor for OPMDs such as oral leukoplakia and OSF, and cigarette smoking was shown to have a modifying effect on chewing, based on an additive-interaction model in oral leukoplakia patients [7].

The fourth most frequently consumed psychoactive substance worldwide after caffeine, nicotine, and alcohol is BQ, a masticatory mixture that combines the areca nut (AN), betel leaf, slaked lime, and various local flavorings [10]. Approximately 10% of the global population (approximately 600 million users) chew some form of BQ, primarily in the Indo-Pakistan subcontinent, South and Southeast Asia, and the South Pacific islands, and a large number of South Asian immigrants to the United Kingdom, Africa, Australia, and the United States are also BQ chewers [10–12]. In Taiwan, chewing BQ is a popular habit, particularly among males, with approximately 2 million regular users [13]. In a previous study, we found that male Taiwanese oral and pharyngeal cancer patients with a history of high-frequency BQ use had poor survival [14, 15].

By 2004, the International Agency for Research on Cancer (IARC) declared that BQ without tobacco is carcinogenic to humans (group 1) and increases the risk of oral cancer [16]. Although the masticatory practices and ingredients in BQ differ in different regions of the world, the AN is a major component of BQ worldwide, and the IARC has reported that AN alone is a group 1 carcinogen in humans. In the presence of slaked lime, the most abundant alkaloid of AN, arecoline, is hydrolyzed to arecaidine during the chewing process [17]. Arecoline has been shown to be cytotoxic to mammalian cells in vivo and in vitro [18–20]. In vitro studies the mutagenicity and genotoxicity of arecoline and arecaidine have been examined primarily in short-term experiments [21]. However, in carcinogenicity studies in animals, the IARC (2004) reported that evidence indicating that arecoline may cause cancer is limited, but inadequate evidence for the carcinogenicity of arecaidine [16].

In general, exposure to AN-derived carcinogens, particularly the alkaloids and the AN-derived *N*-nitrosamines, increases the risk of OPMDs and cancers of the oral cavity and pharynx in BQ chewers. Cytochrome P450 (*CYP*) enzymes are monooxygenases that catalyze many reactions involving carcinogens [22, 23]. During phase I metabolism, *CYP* families play important roles in detoxifying AN-derived compounds, such as arecoline [24], and are involved in the metabolic activation of arecoline-related *N*-nitrosamines [25]. A previous study suggested that arecaidine and three *N*-oxide metabolites are generated by the CYP enzyme system [26].

Environmental carcinogens and genetic polymorphisms, either separately or jointly, play an important role in the occurrence of oral and pharyngeal cancers. Environmental factors, such as alcohol use, BQ chewing, and cigarette smoking, were significantly associated with the risk of oral and pharyngeal cancers and OPMDs, and a synergistic effect among the use of these substances was also observed [6, 7, 16]. The interactions of environmental and genetic factors in the tumorigenesis of oral and pharyngeal cancers have been shown to be affected by various CYP enzyme-mediated metabolic processes [27–30].

Several studies have indicated that *CYP* polymorphisms affect the metabolism of tobacco-derived carcinogens and the risk of oral cancer [31–33]. However, reports of the risk of oral and pharyngeal cancers and OPMDs associated with AN-derived carcinogens are scant. Our review focuses on the role of the CYP enzyme-mediated metabolism in OPMDs and oral and pharyngeal cancers among BQ users and evaluates emerging data that potentially implicate arecoline- and arecoline-derived *N*-nitrosamines in tumorigenesis. The effects of *CYP* polymorphisms are worthy of investigation to further understand the role of genetic factors in susceptibility to OPMDS and cancers of the oral cavity and pharynx and to aid the development of prevention strategies for cancers related to BQ use.

2. AN-Derived *N*-Nitrosamines

2.1. Carcinogenicity of N-Nitrosamines In Vitro. The chewing of AN is believed to produce carcinogenic *N*-nitrosamines. Arecoline is the major compound of AN. The *N*-nitrosation of arecoline has been shown to form *N*-nitrosoguvacoline (NGL), 3-methylnitrosaminopropionaldehyde (MNPA), and 3-methylnitrosaminopropionitrile (MNPN) in vitro [34]. Based on studies of *Salmonella typhimurium* YG7108, CYP2A6 was found to be the most efficient activator of MNPN, followed by CYP1A1, and NGL was activated by CYP2A6. The genotoxicity of NGL was observed to be substantially lower than that of MNPN or MNPA [35]. Thus, that the human *CYP2A6* gene may play an important role in the mutagenic activation of AN-related *N*-nitrosamines has been suggested [35]. Studies on rodents have shown that MNPN, MNPA, and NGL are carcinogenic. In carcinogenicity studies on animals, the IARC (2004) determined that evidence of MNPN carcinogenicity is sufficient [16]. The carcinogenicity

of MNPN may be caused by DNA methylation, which has been observed in rats treated with MNPN [21, 36].

2.2. Endogenous Nitrosation and N-Nitrosamines Carcinogenicity. Endogenous nitrosation occurs during BQ chewing, exposing BQ chewers to four *N*-nitrosamines derived from arecoline [37]. These AN (arecoline)-derived *N*-nitrosamines include MNPN, MNPA, NG, and *N*-nitrosoguvacine (NGC). These arecoline-derived *N*-nitrosamines are undetectable in the AN before chewing and are formed by the endogenous nitrosation of arecoline. Table 1 lists the maximum levels of NGL (142 ng/mL), NGC (26.6 ng/mL), and MNPN (11.4 ng/mL) in the saliva during BQ chewing without tobacco.

Many BQ chewers often swallow the quid juice, which contains the precursors of the nitrosamines. The pH of stomach acid likely facilitates the nitrosation of secondary and tertiary amines from the quid. A modified *N*-nitrosoproline test showed that the urinary levels of *N*-nitrosoproline, an endogenous nitrosation marker, are 2.4- to 6.5-fold higher in BQ chewers, with or without tobacco, compared to nonchewers [42, 43]. Urinalysis of Syrian hamsters fed AN and a nitrite source detected NGL and its metabolite, *N*-nitrosonipecotic acid [44, 45], indicating that exposure to nitrosamine carcinogens formed by endogenous nitrosation is likely higher among BQ chewers who swallow the BQ juice [46]. Several case-control studies have also indicated that swallowing the BQ juice is associated with a significant increase in the risk of oral cancer [6, 47, 48].

2.3. CYP1A1-Mediated Metabolism of N-Nitrosamines. In a study of *S. typhimurium* YG7108, CYP1A1 was the second most efficient activator of MNPN, after CYP2A6, and MNPA activation was catalyzed to a lesser extent by CYP1A1 [35]. Previous studies have demonstrated that *CYP1A1* polymorphisms are associated with susceptibility to tobacco-related oral cancers [31–33, 49, 50]. Studies of the association between *CYP1A1* polymorphisms and BQ-related oral cancers are scant.

Kao et al. found that people with the *CYP1A1* Exon 7 polymorphism G/G genotype (val/val) are susceptible to BQ-related oral cancer and OPMDs [29]. They found that people who have the G/G and A/G (ile/val) genotype have significantly higher rates ($P < .0001$) of oral cancer (7.6% and 79.2%, resp.) and OPMDs (10% and 68.3%, resp.) than controls (1.4% and 53.4%, resp.). Kao et al. calculated odds ratios for the development of oral cancer of 18.86 and 5.08 for those with the G/G (95% CI, 3.61–98.52) or A/G (95% CI, 2.64–9.76) genotype of *CYP1A1*, respectively, and also reported odds ratios for the development of OPMDs of 15.23 and 2.67 for those with the G/G (95% CI, 2.76–83.98) or A/G (95% CI, 1.32–5.40) genotype, respectively. These novel findings indicated that people with the G (val) allele may have an earlier onset age of oral cancer [29]. Another study showed that people with the *CYP1A1* m2 polymorphism within the *Nco*I restriction site (−/−) or the *CYP1A1* m1 polymorphism at the *Msp*I site (+/−) and (−/−) had a significantly higher risk of oral submucous fibrosis (OR = 8.25; 95% CI, 4.31–15.80; OR = 2.88; 95% CI, 1.57–5.24; and OR = 3.16; 95% CI, 1.10–9.04, resp.) [51].

2.4. The N-Nitrosamine-Metabolizing CYP2A6 Gene. Based on previous studies, we conclude that human *CYP2A* and *CYP2E* subfamily members play important roles in the metabolic activation of arecoline-related *N*-nitrosamines [52–54]. Located on human chromosome 19, the *CYP2A6* gene consists of 350 kilobases located at 19q 12–19q 13.2 [55–57]. Thirteen alleles of the *CYP2A6* gene have been identified (*CYP2A6**1 through *CYP2A6**11 and *CYP2A6**1 × 2; Table 2). The *CYP2A6**1 allele has 2 forms, *CYP2A6***1A* and *CYP2A6***1B*, that produce a gene conversion with the *CYP2A7* gene in the 3′-untranslated region [58] and exhibit similar enzyme activity [58]. The *CYP2A6**2, *CYP2A6**3, *CYP2A6**5, *CYP2A6**6, *CYP2A6**7, *CYP2A6**8, *CYP2A6**9, *CYP2A6**10, and *CYP2A6**11 genetic variants contain a different point mutation. In addition, the *CYP2A6**10 variant contributes to variations in *CYP2A6**7 and *CYP2A6**8. The existence of *CYP2A6**3 has been debated, but a previous study indicated that the *CYP2A6**3 genetic variant was the result of multiple *CYP2A6* and *CYP2A7* gene conversions [59]. The *CYP2A6**4 is from a deletion in the *CYP2A6* gene. The *CYP2A6**1 × 2 comprises a variation at 2 sites in the *CYP2A6* gene, and the *CYP2A6***1B* allele is caused by gene conversion in the 3′-untranslated region of *CYP2A7*.

The various alleles of *CYP2A* express at least 13 different isoenzymes, among which *CYP2A6* metabolically activates the *N*-alkylnitrosamines, *N*-nitrosonornicotine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, which have relatively long alkyl chains [62, 63]. Miyazaki et al. first reported that *CYP2A* subfamilies play important roles in the mutagenic activation of AN-derived *N*-nitrosamines [35]. The CYP2A6 P450 enzymes are the primary activators of MNPN. In Asia, the most common variant of *CYP2A6* is *CYP2A6**4 (the *CYP2A6* deletion). The frequency of *CYP2A6**4 is approximately 6.6% to 15.1% in the Chinese population. It is the most common genetic variant in the Japanese population, occurring at a frequency of 20.0% to 31.0% (Table 2).

People are classified as poor (PM), extensive (EM), or ultrarapid metabolizers (UM) based on their type of genetic variation [64]. The UMs have 2 active alleles of the *CYP2A6* gene, including the *CYP2A6**1 × 2 variant. Phenotyping assays have indicated that 2 or more copies of active *CYP2A6* alleles may result in a rapider nicotine metabolism. People with 1 or 2 copies of active gene alleles, such as CYP2A6*1/*1, are extensive metabolizers [60, 65], whereas PMs are those with null alleles, such as *CYP2A6**2/*2 or *CYP2A6**4/*4, with no enzyme function or less activity regarding probe substrates. People who are homozygous for the *CYP2A6**2 allele have little coumarin-hydroxylation activity (<0.1%) [60, 61], and EMs exhibit low activity (<15%) when nicotine is used as the probe substrate [58, 60, 66, 67].

Previous reports have indicated that the deletion of *CYP2A6* (*CYP2A6***4C*) may reduce the risk of lung cancer [68–71], suggesting that people with *CYP2A6***4C* may not activate tobacco nitrosamines from smoking. Because a genetic variation within the *CYP2A6* gene appears to reduce

Table 1: Detected saliva levels (ng/mL) of nitrosamines such as MNPN, NGL, and NGC in chewers with tobacco and without tobacco.

BQ-specific *N*-nitrosamines	BQ alone (without tobacco)	BQ + tobacco	References
MNPN	0.5–11.4	—[a]	Prokopczyk et al., 1987 [36]
NGL	0–5.9	0–7.1	Nair et al., 1985 [38]
	0.6–8.8	3.1–23.5	Nair et al., 1987 [39]
	2.2–9.5[b]	4.3–45[b]	Wenke et al., 1984 [40]
	0–142	—	Stich, 1986 [41]
NGC	0–26.6	0–30.4	Nair et al., 1985 [38]

Adapted from [16].
BQ: betel quid.
[a]The data not reported.
[b]In ppb.

xenobiotic activation, *CYP2A6* polymorphisms may also reduce the metabolic activation of AN-derived nitrosamines. In Sri Lanka, a study found that the deletion polymorphism, *CYP2A6*4C/*4C*, reduces susceptibility to oral squamous cell carcinoma (OR = 0.14; 95% CI, 0.03–0.72) among habitual BQ chewers with oral lesions, suggesting that BQ chewers with reduced *CYP2A6* activity because of polymorphisms may be at lower risk for oral cancer [30].

2.5. The N-Nitrosamine-Metabolizing CYP2E Gene. The *CYP2E* subfamilies include *CYP2E1* and *CYP2E2*. The *CYP2E1* gene has been shown to be the primary activator of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine, which are *N*-nitrosamines with relatively short alkyl chains [62, 63]. A case-control study of 41 male oral cancer patients and 123 healthy controls found that people with the *CYP2E1* c1/c2 or c2/c2 genotype had a higher risk of oral cancer (multicovariate-adjusted OR = 2.0; 95% CI, 0.8–5.4) than those with the c1/c1 genotype [27]. However, the association was not statistically significant. Hung et al. also reported a significant association between *CYP2E1* polymorphisms and oral cancer among those without BQ-chewing habits (OR = 4.7; 95% CI, 1.1–20.2), but not among BQ chewers [27]. All BQ chewers were also cigarette smokers, and the chewing habits had a significant effect on the risk of oral cancer. Thus, Hung et al. suggested that the risk of oral cancer associated with combined habits of BQ chewing and smoking may be too striking to have modified effects by the *CYP2E1* genotype. A more recent study showed that polymorphisms in *CYP2E1* within the PstI site (+/−) were significantly associated with oral submucous fibrosis (OR = 3.14; 95% CI, 1.14–8.62) [51].

2.6. Arecoline-Induced, CYP26B1-Mediated Retinoic Acid Metabolism. In a previous study, we treated normal human gingival fibroblasts (HGFs) with arecoline and screened for the presence of a novel *CYP26B1* by using a microarray [72]. The expression of CYP26B1 was subsequently confirmed using quantitative reverse transcription and real-time polymerase chain reaction [48]. The *CYP26B1* gene is located at the human 2p13.2 region and may play an important role in variations in retinoic acid (RA) metabolism associated with oral lesions. Hyperkeratosis and hyperplasia of the oral mucosa can be caused by insufficient retinol intake [73], and the findings of previous studies have indicated that remission of oral leukoplakia in BQ chewers treated with RA may result from the suppression of AN-related metabolism, rather than the inhibition of tumorigenesis [74–76]. Because it is a critical regulator of cell proliferation, cell differentiation, and apoptosis, RA deficiency may play an important role in carcinogenesis [73, 77–79].

At an RA concentration of 100 μg/mL, the repression level of *CYP26B1* was approximately 15-fold in cultured primary HGFs obtained from a healthy volunteer in our previous study [72]. We suggested that *CYP26B1* may be involved in detoxification processes, and arecoline treatment in normal HGFs was shown to repress susceptibility [72]. We detected higher levels of CYP26B1 mRNA and protein expression in human oral cancer cells compared with adjacent noncancerous tissues. The findings of another previous study suggested that CYP26B1 mRNA is predominantly expressed in the adult human cerebellum and may be associated with the protection of specific human tissues from exposure to RA [80]. We found that the *CYP26B1* polymorphism AA significantly correlated with the risk of oral cancer (OR = 2.26; 95% CI, 1.35–3.80), and BQ chewers with the AA genotype had a significantly increased risk of oral cancer (OR = 70.04; 95% CI, 13.62–360.11). We concluded that *CYP26B1* is a novel candidate gene in the development of BQ-related oral cancer and speculated that *CYP26B1* may be involved in the metabolism of arecoline-related compounds [48].

In the oral mucosa of BQ chewers, *CYP26B1* induction alters the RA metabolism [79]. A previous study demonstrated that *CYP26* members may play a role in RA accumulation in human epidermal keratinocytes [81]. Klaassen et al. [82] found higher levels of RA-inducible CYP26 mRNA expression and higher RA turnover rates in oral squamous cell carcinoma cell lines, compared with noncancerous oral KB cells, and that oral KB cells from cancer patients exhibit a 15-fold higher RA turnover rate compared with noncancerous oral KB cells [83].

A previous study indicated that high-level CYP26 expression may be associated with head and neck cancer [82]. The expression of CYP26 was induced through an RA-receptor-mediated mechanism in breast and colon carcinoma cells [84]. A higher expression of CYP26 caused intracellular RA depletion in Barrett-associated adenocarcinoma [78], whereas other studies have indicated that the expression of CYP26 is downregulated in noncancerous human epidermis

Table 2: The nomenclature of *CYP2A6* and allele frequencies in population.

Allele	Frequencies in population							Nucleotide change	Effect	Enzyme activity	
	Caucasian (%)	African American (%)	Swedes (%)	Finns (%)	Spaniards (%)	Chinese (%)	Japanese (%)			In vitro	In vivo
CYP2A6∗1A	66.5	—[a]	98.9	98.6	97.0	43.2	40.0–42.0	None	—	Normal	Normal
CYP2A6∗1B	30.0	—	—	—	—	40.6	38.0–41.0	Gene conversion at 3′-flanking region	—	—	—
CYP2A6∗1 × 2	0.7	—	—	—	—	0.4	0.0	—	Duplication of CYP2A6	—	—
CYP2A6∗2	1.1–3.0	0.3	1.1	1.4	3.0	0.0–0.7	0.0	488 T → A	L160H	None	None
CYP2A6∗3	—	—	—	—	—	—	—	CYP2A6/CYP2A7 hybrid	—	—	—
CYP2A6∗4A	0.5–4.9	—	—	—	—	6.6–15.1	20.0–31.0	CYP2A6 deletion	CYP2A6 deletion	—	None
CYP2A6∗4B	—	—	—	—	—	—	—	CYP2A6 deletion	CYP2A6 deletion	—	None
CYP2A6∗4C	—	—	—	—	—	—	—	—	—	—	—
CYP2A6∗4D	—	—	—	—	—	—	—	CYP2A6 deletion	CYP2A6 deletion	—	None
CYP2A6∗5	0.0–0.2	—	—	—	—	1.0	0.0	1436 G → T	G479V	None	None
CYP2A6∗6	—	—	—	—	—	—	0.4	383 G → A	R128Q	Down	—
CYP2A6∗7	1.0	—	—	—	—	2.2	6.3	1412 T → C; gene conversion at the 3′-flanking region	I471T	Down	Down
CYP2A6∗8	0.0	—	—	—	—	3.5	1.6	1454 G → T; gene conversion at the 3′-flanking region	R485L	—	Normal
CYP2A6∗9	5.2	—	—	—	—	15.7	—	−48 T → G	TATA box	Down	—
CYP2A6∗10	0.0	—	—	—	—	0.4	1.6	1412 T → C; 1454 G → T; gene conversion at the 3′-flanking region	I471T; R485L	—	Down
CYP2A6∗11	—	—	—	—	—	—	—	670 T?C	S224P	Down	Down

Adapted from [57, 60, 61].
[a]The data not reported.

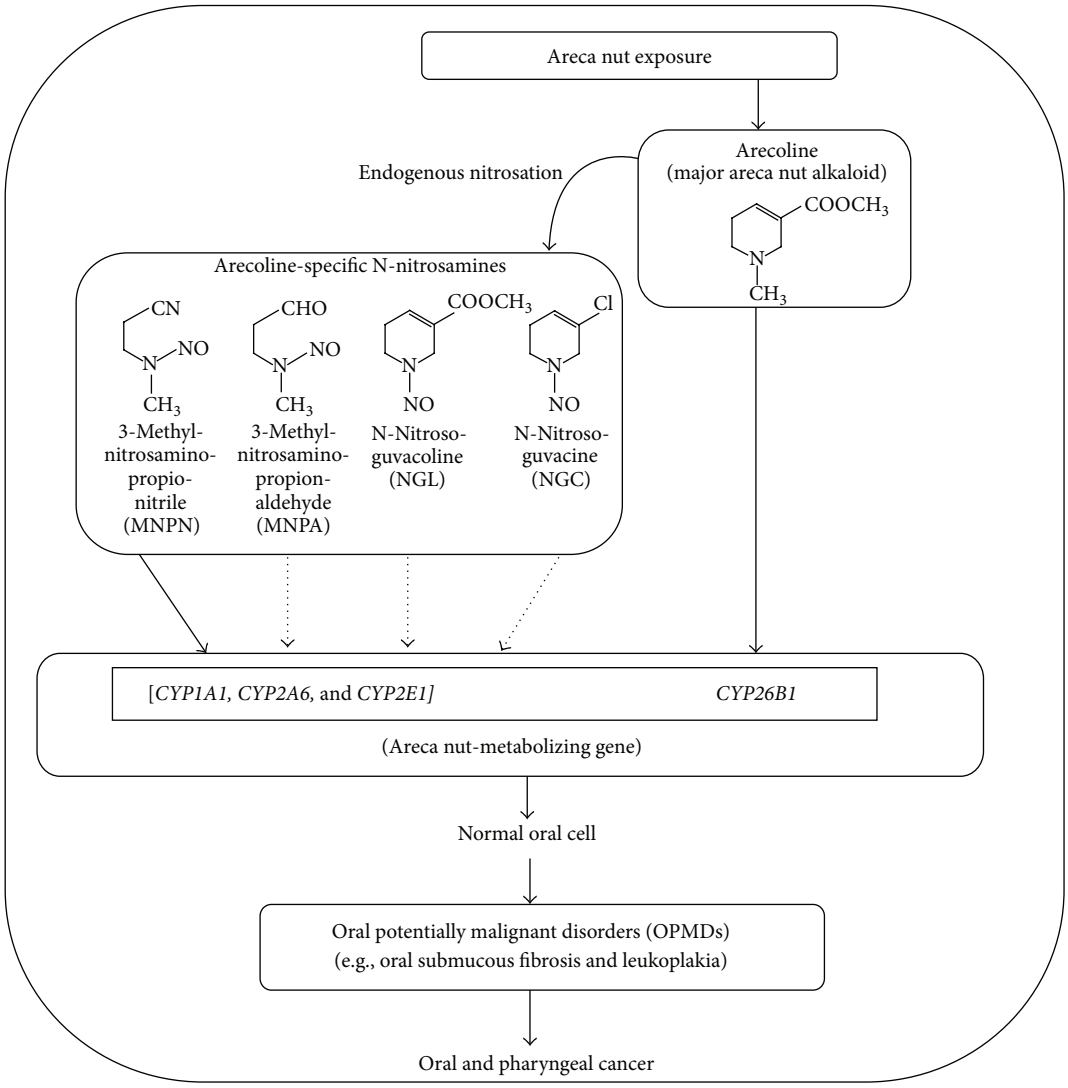

Figure 1: Simplified flow chart for postulated main effects of areca nut induced oral carcinogenesis *via* cytochrome P450 (*CYP*) gene.

[85, 86]. These findings indicate that the RA metabolism is an important factor in the development of oral cancer.

3. Discussion

Oral cancer is one of the most common cancers worldwide and was ranked the eighth leading cause of cancer-related deaths in 2000 [1]. Incidence rates of 8.8 and 5.1 per 100 000 cases and mortality rates of 7.3 and 3.2 per 100 000 cases have been reported for oral cancer among males and females, respectively [1]. In Taiwan, BQ production has increased approximately 44-fold [16]. An international study found that Taiwan had the fourth highest prevalence of oral and pharyngeal cancers, preceded by Papua New Guinea, the Solomon Islands, and Sri Lanka [2]. In BQ-endemic areas, oral and pharyngeal cancers and the most common OPMDs (such as OSF and oral leukoplakia) appear to be associated with BQ use, whereas cigarette smoking and heavy alcohol drinking are the major risk factors in western countries.

Numerous genes are involved in carcinogen metabolism, and most studies have found that *CYP* polymorphisms affect the risk of oral cancer associated with variations in the metabolism of tobacco-derived carcinogens. Arecoline, arecaidine, and other BQ-related metabolites may exist at nanogram concentrations in human blood, and the level of arecoline is significantly associated with the quantity of BQ used [87]. We summarized the findings of studies of the effects of *CYP* polymorphisms on BQ chewing-related symptoms, including cancer of the oral cavity and pharynx and OPMDs, in Table 3. Among xenobiotic metabolizing enzymes, *CYP1A1*, *CYP2A6*, and *CYP2E1* may activate AN-derived nitrosamines. In addition, the expression of CYP26B1 may be induced by arecoline and may be related to RA metabolism. The flowchart in Figure 1 depicts the main effects of *CYP* genetic variants on AN-induced carcinogenesis.

To the best of our knowledge, only 2 studies have reported a relationship between the genetic polymorphism of *CYP1A1* and the risk of oral cancer and OPMDs [29, 51].

Table 3: The association studies between cytochrome P450 (*CYP*) polymorphism and betel quid-related oral disorders.

CYP gene	Cases/number Controls/ number	Chewing habit of cases/controls	OR (95% CI)	Conclusion	Population/ reference
CYP1A1	Oral cancer/106 Controls/146	BQ, 62.3%/15.0%	Gene effects: Exon 7 A/G (ile/val) A/G versus A/A, 5.08 (2.64–9.76)* G/G versus A/A, 18.86 (3.61–98.52)*	Subjects with *CYP1A1* carrying G allele increased the risk for OPMDs and oral cancer	Taiwan/[29]
	OPMDs/60 Controls/146	BQ, 75.0%/15.0%	Gene effects: Exon 7 A/G (ile/val) A/G versus A/A, 2.67 (1.32–5.40)* G/G versus A/A, 15.23 (2.76–83.98)*		
			Gene effects: 3′UTR *Msp*I site	No significant association	
	OSF/75 Controls/150		Gene effects: m1 at *Msp*I site (+/−) versus (+/+), 2.88 (1.57–5.24)* (−/−) versus (+/+), 3.16 (1.10–9.04)* Gene effects: m2 at *Nco*I site (−/−) versus (+/+), 8.25 (4.31–15.80)*	Subjects with *CYP1A1* polymorphisms had significantly increased risks of OSF	India/[51]
CYP2A6	Oral lesions/286 (15 oral cancer, 62 OSF and 209 leukoplakia) Controls/135	betel, 100%/100%	Gene effects **1B/*4C* versus **1A/*1A*, 0.21 (0.05–0.88)* **4C/*4C* versus **1A/*1A*, 0.14 (0.03–0.72)*	BQ chewers with activity deficient of *CYP2A6* deletion decreased the risk of oral cancer	Sri Lanka/[30]
CYP2E1	Oral cancer/41 Controls/123	BQ, 73.2%/12.2%	Gene effects c1/c2 + c2/c2 versus c1/c1, 2.0 (0.8–5.4) Among nonchewers c1/c2 + c2/c2 versus c1/c1, 4.7 (1.1–20.2)* Among chewers c1/c2 + c2/c2 versus c1/c1, 0.8 (0.2–3.3)	A significant relationship between *CYP2E1* polymorphisms and oral cancer risk was found among non-BQ chewers	Taiwan/[27]
	Oral cancer/106 Controls/146		Gene effects: at *Pst*I site (+/−) versus (+/+), 3.14 (1.14–8.62)*	Individuals with *CYP2E1* at *Pst*I site polymorphism (+/−) may confer a significantly increased risk for oral cancer	India/[51]
CYP26B1	Oral cancer/247 Controls/338	BQ, 85.4%/22.5%	rs707718 Gene effects A versus C, 1.48 (1.16–1.87)* AA versus CC, 2.26 (1.35–3.80)* Gene-BQ (+/−) interplay AA-BQ (+) versus CC-BQ (−), 70.04 (13.62–360.11)*	BQ chewing interacted with *CYP26B1*-AA significantly increased the risk of oral cancer	Taiwan/[48]
			Gene effects rs2241057, rs2286965, rs3768641	No significant findings	

OPMDs: oral potentially malignant disorders; OSF: oral submucous fibrosis; betel: betel quid chewing with or without tobacco; BQ: betel quid without tobacco; OR: odds ratios; CI: confidence interval; *statistical significance.

Higher-risk patients with the *CYP1A1* G (val) allele should be strongly encouraged to avoid BQ use and maintain good oral hygiene. People with the *CYP2A6*4C/*4C* genetic variant may be at lower risk of oral cancer because their genotype suppresses the activation of AN-related procarcinogens [30]. The *CYP2E1* polymorphisms may increase the risk of oral cancer [27, 51]. In addition, the findings of our previous study suggest that the combination of higher CYP26B1 expression and polymorphism is associated with an increased risk of oral cancer [48].

Future studies on *CYP26B1* antagonists may identify novel RA-related methods of chemoprevention or treatment for OPMDs. Patients with high-risk alleles who chew BQ have an elevated risk for oral and pharyngeal cancers, and the risk is particularly high for OPMDs patients. We propose that the high-risk genotypes, such as *CYP1A1*, *CYP2A6*, *CYP2E1*, and *CYP26B1*, should be targeted for the development of a single-nucleotide polymorphism (SNP) gene chip for risk assessment in OPMDs patients, especially for those with BQ chewing habits.

4. Conclusion

The accumulation of such findings will be useful for the identification of high-risk patients and the development of novel therapeutic strategies for blocking the activation of AN-related compounds and targeting the *CYP* gene. Large-scale studies on the polymorphisms of *CYP* genes in BQ chewers and the genetic variants related to oral and pharyngeal cancers or OPMDs are warranted. The identification of molecular mechanisms elucidated by future pharmacogenomics studies will establish a strong foundation for the development of chemoprevention strategies, SNP-based clinical diagnostic tools (e.g., specific SNP barcodes for BQ-associated oral disorders), and effective treatments for BQ-related oral disorders.

Abbreviations

OPMDs: Oral potentially malignant disorders
BQ: Betel quid
AN: Areca nut
NGL: *N*-Nitrosoguvacoline
MNPA: 3-Methylnitrosaminopropionaldehyde
MNPN: 3-(Methylnitrosamino)propionitrile
NGC: *N*-Nitrosoguvacine
RA: Retinoid acid.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Jen-Yang Tang and Ping-Ho Chen made equal contributions to this work.

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Research Article

Functional Ginger Extracts from Supercritical Fluid Carbon Dioxide Extraction via *In Vitro* and *In Vivo* Assays: Antioxidation, Antimicroorganism, and Mice Xenografts Models

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Supercritical fluid carbon dioxide extraction technology was developed to gain the active components from a Taiwan native plant, *Zingiber officinale* (ginger). We studied the biological effects of ginger extracts via multiple assays and demonstrated the biofunctions in each platform. Investigations of ginger extracts indicated antioxidative properties in dose-dependant manners on radical scavenging activities, reducing powers and metal chelating powers. We found that ginger extracts processed moderate scavenging values, middle metal chelating levels, and slight ferric reducing powers. The antibacterial susceptibility of ginger extracts on *Staphylococcus aureus*, *Streptococcus sobrinus*, *S. mutans*, and *Escherichia coli* was determined with the broth microdilution method technique. The ginger extracts had operative antimicroorganism potentials against both Gram-positive and Gram-negative bacteria. We further discovered the strong inhibitions of ginger extracts on lethal carcinogenic melanoma through *in vivo* xenograft model. To sum up, the data confirmed the possible applications as medical cosmetology agents, pharmaceutical antibiotics, and food supplements.

1. Introduction

Antioxidative components are of importance due to the abilities to reduce free radical-mediated degradations of cells, tissues, and organisms in humans [1]. There are many diet sources to decrease oxidative stress, including legumes, cereals, fruits, various vegetables, and other herbal medicines all over the world [2].

With the long-term exposure in high oxidative stress, the normal cells often accompany with carcinomas and transfer into sick status. The strategy of diminishing oxidative stress is a good solution to keep human being physiologically healthy.

Therefore, natural antioxidants from plant species which decrease oxidative stress from intrinsic and external sources have huge applicable biofunctions in human health care [3, 4].

A worldwide troubling public health issue emerged from hospital-acquired or nosocomial illness is caused by infections microorganisms in recent decades. Pathogenic bacteria are able to survive for extended periods on human superficial skin, mucosa, or environmental surfaces and have been implicated in infectious outbreaks within hospitals, medical facilities, and institutions in many countries [5]. With the abuse of broad-spectrum antibiotics, the most alarming characteristic of these microorganisms is their resistance to almost all commercially available antimicrobial drugs. As a result, many of these microorganisms were now classified as highly antibiotic-resistant microorganisms. Therefore, the research requirements for new anti-infection therapeutic agents have increased in natural medicinal therapies. The utilization of plant-derived constitutes as preservatives, cosmetics, and pharmaceuticals has recently attracted increased interests [6].

Human skin is normally contacted with damage stress, which is produced by external and intrinsic sources, such as ultraviolet radiation, free radicals, and reactive oxygen species. There are many studies about the ultraviolet radiation which are responsible for skin aging or tumorigenesis. Melanoma, a malignant tumor of epidermal melanocytes, is one of the most deadly skin cancers [7]. During the past several decades, the occurrences of cutaneous melanoma have increased because it has a strong propensity to metastasize and, therefore, is one of the most aggressive skin cancers. Unlike other cancers, the malignant melanoma is not easy to treat with surgery, chemotherapy, or radiotherapy [8]. Clinically, recent treatments for advanced melanoma do not significantly improve the mortalities and prolong survivals of patients due to the exceeding metastasis of melanoma [9]. Fortunately, numerous widely used Chinese medicinal herbs have been shown to exert anticancer bioactivities [10, 11]. For this reason, the development of herbal medicine has the possibility of the therapeutic effects against melanoma cells.

Traditionally, natural matrices have been obtained by means of conventional extraction with organic solvents. Nevertheless, the extraction changes in the physicochemical properties of extracts could alter their functionalities. Thus, the extraction processes should be performed at suitable and mild conditions. Supercritical carbon dioxide extraction is an advanced technology that has many advantages, including low environmental impacts due to no residue of harmful solvents, noncorrosiveness, nontoxicity, and easy separation from extracts [12, 13]. It was widely used in pharmaceutical and food industries in modern times [14].

Ginger, the powdered rhizomes of the herb *Zingiber officinale* Roscoe (Zingiberaceae, dietary ginger), is used widely as a spice throughout the world. In Chinese medicine, ginger has been used traditionally as a treatment for allergy, constipation, asthma, diabetes, nervous diseases, rheumatism, toothache, stroke, and antimicroorganism infection [15]. In addition, ginger could be as the treatment of chemotherapy-associated nausea, the suppression of platelet aggregation, the inhibition of tyrosinase, cyclooxygenase, or nitric oxide synthase and prevent lipid peroxidation which sets in a variety of biofilm by oxidation to cause injury [16–18]. The previous literature reported about inhibiting skin tumor promotion in imprinting control regions mice [19]. The present work analyzed ginger extracts to identify the antioxidative, antimicrobial, and anticancer activities.

2. Materials and Methods

2.1. Reagents and Samples. Dimethyl sulfoxide (DMSO) and Luria-Bertani broth were purchased from Sigma-Aldrich Chemical Inc. (St Louis, MO, USA). Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies CO., Ltd. (Gibco, Grand Island, NY, USA). The plant specimen was authenticated by Dr. Chih-Chen Lee, Derlin Biotech Corporation (Nantou, Taiwan), where voucher specimens were kept. The air-dried extracts of ginger were cultivated and obtained in the district of Nantou, Taiwan (2012). All buffers and other reagents were of the highest purity commercially available.

2.2. Supercritical Extraction. The supercritical carbon dioxide fluid extraction was performed on United States supercritical fluid extractor (Applied Separation Inc. Co. Ltd.; Derlin Biotech Corp. Taiwan) with the extractor volume of 5.0 L. The liquid ethanol from a cylinder was pressured to reach the supercritical state with a piston pump and cooling at −4°C in a water bath before it passed into the extraction vessel (with 5.0 L inner extraction container). The extraction pressure was adjusted by needle and micrometric valves. The extraction temperature was controlled by a thermostatic batch. The flow rate of ethanol was regulated by a rotameter. The container was placed into the extraction vessel, and the consumption volume of ethanol was recorded by a gas meter. After the temperature of the extraction vessel reached the set point, the micrometric valves were closed. Then, ginger extracts (500 g) were loaded into a 5.0 L stainless steel extraction container (9.2 cm ID) with stainless steel filters placed at both ends to prevent carryover of the particles. The pressure of extraction vessel was controlled at 5,800 psi by a needle valve and set at 2 hours of static extraction time with a timer. The solute-rich fluid departing from the extractor was expanded through micrometric valves to atmospheric pressure. The flow rate was set at 6 NL/min and the total volume of gas was measured with a gas meter. The carbon dioxide discharge was recycled to cultivate ginger extracts to reduce carbon dioxide emission to the environment.

2.3. Determination of DPPH Radical Scavenging Capacity. DPPH is a stable free radical with violet color (absorbed at 517 nm), and DPPH solution changes its color to light yellow when free radicals are scavenged. Various concentrations of ginger extracts (100~5000 μg/mL) were added to 1 μL of stable DPPH (60 μM) solution and measured with a 96-well plate. When DPPH radicals react with antioxidative agents donating hydrogen, the solution color is reduced resulting in a decrease in absorbance at 517 nm (Table 1). The analyzed

Table 1: Antioxidant properties of ginger extracts on DPPH free radical scavenging, ferrous ion chelating, and reducing power ability assays.

Ginger (μg/mL)	DPPH (%)	Reducing power (OD_{700} nm)	Chelating (%)
100	NS	NS	NS
250	4.5	NS	NS
500	18.1	12.7	NS
2500	31.9	21.5	0.17 ± 0.00
5000	66.0	52.2	0.35 ± 0.01
Vitamin C[a]	87.5	—	—
BHA[b]	—	95.4	—
EDTA[c]	—	—	1.92 ± 0.04

(—): no testing.
Vitamin C[a] was used as a positive control on DPPH assay at 100 μM.
EDTA[b] was used as a positive control on metal chelating ability at 100 μM. BHA[c] was used as a positive control on reducing power at 100 μM. Mean ± SD.

time interval was 5 min per point, up to 30 min by using UV-vis spectrophotometer (BioTek Co.). Vitamin C was used as a positive control. The DPPH radical scavenging activity (%) was determined as

$$\text{scavenging activity\%} = \frac{\left(A_{\text{control}} - A_{\text{samples}}\right)}{A_{\text{control}}} \times 100\%. \quad (1)$$

2.4. Metal Chelating Activity. The ferrous ion chelating potential from ginger extracts (100~5000 μg/mL) was investigated according to a previously described method [20]. Briefly, testing samples dissolved in DMSO were added to a solution of 2 mM $FeCl_2 \cdot 4H_2O$ (10 μL). The reaction was initiated by the addition of 5 mM ferrozine (20 μL), and the mixture was vigorously shaken and left standing at 25°C for 10 min. The absorbance of the mixture was read at 562 nm against a blank. EDTA was used as a positive control, and the chelating activity calculation formula was similar to (1).

2.5. Reducing Power Assay. The reducing powers of ginger extracts (100~5000 μg/mL) were determined according to the method of [21]. In brief, testing compounds were mixed with 85 μL of 67 mM phosphate buffer (pH 6.8) and 2.5 μL of 20% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min, and then 160 μL of trichloroacetic acid (10%) was added to the mixture to centrifuge for 10 min at 3,000 ×g. The upper layer of the solution (75 μL) was mixed with 2% $FeCl_3$ (25 μL), and the absorbance was assayed with a 96-well plate at 700 nm. A higher absorbance demonstrates a higher reductive capability. BHA was used as a positive control.

2.6. Microorganism Strains. The four microorganismic strains used within this study, which were purchased from American Type Culture Collection (ATCC), included *Staphylococcus aureus* (ATCC 29213), *Streptococcus mutans* (ATCC 25175), *S. sobrinus* (ATCC 33478), and *Escherichia coli* (ATCC 35218). The most common species of *staphylococcus* is *S. aureus*, which causes staphylococcus infections and is frequently found in the human respiratory tract and on the skin surfaces. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g., methicillin-resistant *Staphylococcus aureus*, MRSA) is a worldwide problem in clinical medicine [22]. Another species in the *Streptococcus* species is *S. sobrinus* which is a spherically shaped anaerobic and Gram-positive bacterium. They grow in pairs or chains, and they are not motile and do not form spores. *S. mutans* is a facultatively anaerobic, Gram-positive, coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay. The most intensively studied prokaryotic model organism is *E. coli*, a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms. This bacterium is easily and inexpensively grown in a laboratory setting and has been studied intensively in the past half century [23].

2.7. Determination of Antibacterial Properties. The antimicrobial properties of the ginger extracts were investigated using previously described methods. Briefly, 10^5 CFU/mL microbial suspensions of four bacteria, *S. aureus*, *S. mutans*, *S. sobrinus,* and *E. coli*, were incubated in each well at 37°C for 24 hours. The microbial bacteria were then harvested in normal saline and adjusted to McFarland 0.5 (1.5×10^8 CFU/mL). One milliliter of each bacterial sample suspension was centrifuged at 9,000 rpm for 5 minutes and then treated with 0.3 mL of an extract to obtain a final concentration of 10% v/v. Reactions were compared at 25°C at intervals of 5, 30, 60, 180, 300, and 900 seconds, respectively. A well containing DMSO was used as the growth blank vehicle control; a well containing medium only was used as the negative control; all other wells contained the experimental groups treated with ginger extracts. The examination was considered valid if the well for the growth control group was positive and those for other groups were negative. After the specified reaction times, the bacteria were centrifuged for 2 minutes, washed once, and suspended in sterile saline water. A 10^4-fold dilution of the bacterial suspension (100 μL) was then plated on blood agar plates. After 24-hour incubation period time, the bactericidal effects of the extracts were determined in each sample by measuring bacterial growth in cultures. The inhibition of bacterial growth was then measured by comparison with normal growth observed in microbes not treated with the testing samples.

Table 2: Inhibition percentage of colony growth (CFU per milliliter) in *S. aureus*, *S. mutans*, *S. sobrinus,* and *E. coli.*

Ginger extract	Inhibition of bacterial growth (%) Exposure time (seconds)						
	0	5	30	60	180	300	900
S. aureus	0	45.5 ± 5.9	58.5 ± 4.4	63.2 ± 9.2	64.1 ± 8.7	69.3 ± 10.2	74.8 ± 8.5
S. mutans	0	2.7 ± 1.5	9.3 ± 1.5	9.7 ± 3.2	14.4 ± 12.1	16.9 ± 4.3	19.6 ± 3.6
S. sobrinus	0	23.8 ± 9.2	34.6 ± 5.5	38.0 ± 5.5	43.2 ± 4.2	50.8 ± 6.7	56.2 ± 7.7
E. coli	0	15.6 ± 6.2	36.7 ± 4.0	45.4 ± 3.1	59.1 ± 2.9	75.7 ± 7.9	86.7 ± 7.7

2.8. Cell Cultures. The human skin cancer A375 cell line was purchased from the Bioresource Collection and Research Center (BCRC number: 60039, Hsinchu, Taiwan). This malignant melanoma A375 cell line was derived from a 54-year-old female. The cells were seeded in 96-well plates at a density of 7×10^3 cells/well. The medium was then changed, and cells were maintained in either solvent alone (control cells) or in the presence of the indicated ginger extracts (50~1000 μg/mL) in a final volume of 100 μL within 10% FBS cellular culture medium. Each sample was added to a microplate and incubated under the same conditions as aforementioned.

2.9. Animal Material. In this study, the use of animals complied with the Guiding Principles in the Care and Use of Animals of the American Physiology Society and was approved by the National Kaohsiung Medical University and Use Committee. BALB/c nu/nu female mice (4-5 weeks) were purchased from BioLASCO Experimental Animal Center (Taiwan Co., Ltd). The mice were housed in Plexiglas cages in a temperature-controlled room (22 ± 1°C), on a 12-hour/12-hour light/dark schedule, and with free access to food and water. After one week, ten mice were randomly divided into 2 groups, control and drugs treatment groups.

2.10. Xenograft Tumor Assay. The performance of xenograft tumor assay was described previously with minor modifications [24]. In brief, BALB/c nu/nu female mice were housed and the *in vivo* experiments were performed at the animal center (Kaohsiung Medical University, Kaohsiung, Taiwan). Mice were implanted subcutaneously with 1×10^7 of A375 cells in 0.1 mL PBS injected subcutaneously in each mouse. Mice were treated four times a week with a subcutaneous injection of ginger extract (300 mg/kg) until sacrifice at day 35. The diameters of xenograft tumor were measured at 4 days intervals with vernier calipers and calculated as length × width^2/2 in mm^3 [25].

2.11. Blood Flow Measurement Using Laser Doppler Flowmetry. Mice used the tape fixed under test location and the moorLDI2 laser Doppler blood perfusion imagers (Moor Instruments Ltd, USA) was used to measure in the tumor periphery, and the digital color-coded images were analyzed to quantify blood flow in the back [26].

2.12. Statistical Analysis. The data were expressed as the mean value obtained in three experiments. Statistical comparisons were performed by Student t-test for paired values.

3. Results and Discussion

3.1. Determination of DPPH Radical Scavenging Capacity. The following purpose of this section was to survey the antioxidative properties of ginger extracts from supercritical fluid carbon dioxide extraction, and the testing samples were added at various concentrations (ranging from 100 μg/mL to 5,000 μg/mL). DPPH free radical scavenge testing mechanism is an acknowledged system by which antioxidants act to inhibit oxidation products; hence, this DPPH scavenging platform has been widely applied as one of the indicators for antioxidant abilities. In this DPPH assay procedure, antioxidants are able to decrease the stable purple DPPH radicals to the light yellow colored diphenyl-picrylhydrazine. The inhibition values of ginger extracts were listed in Table 1, and vitamin C at 100 μM was as a positive control within this assay. It showed a dose-dependent manner that ginger extracts had moderate-high scavenging capability in DPPH assay.

3.2. Metal Chelating Activity. The ferrous ion chelating activity of ginger extracts was described in Table 1. In ferrous ion chelating activity assay, ferrozine and Fe^{2+} can quantitatively form complexes and EDTA at 100 μM applied as a positive control to possess ion chelating capacity of 95.4%. In the presence of chelating agents, the reagent complex formation is disrupted, resulting in a reduction in the dark red color of the complex. Ginger extracts at the dosage of 5,000 μg/mL presented a middle-strong level on Fe^{2+} scavenging effects of 52.2%.

3.3. Reducing Power. Ferric reducing antioxidant power assay is a simple and reliable platform, measuring the reducing potential of an antioxidant reacting with a ferric 2,4,6-tripyridyl-s-triazine Fe(III)-TPTZ complex, producing a dark blue colored ferrous Fe(II)-TPTZ complex by an adopted reductant. This complex has a conspicuous blue color which can be monitored at 700 nm. A higher absorbance in spectrogram indicates a higher ferric reducing power. In Table 1, ginger extracts demonstrated a minor ferric reducing power at the high dosage of 5,000 μg/mL (0.35 ± 0.01), and the positive control was BHA at 100 μM (1.92 ± 0.04).

3.4. Antibacterial Effects of Extracts. The next experiment was to compare the antibacterial effects of ginger supercritical carbon dioxide fluid extracts. Table 2 showed that ginger extracts were with efficient inhibition for the growth of *S. aureus*, *S. sobrinus,* and *E. coli*, but not *S. mutans*. The antibacterial

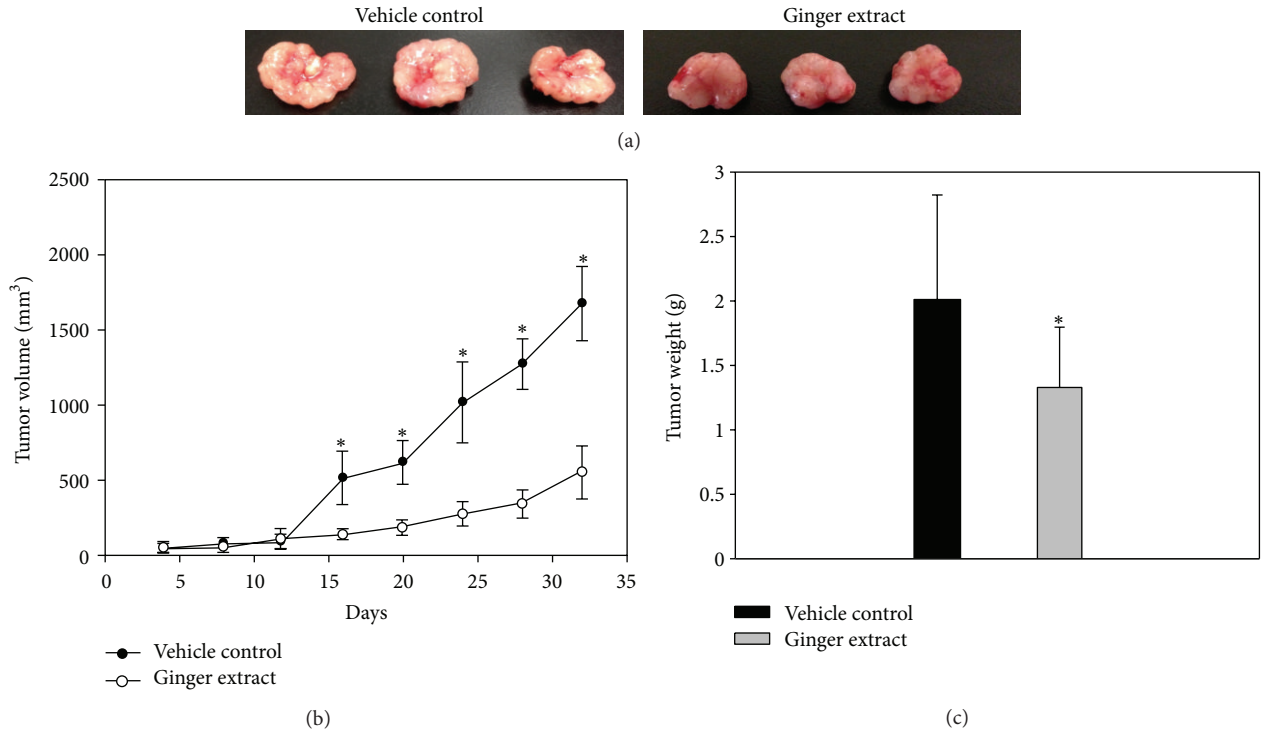

Figure 1: Ginger extract inhibits tumor growth in xenografts tumor assay. (a) Photo of mice and dissected tumors from vehicle control group and treated with ginger extracts 300 mg/kg. (b) Average tumor volume of vehicle control group versus ginger extracts and (c) average tumor volume weight were measured at the end of experiment. Five the number of samples were analyzed in each groups, and value represent the mean ± SD. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

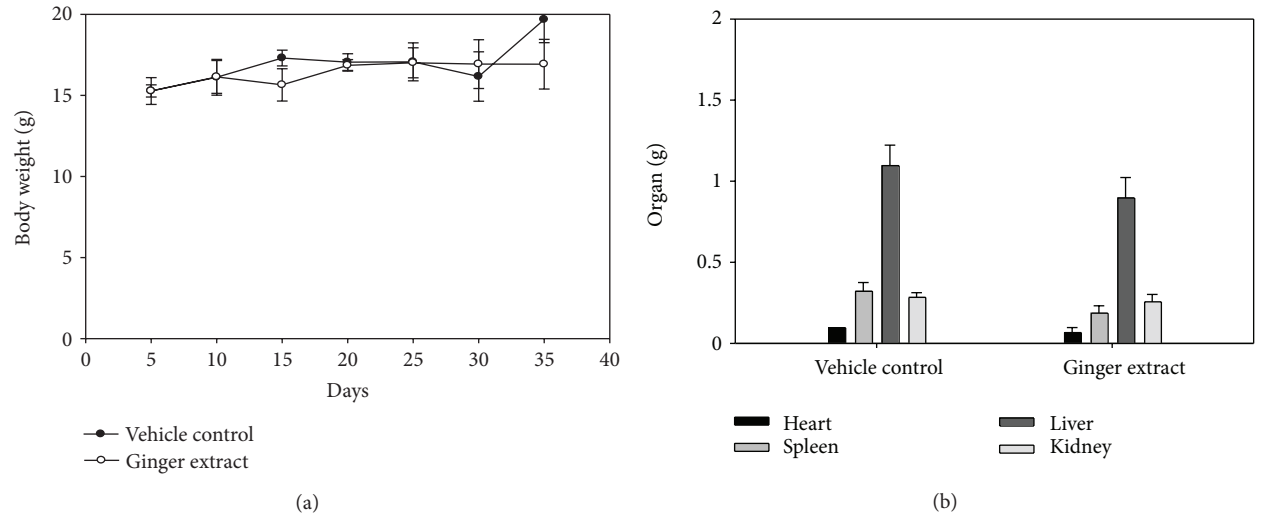

Figure 2: Effect of ginger extracts in organs in xenograft assay. (a) Average body weight of vehicle control group versus ginger extracts and (b) average organs (heart, spleen, liver, and kidney) of vehicle control group versus ginger extracts.

assay illustrated that ginger extracts effectively inhibited both Gram-negative and Gram-positive bacteria. Generally, for each microbe, whether Gram-negative or Gram-positive, the growth phenomenon was time-dependently inhibited by the exposures of 5, 30, 60, 180, 300, and 900 seconds.

3.5. The Melanoma Growth Inhibition on Xenograft Tumor by Ginger Extracts. The cellular proliferation assay illustrated antigrowth effects in nude mice on skin cancer cells (Figure 1). In this study, we examined the therapeutic efficacy of supercritical carbon dioxide fluid ginger extracts *in vivo*

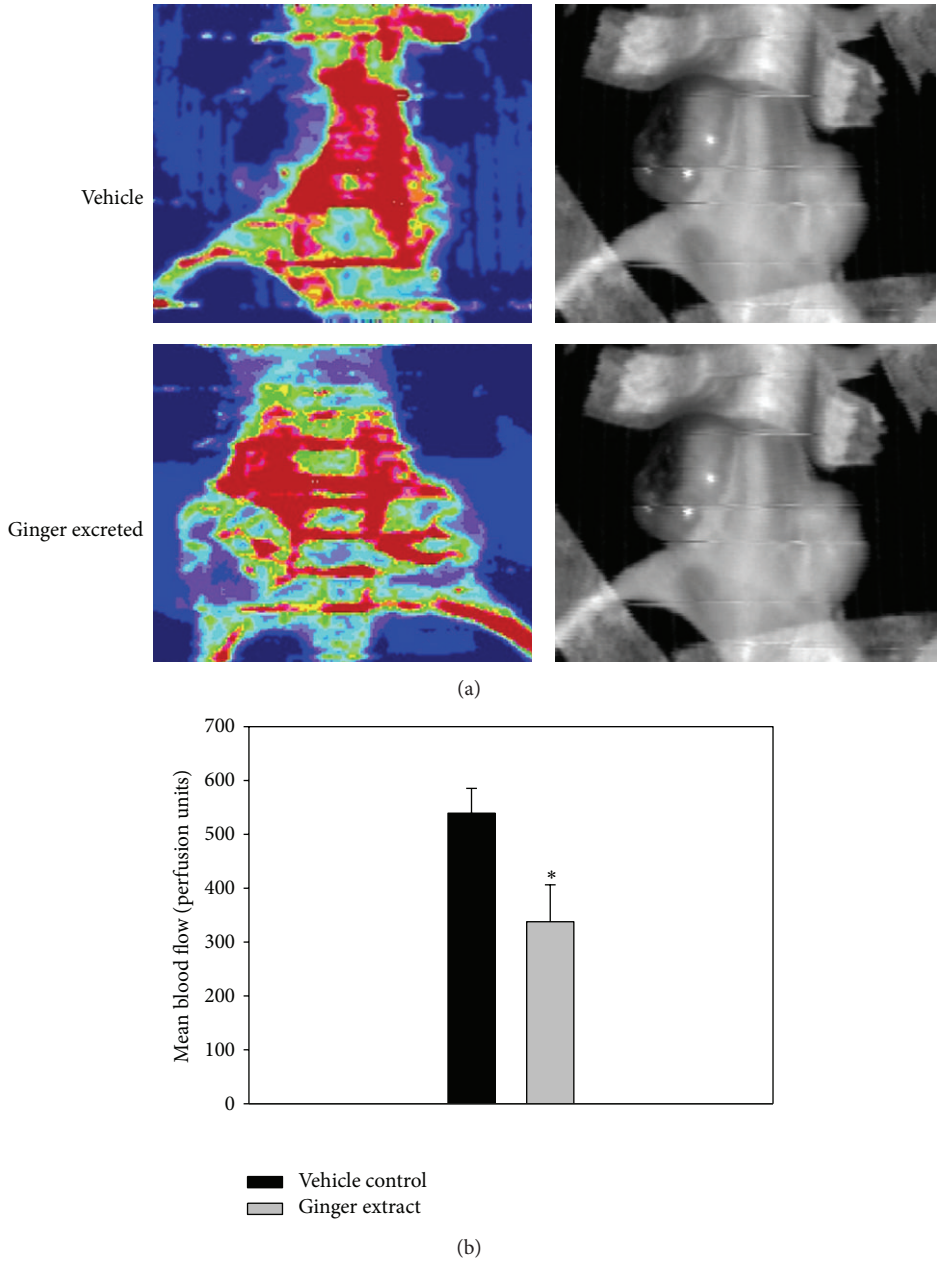

FIGURE 3: Effect of ginger extracts shows a laser Doppler scan of blood flow *in vivo*. (a) Photo of mice blood flow from vehicle control group and treated with ginger extracts 300 mg/kg. (b) Average blood flow of vehicle control group versus ginger extracts. Comparisons were subjected to Student's t-test. Significantly different at $^{*}P < 0.05$.

tumor xenograft by treating BALB/c nu/nu female mice bearing human malignant melanoma A375 cell lines, at a concentration of 300 mg/kg. After the establishment of palpable tumors (the mean tumor volume was around 50~100 mm^3), animals received subcutaneous injections of ginger extracts four times for one week. After 35 days, we found out that the cellular A375 proliferation was reduced significantly compared to the vehicle control, and photographs in Figure 1(a) were the malignant cells of human skin cancer to show the inhibitory effects of ginger extracts. Obvious reducing properties were discovered at treatment conditions, and the quantification analysis of tumor volume and tumor weight was presented in Figures 1(b) and 1(c). We confirmed the inhibitory effect of ginger extracts on xenograft tumor assay, significantly.

3.6. Effects of Ginger Extracts on the Mice Organ Weights and the Scanning Images of Laser Doppler Flowmetry In Vivo. Within mice receiving the treatment regimens, no gross signs of toxicity were observed (body weight, visible control inspection of general appearance, and microscopic examination of individual organs) (Figures 2(a) and 2(b)). In the tumors periphery, the blood flow was higher than that in the normal stomach tissues [27]. We analyzed the

tumor blood flow around the experimental areas and showed that the blood flow of ginger extracts group was reduced compared to the vehicle control group (Figures 3(a) and 3(b)). Therefore, all of the results pointed out the promising potential of ginger extracts on antimelanoma treatment in the future.

4. Conclusion

Our research results showed that ginger extracts from supercritical fluid carbon dioxide extraction demonstrated DPPH free radical scavenging ability, reducing power and chelating property in dose-dependent manners. This research directly confirmed that the importance of ginger extracts had positively influencing levels of dietary antioxidants in the human body. Ginger extract proved the antibacterial activities against both gram-negative bacteria (*E. coli* and *S. sobrinus*) and gram-positive bacteria, (*S. aureus* and *S. mutans*), but *S. mutans* has no obvious effect in antibacterial activities. In animal experiments, the nude mice were pretreated with A375 melanoma cells, and the ginger extracts illustrated an obvious reduction in cell proliferations *in vivo* xenografts. To sum up, it was the first study of ginger extracts from supercritical fluid carbon dioxide to be potentially applied in food additive, infectious inhibition, or anticancer agents.

Conflict of Interests

No contributing author has a conflict of interests in the publication of this study.

Acknowledgments

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Research Article

Online Monitoring Oxidative Products and Metabolites of Nicotine by Free Radicals Generation with Fenton Reaction in Tandem Mass Spectrometry

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In general, over 70% absorbed nicotine is metabolized to cotinine and trans-3′-hydroxycotinine by cytochrome oxidase P450, and nicotine is also a major addictive and the psychoactive component in cigarettes. As a xenobiotic metabolism, hydrophobic compounds are usually converted into more hydrophilic products through enzyme systems such as cytochrome oxidase P450, sulfotransferases, and UDP-glucuronosyltransferases to deliver drug metabolites out of the cell during the drug metabolic process. In this study, an electrodeless electrochemical oxidation (EEO) reaction via Fenton reaction by producing free radical to react with nicotine to immediately monitor the oxidative products and metabolic derivatives of nicotine by tandem mass spectrometer (MS) is done. Fenton reaction generates free radicals via ferrous ion (Fe^{2+}) and hydrogen peroxide (H_2O_2) to oxidize DNA and to degrade proteins in cells. In the EEO method, the oxidative products of nicotine including cotinine, cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nornicotine, norcotinine, 4-oxo-4-(3-pyridyl)-butanoic acid, 4-hydroxy-4-(3-pyridyl)-butanoic acid, and nicotine-*N*′-oxide were detected by tandem mass spectrometer to simulate the changes of nicotine and its derivatives in a time-dependent manner.

1. Introduction

A free radical can be defined as an atom, molecule, or ion with an unpaired valence electron [1] and has a strong reactivity to attack other molecules or generate new free radicals by atom transfer radical polymerization (ATRP) [2]. Free radicals, such as superoxide, nitric oxide (NO), thyl, peroxyl, and hydroxyl radical, play important roles in biological processes [3], and these oxygen-containing free radicals usually originate from losing a partial valence electron in electron transport chain at mitochondria. The terms "reactive oxygen species (ROS)" and "reactive nitrogen species (RNS)" contain not only free radicals but also active reagents such as hydrogen peroxide (H_2O_2), singlet oxygen, and ozone (O_3) in living organisms.

ROS- and RNS-related oxidative stress resulted in disorders including serious aging, cancers, stroke, and diabetes [4, 5]. According to the previous reports, free radicals were also involved in neurodegenerative disease such as Alzheimer's disease and Parkinson's disease [6]. However, not all of the free radicals play harmful roles in human health. Nitric oxide (NO), generated by nitric oxide synthases (NOSs) when L-arginine is converted to citrulline, serves as a cellular signaling molecule to regulate vasodilatation in blood vessels by

activating guanylate cyclase, guanosine 3′,5′-monophosphate (cyclic GMP), and protein kinase G to relax smooth muscle via proteins phosphorylation [7].

In the last decade, metabolomics has progressed at a marvelous rate in the omics field. At an early stage of drug development, rat liver microsomes (RLMs) with specific cytochrome P450 (CYP450) activity were employed as an approach for the investigation of drug metabolism [8, 9]. However, in drug discovery, it is time consuming, labor consuming, and expensive for the target molecules screening, pharmacodynamics, and pharmacokinetics followed by a series of *in vivo* and *in vitro* experiments. Fortunately, Volk et al. utilized on line electrochemical cell coupled with tandem mass spectrometer (EC-MS) to proceed with new instrumental analyses for oxidative metabolic molecules [10–13]. Furthermore, Karst's group had extended the application of EC-MS to connect with liquid chromatographic (LC) system by a switch valve to be an EC-LC-MS [14]. The EC-LC-MS system is not described as a continuous analysis but as an online sequential separation [14, 15]. Moreover, Karst's group had utilized EC-MS techniques to simulate the metabolic pathways of drug such as a muscle relaxant tetrazepam and an antiarrhythmic drug procainamide [14, 16], to investigate and identify nucleotide oxidative products [17], and to analyze the oxidation of aniline with the formation of protein adducts [18]. Moreover, the cyclic voltammetry (CV) technique has been utilized to evaluate redox reaction of various biomolecules with their oxidative derivatives and to compare the oxidative products by different electrodes [19–25].

In this study, we generated free radicals by Fenton reaction (Figure 1) to investigate the oxidative products or possible metabolites of nicotine with an online sequential analytic mass spectrometer system. Besides, Fenton reaction is an oxidative reaction occurring in mitochondria when ferrous ion (Fe^{2+}) reacts with H_2O_2 to generate free radicals such as the hydroxyl radical ($OH^{\bullet}$) and superoxide anion. Meanwhile, superoxide dismutase (SOD) converts superoxide anion to H_2O_2. The total reaction is described as Haber-Weiss reaction (Figure 1), and the net reaction shows that superoxide anion and H_2O_2 are converted to oxygen, hydroxide ion, and hydroxyl radical. Avoiding contamination of electrodes and crack of electrochemical flow cell (max. pressure: 40 psi, Antec Leyden, Zoeterwoude, The Netherlands) [14, 15, 17, 18] caused by high pressure of HPLC, online electrodeless electrochemical oxidation (EEO) of free radicals generated by Fenton reaction was a novel technique to monitor the oxidative products and metabolites of nicotine. The EEO/HPLC separated system was equipped with electrospray ionization (ESI) and tandem mass spectrometer (MS/MS) to be an online EEO/HPLC ESI-MS/MS monitoring system. According to this EEO/HPLC ESI-MS/MS electrochemical equipment, the metabolic derivatives of nicotine [26] including cotinine, cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nornicotine, norcotinine, nicotine-*N*′-oxide, 4-oxo-4-(3-pyridyl)-butanoic acid, and 4-hydroxy-4-(3-pyridyl)-butanoic acid were detected by tandem mass spectrometer.

Haber-Weiss reaction

$$Fe^{3+} + O_2^{-\bullet} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Fenton reaction)}$$

$$\text{Net reaction: } O_2^{-\bullet} + H_2O_2 \longrightarrow OH^- + OH^{\bullet} + O_2$$

Figure 1: The Haber-Weiss Reaction and Fenton reaction for hydroxyl free radical generation.

2. Materials and Methods

2.1. Chemicals. The reagents including hydrogen peroxide (H_2O_2), nicotine, sodium acetate (CH_3COONa), and potassium titanium oxide oxalate dehydrate ($C_4O_9K_2Ti\cdot 2H_2O$) were provided by Sigma-Aldrich (St. Louis, MO, USA). The concentrated sulfuric acid, anhydrous methanol (MeOH), and ferrous sulfate heptahydrate ($FeSO_4\cdot 7H_2O$) were purchased from J. T. Baker (Phillipsburg, NJ, USA). The chemicals such as 1, 10-phenanthroline monohydrate ($C_{12}H_8N_2\cdot H_2O$), acetic acid, and ammonium acetate (NH_4CH_3COO) were purchased from Aldrich (Milwaukee, WI, USA). Distilled water was prepared to 18.2 MΩ cm resistivity at 25°C by a Milli-Q system (Millipore, Bedford, MA).

2.2. The Concentration of H_2O_2 and Fe^{2+} Solutions. The concentration of H_2O_2 was determined by Sellers' method [27, 28]. The reagent of potassium titanium (IV) oxalate ($K_2TiO(C_2O_4)\cdot 2H_2O$ 354 mg was mixed with 2.72 mL of concentrated sulfuric acid and 3 mL of deionized water. After the diluted sulfuric acid solution was cooled to room temperature, it was adjusted to 10 mL by distilled water. Titanium(IV) solution 500 μL was composed of different concentration of H_2O_2 solutions (500 μL) and displayed yellow-orange complex detected by spectrophotometer at 400 nm. The calibration curve was plotted by the intensities of spectrophotometer of different H_2O_2 concentration.

The concentration of ferrous ion was determined by ferroin indicator. Ferroin indicator includes both solution A (100 mg of 1,10-phenanthroline monohydrate dissolved in 100 mL distilled water) and solution B (25 g of ammonium acetate mixing with 15 mL distilled water and 70 mL acetic acid). The different concentrations of ferrous solutions (1 mL) were prepared with 40 μL solution A and 100 μL solution B, respectively. After 25 mins, solutions were detected by spectrophotometer at 510 nm. The calibration curve was plotted by the intensities of spectrophotometer at different ferrous sulfate concentrations.

2.3. The Preparation of Fenton Reaction and Nicotine. The reactive solution composed of 10 μL nicotine (20 ppm), 90 μL H_2O_2 (3%), 1890 μL sodium acetate buffer (50 mM, pH 5.6), and 10 μL ferrous sulfate heptahydrate (100 mM) solutions was adjusted to 200 ppb nicotine, 0.135% H_2O_2, and 0.5 mM $FeSO_4$. The ultrahigh performance liquid chromatography (UHPLC) gradient was set at 30 mins in one experiment. The sequential analyses were performed by injection of 10 μL nicotinic oxidative mixture via the syringe of autosampler every 30 mins.

Table 1: Metabolic derivatives of nicotine in EEO/ESI-MS/MS. The molecular formula, molecular weight, and m/z of parent ion and daughter ions are listed.

Name	Molecular formula	Molecular weight (Da)	Parent ion	Daughter ions
Nicotine	$C_{10}H_{14}N_2$	162.12	162.2	117, 130, 132
Cotinine	$C_{10}H_{12}N_2O$	176.22	177.7	80, 119.5, 134.5
trans-3′-Hydroxyl cotinine	$C_{10}H_{12}N_2O_2$	192.21	194.0	106, 118, 134
Nornicotine	$C_9H_{12}N_2$	148.20	148.7	80, 130
Nicotine-*N*-oxide	$C_{10}H_{14}N_2O$	178.23	179.0	120, 130, 148
Norcotinine	$C_9H_{10}N_2O$	162.19	162.5	67, 93, 119
4-Oxo-4-(3-pyridyl)-butanoic acid	$C_9H_9NO_3$	179.17	180.2	81, 99.5, 108.6
4-Hydroxy-4-(3-pyridyl)-butanoic acid	$C_9H_{11}NO_3$	181.07	182.2	84, 91, 122, 136
Cotinine-*N*′-oxide	$C_{10}H_{12}N_2O_2$	192.21	192.9	80, 121

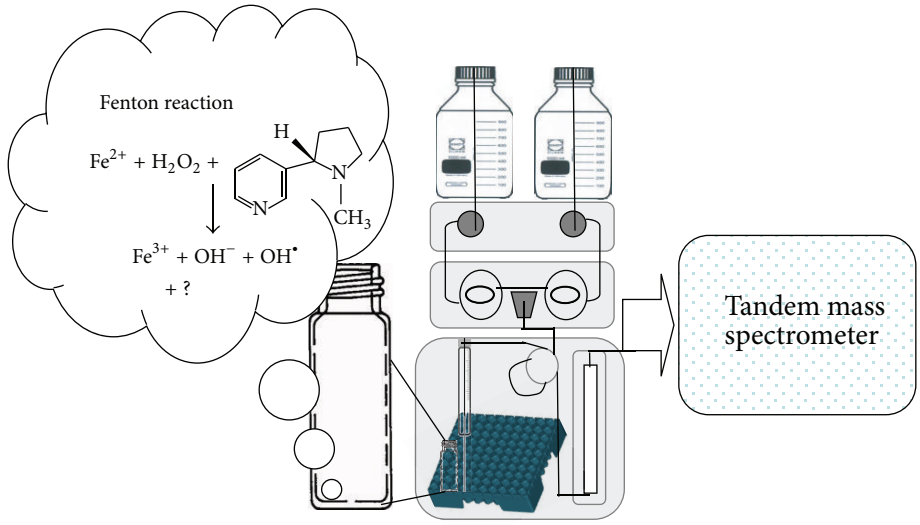

Figure 2: The schematic representations of experimental apparatus and Fenton reaction oxidative processes. Nicotine reacted with hydroxyl free radical in sample bottle and nicotinic oxidative products or metabolic derivatives were injected via syringe into analytic column in autosampler.

2.4. UHPLC and ESI-MS Analytic Conditions. Immediate online electrospray ionization mass spectrometry (ESI-MS) analyses of mixture with nicotine and Fenton reaction reagents were detected by a Thermo Finnigan TSQ Quantum Ultra Mass Spectrometer Analytic System (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with the Micro ESI ion source which was set at 3.0 kV coupled with Acella 1250 UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The oxidative mixture was subject directly into the UHPLC via Acella 1250 autosampler and was separated by Shiseido HPLC CAPCELL PAK C18 MGII column (150 mm × 1.5 mm, 3.0 μm, Tokyo, Japan). The UHPLC flow rate was set at 250 μL/min (gradient pump). The mobile phases were composed of (A) 10 mM NH_4CH_3COO in water and (B) 10 mM NH_4CH_3COO in 100% MeOH with a linear gradient followed from 5% (B) in 2 min, 5%–40% (B) in 20 min, 40%–98% in 5 min, 98% (B) in 2 min, 98%–5% (B) in 0.1 min, and 5% (B) in 2.9 min. The nicotine, oxidative chemicals, and its metabolic derivatives were detected by mass spectrometer with applying voltage of 3.0 kV in the positive ion mode, vaporizing and capillary temperature set at 300°C and 350°C, respectively, sheath gas and aux gas pressure set at 35 and 10, respectively, collision pressure at 1.5, and collision energy adjusted at 25 V. The survey scan mode was set at m/z 50–250 Da in the Quadrupole I chamber and nicotinic oxidative ions were detected and selected (intensity > 10^4) in MS mode with three high intensity signals (data dependant scan), transferred into collision-induced dissociation (CID) chamber for MS/MS fragmentation and further detected in Quadrupole III chamber. The Xcalibur software (version 2.2, Thermo-Finnigan Inc., San Jose, CA) was utilized to control and adjust mass spectrometry instrument and data acquisition.

3. Results and Discussion

In this study, Fenton reaction generated free radical to react with nicotine and produced nicotinic oxidative products

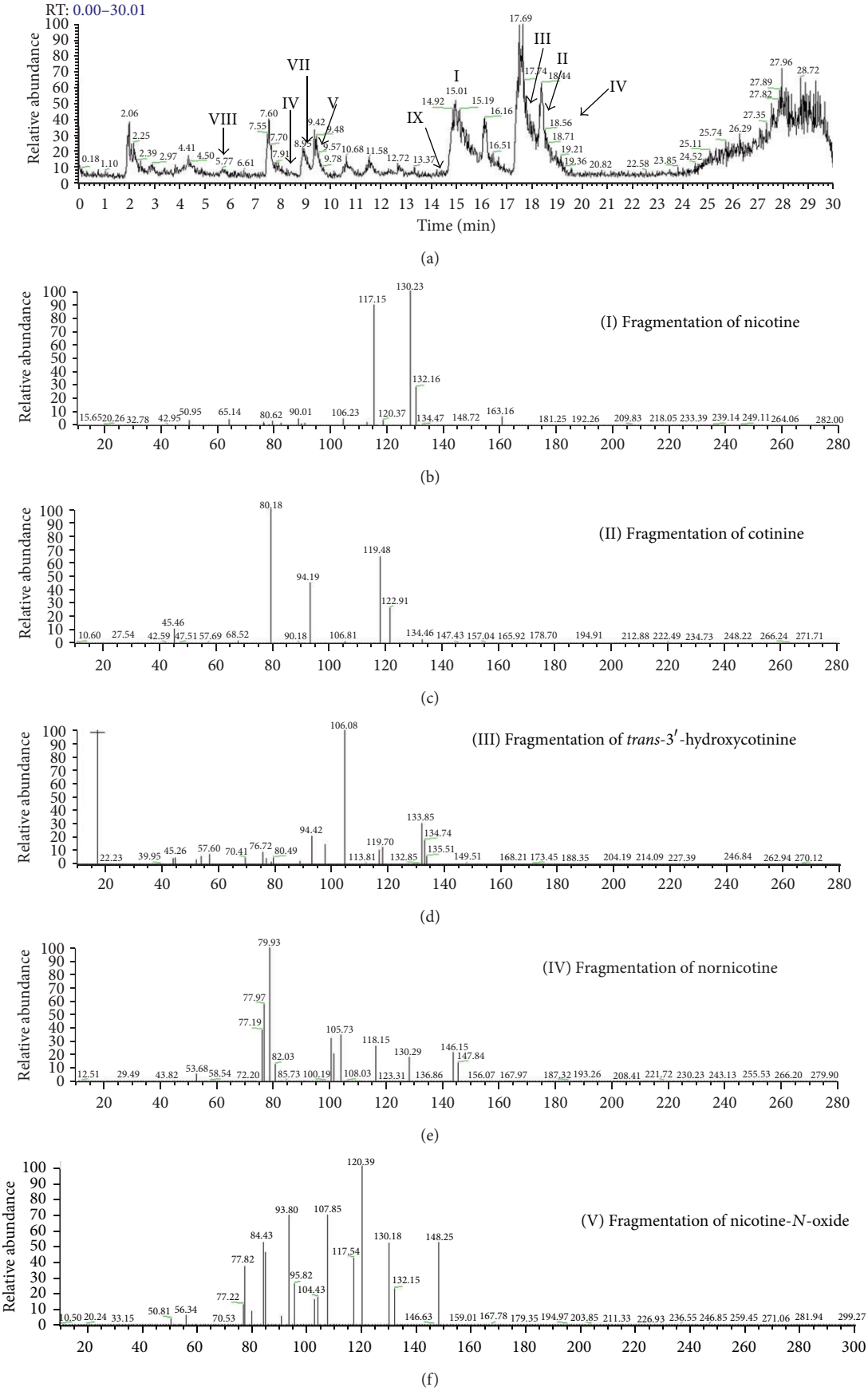

Figure 3: Continued.

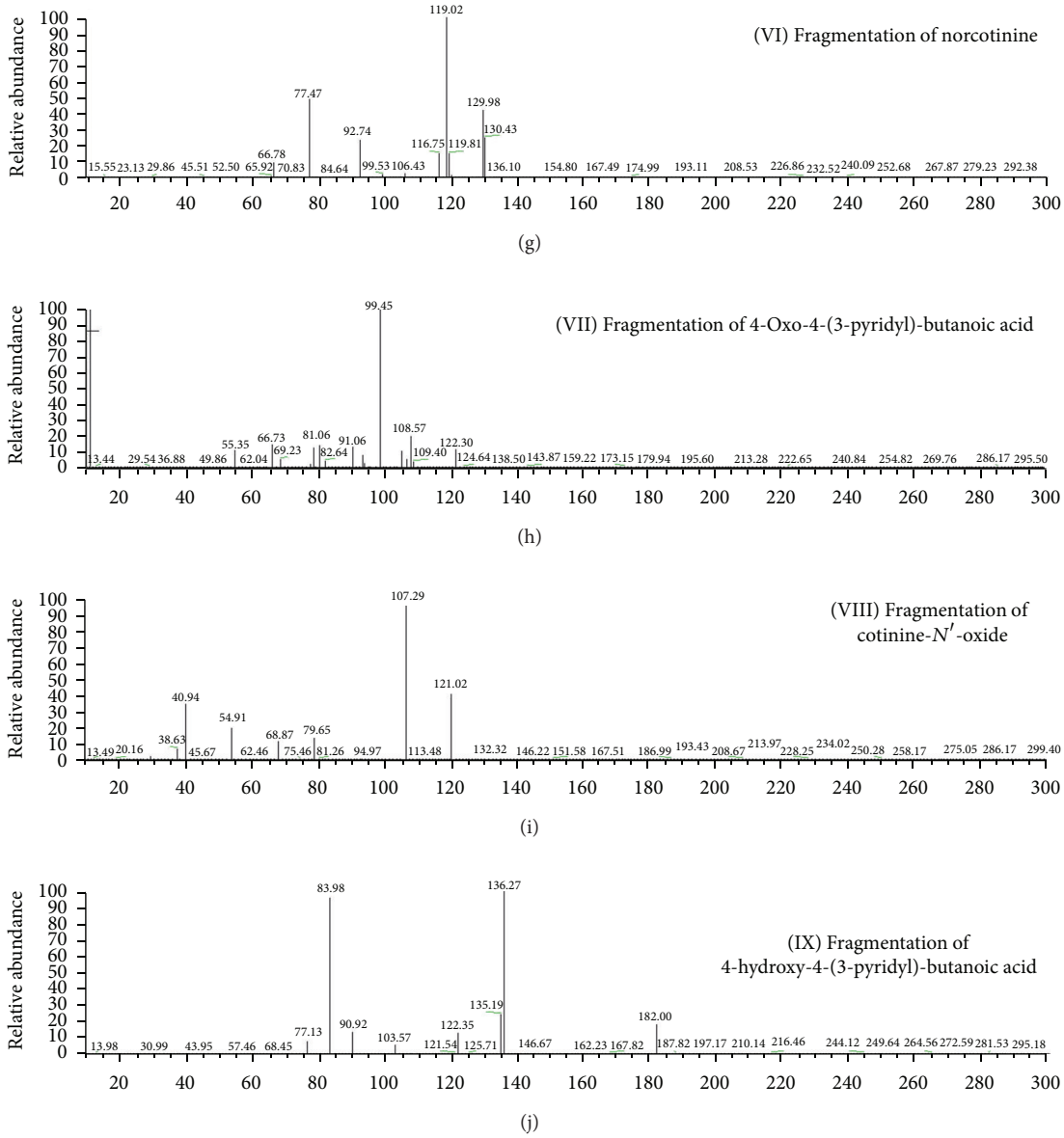

Figure 3: Electrodeless electrochemical oxidation (EEO) method coupled with UHPLC-MS/MS showed the spectra of the nicotine and its derivatives labeled with I–IX. The spectra displayed (a) base peak chromatogram and fragmentation pattern of nicotine (b), cotinine (c), *trans*-3′-hydroxyl cotinine (d), nornicotine (e), nicotin-*N*-oxide (f), norcotinine (g), 4-oxo-4-(3-pyridyl)-butanoic acid (h), 4-hydroxy-4-(3-pyridyl)-butanoic acid (i), cotinine-*N*′-oxide (j).

and derivatives. The electrodeless electrochemical oxidation (EEO) technique integrated with tandem mass spectrometer (EEO/UHPLC-ESI-MS/MS) for oxidative derivatives monitoring. By MS characterization, the results of nicotine and its derivatives were listed in Table 1.

3.1. Free Radical Generation from Haber-Weiss Reaction. As a catalytic reagent, Fe^{2+} reacted with H_2O_2 for producing hydroxyl free radical and oxygen. In the process of free radical generation, bubbles (O_2) were produced from the sample bottle. The Fenton reaction generating hydroxyl free radical was a side reaction of Haber-Weiss reaction. The chemical reaction formulas of Haber-Weiss reaction and Fenton reaction were listed in Figure 1.

3.2. EEO/UHPLC ESI-MS/MS Equipment for Nicotine and Its Oxidative Derivatives Monitoring. The schematic representation of experimental apparatus was showed in Figure 2. The mixture of free radical and nicotinic derivatives was injected by the syringe of autosampler into UHPLC system. In this technique, nicotine and its oxidative derivatives could be monitored in a run-to-run and time-dependent manner (data not shown). According to the previous studies [29–31], nicotinic metabolites detected in hair, urine, and plasma, and

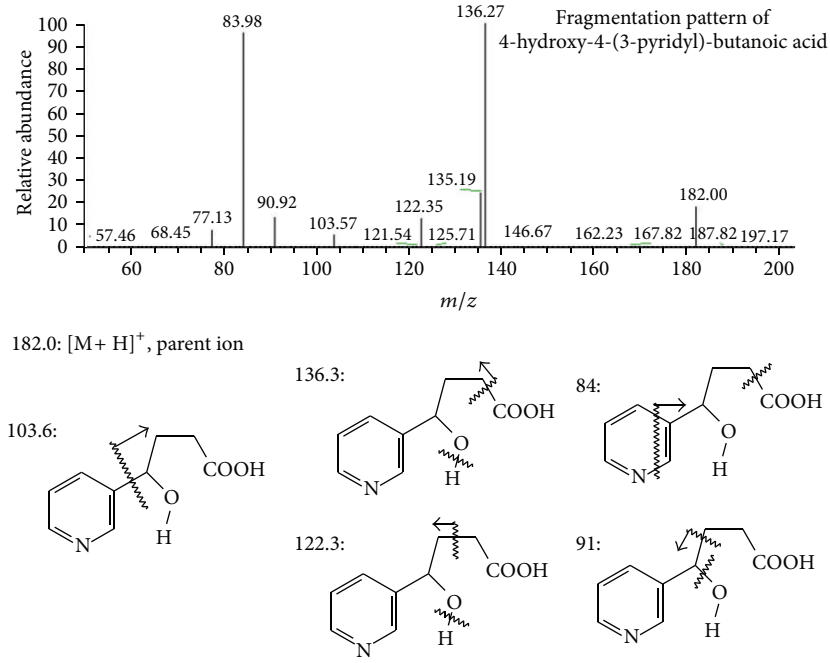

Figure 4: ESI-MS/MS fragmentation pattern spectrum of nicotinic derivative: 4-hydroxy-4-(3-pyridyl)-butanoic acid.

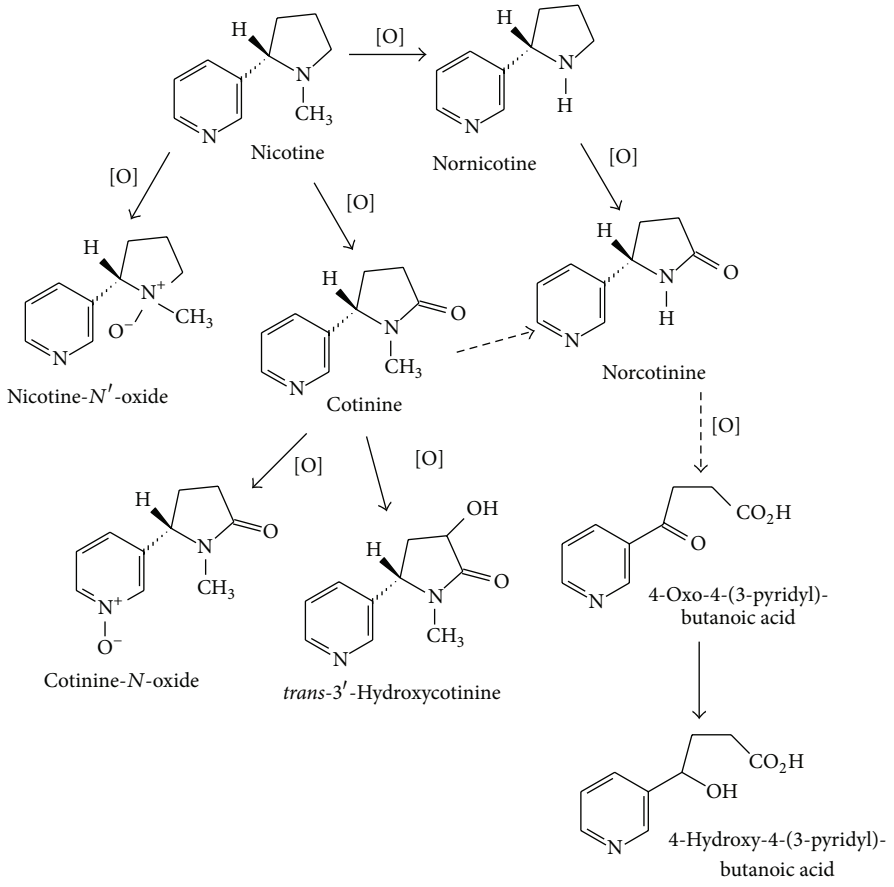

Figure 5: Pathways establishment of nicotine metabolism in electrodeless electrochemical oxidation (EEO) method coupled with UHPLC ESI-MS/MS.

nicotine, 3-hydroxycotinine, or cotinine were selected as candidates. However, in the EEO/UHPLC ESI-MS/MS method, nicotinic metabolites including cotinine, cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nornicotine, norcotinine, nicotine *N*′-oxide, 4-oxo-4-(3-pyridyl)-butanoic acid, and 4-hydroxy-4-(3-pyridyl)-butanoic acid were identified. The characterization of nicotine and its derivatives with name of derivatives, molecular formula, molecular weight, *m/z* of parent ion, and daughter ions was listed in Table 1. Furthermore, UHPLC base peak chromatogram and the fragmentation pattern of each metabolite were shown in Figure 3. The fragmentation patterns of nicotine and its derivatives showed that each derivative was selected by mass spectrometer (depending on different molecular weights) and fragmentized by collision gas with kinetic energy in collision chamber. The fragments (daughter ions) of MS/MS data labeled with English vocabulary from (b) to (j) were shown in Figure 3 and Table 1. We selected nicotinic metabolic reagent, 4-hydroxy-4-(3-pyridyl)-butanoic acid, as a representative; the fragmentation pattern and predictive fragments of this metabolite were shown in Figure 4. According to Figure 3, in the fragmentation patterns of nicotine, cotinine, *trans*-3′-hydroxycotinine, nornicotine, nicotine *N*′-oxide, and cotinine-*N*′-oxide, the spectra included the pyridinium ion at *m/z* 80. Unfortunately, the pyridinium ion could not be observed at the fragmentation patterns of 4-hydroxy-4-(3-pyridyl)-butanoic acid and 4-oxo-4-(3-pyridyl)-butanoic acid. However, in the predictive fragments of 4-hydroxy-4-(3-pyridyl)-butanoic acid spectra, we could track the sources of each daughter ion. To sum up, EEO/UHPLC ESI-MS/MS method could be utilized to simulate the generation of metabolites and to monitor the changes in a time-dependent manner.

3.3. Establishment of Nicotinic Oxidative Pathway. According to the previous study [26] and the spectra shown in Figures 3 and 4 and Table 1, we could establish the oxidative pathway of nicotine. Moreover, the nicotinic oxidative metabolites characterized by EEO/UHPLC ESI-MS/MS could be integrated into the metabolism pathway, further shown in Figure 5. The arrow with a solid line directly showed the metabolic processes and the arrow with a dotted line indicated that this pathway required an intermediate. The label of [O] showed that the metabolite was generated via oxidative reaction.

4. Conclusion

The establishment of nicotinic metabolic pathway based on the electrodeless electrochemical oxidation (EEO) utilizing Fenton reaction to produce hydroxyl free radical to react with nicotine was demonstrated. The online EEO/UHPLC ESI-MS/MS analytic system setup could be employed in metabolomics study. Thus, nicotinic metabolic pathway was established via the characterized oxidative nicotinic derivatives. The tandem mass spectrometer is a powerful analytic instrument with characterization of nicotinic derivatives by fragmentation patterns. However, by the sequential analyses, this analytic technique could not immediately monitor the changes of nicotinic metabolites. In the future, Fenton reaction (Fe^{2+}/H_2O_2) has the potential to be utilized in exploring oxidative products of biomolecules including DNA, small molecular drugs, and formation of protein adducts.

Conflict of Interests

The authors declare that they have no conflict of interests in the study.

Acknowledgments

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Review Article

Long Noncoding RNAs-Related Diseases, Cancers, and Drugs

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Long noncoding RNA (lncRNA) function is described in terms of related gene expressions, diseases, and cancers as well as their polymorphisms. Potential modulators of lncRNA function, including clinical drugs, natural products, and derivatives, are discussed, and bioinformatic resources are summarized. The improving knowledge of the lncRNA regulatory network has implications not only in gene expression, diseases, and cancers, but also in the development of lncRNA-based pharmacology.

1. Introduction

Less than 2% of the mammalian genome is in protein-encoded regions, and the remainder is in noncoding RNAss (ncRNAs) [1]. Most long noncoding RNA (lncRNAs) are transcribed by RNA polymerase (Pol) II/Pol I, and some are transcribed by RNA Pol III [2]. The ncRNAs with nucleotide lengths of <200 and >200 are classified as short and long ncRNAs (lncRNAs), respectively. The lncRNAs can be further classified in terms of their orientation and location relative to neighboring genes as sense/antisense, divergent/convergent, and intronic/intergenic [3]. The lncRNAs function as chromatin scaffolds for complex assembly, as enhancers and decoys for improving and inhibiting transcription of target genes, and as *cis*-acting or *trans*-acting regulators of gene expression [4–6]. *Cis*-acting lncRNAs mediate local genes whereas *trans*-lncRNAs mediate multiple targets [6]. By dysregulating target gene expression, abnormal lncRNA expression causes cell dysfunction and disease progression. The official symbols of lncRNAs were designated by the HUGO Gene Nomenclature Committee [7].

2. The lncRNAs and Gene Expressions

The lncRNAs modulate cell cycle distribution and cell differentiation. For example, DNA damage-inducible lncRNA, namely, growth-arrested DNA damage-inducible gene 7 (gadd7), binds to TAR DNA-binding protein (TDP-43). By blocking the interaction between TDP-43 and cyclin-dependent kinase 6 (Cdk6) mRNA, gadd7 regulates cell cycle progression by promoting the decay of Cdk6 mRNA [17]. The lncRNAs reportedly modulate the differentiation of cells [18], the induction of pluripotent stem cell [19], and the induction of embryonic stem cells [20].

Some lncRNAs also have modulating effects on apoptosis [21]. For example, lncRNA, namely, erythroid prosurvival (EPS) is upregulated in terminal differentiation of murine erythroid cells [22] by inhibiting apoptosis [23]. Similarly, a study of melanoma cell lines showed that by downregulating sprouty homolog 4 intronic transcript 1 (SPRY4-IT1), lncRNAs inhibit cell proliferation and apoptosis [24].

In human cells, lncRNAs epigenetically regulate gene expression [25, 26] through chromatin remodeling [27]. For example, the mouse lncRNA, namely, potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) overlapping transcript 1 (Kcnq1ot1) has a chromatin-interacting ability and can downregulate multiple genes in the Kcnq1 domain [28]. This gene silencing was reported to be mediated by DNA methylation at some target genes [29]. Other studies of cancer patients show that silenced tumor suppressor genes are often hypermethylated [30–32]. In the case of tumor suppressor genes, the epigenetic effect may have a role in carcinogenesis. In Hox antisense intergenic RNA (HOTAIR), long intergenic noncoding RNA (lincRNA), which is lncRNAs transcribed from noncoding DNA regions between protein-coding genes [33], may function as scaffolds for assembly of histone modification machinery [34].

Some lncRNAs may function through repeat sequences. For example, some lncRNAs that contain Alu elements [35] may transactivate Staufen 1- (STAU1-) mediated mRNA decay (SMD) by base pairing of Alu elements within both lncRNAs and 3′ untranslated region of the SMD target. These lncRNAs then downregulate several SMD targets [35].

3. The lncRNAs and Diseases

The functions of lncRNAs that are known to have roles in diseases have been reviewed previously [36, 37]. Recent studies suggest that lncRNAs have roles in neurodegenerative disorders [38, 39] and brain development [40]. In Huntington's disease, for example, neural lncRNAs are upregulated in taurine upregulated 1 (TUG1) and in nuclear paraspeckle assembly transcript 1 (NEAT1) but are downregulated in maternally expressed 3 (MEG3). The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) lncRNA is reportedly highly upregulated in neurons. In cultured hippocampal neurons, synaptic density is reduced by MALAT1 depletion but rescued by MALAT1 overexpression. Studies of patients with alcohol addiction reveal upregulated MALAT1 in the cerebellum, hippocampus, and brain stem [41], which suggests that the lncRNA network may have key roles in neurodegenerative processes [42].

Studies of patients with facioscapulohumeral muscular dystrophy (FSHD) involving Polycomb/Trithorax epigenetic regulation show a deregulated copy number in D4Z4 repeat mapping to 4q35 [43]. A recent study of FSHD patients further showed that selective upregulation of DBE-T, a chromatin-associated lncRNA, reverses repression of 4q35 gene transcription [44]. These results suggest that lncRNAs derived from repetitive sequences may contribute to disease development through epigenetic regulation.

Recently, the single nucleotide polymorphisms (SNPs) of lncRNAs have been found to play important roles for disease association studies. For example, the SNP rs1333049 in the lncRNA, namely, antisense noncoding RNA in the INK4 locus (ANRIL) is reportedly associated with myocardial infarction as well as the pharmacogenomic evaluation in hypercholesterolemia [45]. SNP rs2383207 on lncRNA-ANRIL and SNP rs11066001 on protein-coded BRCA1 associated-protein (BRAP) gene were both associated with ankle-brachial index in a Taiwanese population [46]. Three SNPs (rs2067051, rs2251375, and rs4929984) located in 5′ region of the H19 imprinted maternally expressed transcript (H19) genes were reportedly associated with birth weight [47]. Additionally, the rs2839698 TC genotype of H19 was reportedly associated with a low risk for nonmuscle-invasive disease [48].

4. The lncRNAs and Cancers

Aberrant lncRNA expression contributes to tumor development in many cancer types [49–55]. For example, an lncRNA microarray showed that some lncRNAs contribute to glioma carcinogenesis [56, 57]. The lncRNAs also have important roles in the development of lung [58], breast [59], and liver cancers [60].

The accumulating evidence of lncRNA involvement in carcinogenesis includes findings that downregulation of maternally expressed gene 3 (MEG3), an imprinted lncRNA, is associated with carcinogenesis of meningiomas [61] and bladder cancer [62]. The lncRNA, namely, ANRIL also contributes to the development of plexiform neurofibromas in neurofibromatosis type 1 [63]. The ANRIL downregulates tumor suppressor gene p15 (INK4B) expression by binding to and recruiting the suppressor of zeste 12 homolog (*Drosophila*) (SUZ12), a component of the Polycomb Repressive Complex 2 [64]. When DNA damage occurs, ANRIL is upregulated by the ATM-E2F1 signaling pathway [65].

In human colorectal cancer, lncRNA H19 and H19-derived miR-675 are overexpressed in cell lines and primary tissues but not in adjacent noncancerous tissues [66]. Exogenous miR-675 expression also downregulates the tumor suppressor retinoblastoma, which is a direct target of miR-675 and increases tumor cell growth. Upregulation of H19 is also known to contribute to gastric cancer cell proliferation [67] and bladder cancer metastasis [68].

The HOTAIR is overexpressed in breast [69], nasopharyngeal [70], and liver [71] cancers. Loss of HOTAIR moderates the invasiveness of breast cancer, particularly in cells with upregulated Polycomb Repressive Complex 2 (PRC2). In nasopharyngeal and hepatocellular carcinoma, upregulated expression of HOTAIR indicates a poor prognosis [70, 71].

In lung cancer cells, downregulation of MALAT1 by siRNA decreases cell motility and downregulates motility-related genes [72], which suggests that MALAT1 promotes lung cancer metastasis. Similarly, MALAT1 is important in regulating cell proliferation, migration, and invasion of colorectal cancer metastasis [73]. In bladder cancer tissues, MALAT1 is overexpressed. Downregulation of MALAT1 by siRNA, the epithelial-to-mesenchymal transition-related genes, and cell migration of bladder cancer cells are inhibited [74]. After liver transplantation, MALAT1 is overexpressed

in both cell lines and tissues of patients with hepatocellular carcinoma. Additionally, upregulated MALAT1 is associated with increased risk of liver tumor recurrence [75].

An lncRNA of highly upregulated liver cancer (HULC) is reportedly overexpressed in hepatocellular carcinoma [76]. The HULC may downregulate miR-372 and induce phosphorylation of cAMP responsive element binding protein 1 (CREB1) in liver cancer [77]. Similarly, overexpressed lncRNA, namely, urothelial carcinoma associated 1 (UCA1) affects cell proliferation and invasion in bladder cancer [78]. The CREB1 is involved in the UCA1-mediated cell cycle distribution of bladder cancer [79]. Another lncRNA, UCA1a (cancer-upregulated drug-resistant gene, CUDR), reportedly regulates the carcinogenesis of human bladder cancer [80].

Methylation may also have a modulating role in lncRNA expression. For example, a study of triple-negative breast cancer cell lines showed hypermethylation and downregulation in both miR-31 and its MIR31 host gene (MIR31HG) [81]. The lncRNA, namely, colorectal neoplasia differentially expressed (CRNDE) is overexpressed in colorectal cancer and leukemia [82]. In esophageal adenocarcinoma, high-resolution methylome analyses have shown hypomethylated noncoding DNA regions and upregulated lncRNA in actin filament-associated protein 1 (AFAP1) antisense RNA 1 (AFAP1-AS1) [83].

Similar to the disease association studies as described above, the accumulating evidence of SNPs in lncRNAs has been reported in cancer association studies. For example, SNP array-based study reported that several SNPs in lncRNAs were associated with prostate cancer risk [84]. An lncRNA prostate cancer gene expression marker 1 (PCGEM1) is overexpressed in prostate cancer [85]. Two tagSNPs (rs6434568 and rs16834898) of the PCGEM1 were reported to be associated with prostate cancer [86]. Several lncRNAs contain SNPs such as rs7763881 in highly upregulated in liver cancer long noncoding RNA (HULC) and rs619586 in MALAT1 which are reportedly associated with decreased hepatocellular carcinoma risk [87].

5. The lncRNAs and Their Potential Modulators

Chemically engineered oligonucleotides that have proven effectivess for targeting endogenous miRNAs in mice [88] have potential applications in lncRNAs. For example, antisense oligonucleotides targeted at the mouse lncRNA Malat1 correct RNA gain-of-function effects of myotonic dystrophy [89]. Using siRNA treatment to lncRNA, the lncRNA, namely, antidifferentiation ncRNA (ANCR) is downregulated to promote osteoblast differentiation [90]. Similarly, siRNA-based downregulation of lncRNA associated with liver regeneration (LALR1) inhibits hepatocyte proliferation and cell cycle progression during liver regeneration [91]. Data obtained by a recent systematic transcriptome-wide analysis of lncRNA-miRNA interactions [92] may reveal additional regulators of lncRNA expression such as miRNAs that contribute to lncRNA degeneration. For example, in some lncRNAs targeted by breast cancer-related miRNAs, changes in gene expressions differ between women with and without breast tumors [93].

Inhibitors that modulate lncRNA function have also been identified. For example, small molecules such as diazobenzene-related compounds are now known to inhibit the function of miR-21 [94], a polyadenylated lncRNA [95]. 5-aza-2′-deoxycytidine (5-aza-dC), a methylation inhibitor, inhibits the methylation of putative imprinted control region (ICR) of H19 gene and leads to the downregulation of the H19 mRNA expression in blastocysts derived from vitrified two-cell embryos [96]. This finding suggests that epigenetic agents may be the modulators for lncRNA expression as well as their related targeting signals.

The hypothesis that environmental exposures are another cause of ncRNA alterations [97] was tested by exposing aquatic midges to xenobiotics, which revealed upregulation of lncRNAs derived from repetitive sequences [98]. Additionally, telomeric and centromeric ncRNA can be activated by bisphenol A, a synthetic chemical with estrogen-like effects [98]. Based on these findings, some drugs may also modulate lncRNA expression. Therefore, many natural products and their derivatives are likely to prove suitable for screening and identifying these modulators in lncRNAs.

6. Long Noncoding RNA and Bioinformatics Resources

Computational methods for predicting lncRNA function have been well reviewed [99]. Recently, consistently improving computational capability enabled rapid development of functional analyses and bioinformatics resources for lncRNAs [100]. Except for NRED [8], ncFANs [9], and lncRNAdb [10], we summarize the update progression of bioinformatics resources for lncRNAs during 2012-2013 as shown in Table 1.

For example, the NRED [8] database of lncRNA expression includes both microarray and *in situ* hybridization data for human and mouse lncRNAs. The noncoding RNA Function Anotation server (ncFANs) [9], a web server for functional anotation of lncRNAs, includes ten reannotated human and mouse microarray datasets. The lncRNAdb [10] is a comprehensive database of eukaryotic lncRNA anotations. The data contained in the lncRNAdb include sequences, structures, genomic contexts, expressions, and subcellular distributions. Most (~75%) lncRNAs in the database were collected from mammals.

The NONCODE v3.0 [11] is an integrated database of lncRNA anotations obtained from re-annotated and updated microarray data from NONCODE v2.0 [101]. The NONCODE v3.0 database includes a visualized Genome Browser and a BLAST-based sequence alignment search. Since the secondary structure of an lncRNA may affect its protein interactions, the LNCipedia [12] provides helpful information for visualizing the structures of annotated lncRNA sequences. The LNCipedia also uses an algorithm for predicting potential coding scores for each transcript and an HMMER algorithm for searching for RNA sequences in Pfam protein domains. The LncRNADisease [102] provides experimentally validated lncRNA—disease associations for 166 diseases in curated lncRNA interacting partners at the protein, RNA, miRNA, and DNA levels. Similarly, DIANA-LncBase provides experimentally verified and computationally predicted

Table 1: The lncRNA bioinformatics resources.

Name	Description
NRED [8]	Database of lncRNA expression. (http://nred.matticklab.com/cgi-bin/ncrnadb.pl/)
ncFANs [9]	Web server for functional annotation of lncRNAs. (http://ebiomed.org/ncfans/)
lncRNAdb [10]	Database of comprehensive annotations of functional lncRNAs. (http://www.lncrnadb.org/)
NONCODE [11]	Database of integrative annotations of lncRNAs. (http://www.noncode.org/NONCODERv3/guide.htm)
LNCipedia [12]	Database of annotations and structures of lncRNA sequences. (http://www.lncipedia.org/)
LncRNADisease [13]	Database of lncRNA-associated diseases. (http://cmbi.bjmu.edu.cn/lncrnadisease/)
DIANA-LncBase [14]	Database of microRNA targets on lncRNAs. (http://www.microrna.gr/LncBase/)
iSeeRNA [15]	The lincRNA transcripts identified from transcriptome sequencing data. (http://www.myogenesisdb.org/iSeeRNA/)
ChIPBase [16]	Database for annotating and exploring the expression profiles in transcriptional regulation of lncRNAs and other ncRNAs. (http://deepbase.sysu.edu.cn/chipbase/)

miRNA target sites of human and mouse lncRNAs [14]. The iSeeRNA [15] webserver was constructed by using a support vector machine- (SVM-) based classifier to identify lincRNAs from transcriptome sequencing data. Based on next-generation sequencing (ChIP-Seq) data, ChIPBase [16] provides anotations and identifyies information for transcription factor binding sites (TFBS) of lncRNAs and miRNAs from chromatin immunoprecipitation. A database of the regulatory relationships of transcription factors/lncRNA and transcription factors/miRNA is also being considered.

7. Conclusion

Various lncRNA functions are essential for regulating gene expression. This study focused on lncRNA dysregulation associated with disease progression and carcinogenesis and on the development of drugs for modulating lncRNA function. Since lncRNA is rarely studied in natural products, the resources mentioned in the paper may provide helpful information for researchers studying natural products.

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Research Article

Antioxidant and Antimicrobial Properties of the Essential Oil and Extracts of *Zanthoxylum alatum* Grown in North-Western Himalaya

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The essential oil obtained from the fresh leaves of *Zanthoxylum alatum* was analysed by gas chromatography/mass spectrometry (GC/MS). Fourteen components were identified, and linalool (30.58%), 2-decanone (20.85%), β-fenchol (9.43%), 2-tridecanone (8.86%), β-phellandrene (5.99%), Sabinene (4.82%), and α-pinene (4.11%) were the main components. The EO and methanolic extract of *Z. alatum* exhibited potent antifungal activity against *Alternaria alternata*, *Alternaria brassicae*, and *Curvularia lunata*. The EO also showed significant antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Escherichia coli*. Further, antimicrobial constituents of the EO were isolated by bioautography and preparative thin layer chromatography (PTLC) and identified as β-fenchol and linalool using GC/MS analysis. In addition to this, the free radical scavenging activity and antioxidant potential of EO and methanolic extract/fractions of *Z. alatum* were also investigated using *in vitro* assays including scavenging ability against $DPPH^{\bullet}$, reducing power and chelating ability on Fe^{2+} ions. Our results demonstrate that *Z. alatum* could be used as a resource of antioxidant and antimicrobial compounds which may find applications in food and pesticide industries.

1. Introduction

Plant diseases caused by microorganisms are responsible for heavy loss of agricultural crops every year. Synthetic chemicals used to control the diseases of crop plants are generally toxic and have negative impact on the environment and human health [1]. Furthermore, there is a heavy risk of development of resistance to these chemicals by disease causing microorganisms [2, 3]. Therefore, there is a need for development of new natural product based antimicrobial compounds which are less toxic and eco-friendly [4, 5].

Oxidative stress induced by oxygen radicals is reported to be causative agent of various degenerative diseases like arthritis, cancer arthrosclerosis, diabetes, and Parkinson's disease [6, 7]. Oxidation also causes rancidity in food products, leading to degradation of lipids and proteins, deterioration of flavour, taste, colour, and nutritional quality of the processed food [8]. Although, synthetic antioxidants like BHT and BHA are available in the market, but their use is limited due to the side effects caused by them [9]. It has been shown that natural products present in medicinal plants are inhibitory to the deleterious effects of oxidative stress [10]. Plant essential oils (EOs) and their extracts have extensive use in folk medicines, food flavouring, fragrance, and pharmaceutical industry as they are endowed with antimicrobial, antioxidant, and anti-inflammatory properties [11, 12].

The genus *Zanthoxylum* belonging to family Rutaceae comprises over 200 species, among them *Zanthoxylum alatum* Roxb. which is a medicinal shrub, locally known as "Timber" growing in the valleys of sub-tropical Himalayas [13]. Its fruits branches and thorns are generally used as carminative, stomachic, and remedy for toothache [14]. In India, different parts of the *Z. alatum* are used in Ayurvedic practices for the treatment of skin diseases, abdominal pain, anorexia, and ataxia [15]. In this paper we report the chemical composition, antimicrobial and antioxidant activity of EO and extracts of *Z. alatum* growing in north-western Himalaya. To the best of our knowledge, this study can be assumed as first report on isolation and identification of antimicrobial molecules from *Z. alatum* EO.

2. Materials and Methods

2.1. Plant Material and Extraction of EO. Fresh leaves from *Z. alatum* were collected and identified at Herbal Garden and Herbarium Research Institute in ISM, Joginder Nagar, District Mandi (HP), India. They were subjected to hydrodistillation for 2 h using a Clevenger-type apparatus for the extraction of EO. The oil was stored at 4°C in the dark until analyzed.

2.2. Qualitative and Quantitative Analysis of EO. Analysis of the oil using gas chromatography and mass spectrometry was carried out at Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India. A GC-MS 4000 (Varian, USA) system with a HP-5MS agilent column (30 m × 0.25 mm i.d., 0.25 μ film thickness). Injector temperature was 280°C. Oven temperature programme used was holding at 50°C for 5 min, heating to 280°C at 3°C/min, and keeping the temperature constant at 280°C for 7 min. Helium was used as a carrier gas at a constant flow of 1.0 mL/min and an injection volume of 0.20 μL was employed. The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40–500 m/z. The identification of components of the essential oil was based on comparison of their mass spectra with those stored in NIST05 library or with mass spectra from literature [16].

2.3. Fungal Species and Antifungal Assay. The cultures of agricultural pathogenic fungi, namely, *Alternaria brassicae*, *Alternaria alternata* were obtained from Division of plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology, Chatha, Jammu, India, and *Curvularia lunata* from Type Culture Collection Centre, Indian Agricultural Research Institute (IARI), New Delhi, India. Test component (EO/extract) was added to the sterilized potato dextrose agar (containing 0.5% T20 v/v) in 9 cm petri plates. After preparing the plates containing different concentrations of EO, 5 mm bit of test fungus was inoculated in the centre of the agar plate (mycelial surface of the bit was placed upside down). Plates were incubated in dark at 26°C. The extension diameter (cm) of hyphae from the centre to the side of the dish was measured at 24 h interval, till the growth of fungus in the plate without test component (control) reached the edge of the plate. The experiment was repeated thrice and results were expressed as average of three replicates.

Fungal growth diameter in each plate containing different concentrations of test component was determined to calculate per cent growth inhibition [17].

The antifungal indices were calculated as

$$\text{Antifungal index } (\%) = \left(1 - \frac{D_a}{D_b}\right) \times 100, \quad (1)$$

where D_a = diameter of growth zone in the experiment dish (in cm), D_b = diameter of growth zone in the control (in cm).

2.4. Antimicrobial Activity. Bacterial strains, *Bacillus subtilis* MTCC2389, *Staphylococcus aureus* MTCC7443 *Micrococcus luteus* MTCC4821, *Escherichia coli* MTCC2127, and *Pseudomonas aeruginosa* MTCC2642 were obtained from Institute of Microbial Technology (IMTECH, CSIR), Chandigarh, India. Qualitative analysis for screening of antimicrobial activity of EO/methanol extract was carried out by Agar-well diffusion method [18]. 20 mL of sterilized nutrient agar was inoculated with 100 μL of bacterial suspension (10^8 CFU/mL) and then poured on to sterilized petri plate. The agar plate was left to solidify at room temperature. A well of 6 mm was aseptically bored into the agar plate and 20 μL of the EO (diluted with DMSO, 1 : 1) and methanol extract (2 mg) was added in each well. Chloramphenicol (10 μg) was used as a positive reference to determine the sensitivity of bacteria. The plates were kept at 4°C for 2 h to allow the dispersal and then incubated at 37°C for 24 h.

2.5. Determination of MIC by Broth Dilution Technique. Broth dilution technique was used to determine the minimum inhibitory concentration of the EO against five bacterial strains [19]. One millilitre of nutrient broth was kept in each tube and autoclaved. The EO diluted with DMSO (1 : 1) was filtered with (0.22 μm) filter disk before use and then added to each tube to keep the final concentration ranging from 62.5 μg/mL–2000 μg/mL. The test bacterial suspension was added into each tube to yield bacterial density of 10^6 CFU/mL and the inoculated tubes were incubated at 37°C for 24 h. Tubes containing nutrient broth without EO served as positive control, whereas, without bacteria as negative control. After incubation, 50 μL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) was added in each tube to indicate the bacterial growth. The tubes were again incubated for 30 min at 37°C. Development of pink colour in the tube (due to reduction of dye) indicated the bacterial growth, whereas tubes without colour indicated no active bacterial growth. The lowest concentration at which no bacterial growth was observed (as indicated by colour) corresponded to the minimum inhibitory concentration (MIC). All the assays were performed in triplicate.

2.6. Qualitative Antifungal and Antibacterial Activity Assay by Bioautography. Direct bioautography was performed using silica gel 60 F_{254} TLC plates (Merck). Five microlitres of 1 : 10 dilution of EO in dichloromethane were applied to the TLC plates and developed in hexane-ethyl acetate (9 : 1) solvent system.

For assaying antifungal activity, aliquots of 25–50 mL of inoculum spray solution (ca. 3×10^5 conidia/mL) were prepared for test fungi (*A. alternata*) with liquid potato dextrose (potato 200 g, dextrose 20 g, and water to make total volume of litre). 100 mL chromatographic sprayer plate was sprayed lightly (to a damp appearance) three times with spore suspension and incubated for 4 d in a dark moist chamber at 25°C. Fungal growth inhibition appeared as clear zones against a dark background.

For assaying antibacterial activity direct bioautography method was used [20]. TLC plates were developed as mentioned previously. The developed plates were allowed to dry in a stream of air to remove residual solvent which might otherwise inhibit bacterial growth. *Bacillus subtilis* was then sprayed on the TLC plate and incubated at 37°C in humid conditions. After incubation plate was sprayed with 2 mg/mL solution of INT. Clear zones on chromatogram indicated inhibition of growth after incubation.

2.7. Isolation of Antimicrobial Constituents. After identification of the inhibition zones on the TLC plate, PTLC was performed by loading the essential oil onto a preactivated silica gel 60 F_{254} coated glass plate (20 × 20 cm, 500 μm thickness) which was developed in *n*-hexane/ethyl acetate (9 : 1, v/v) solvent system. The separated compounds were visualized under UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid spray reagent. The isolation was carried out by scrapping off the detected zones corresponding to the antimicrobial constituents Za_1 (R_f = 0.40) and Za_2 (R_f = 0.61) and transferring them into percolator. The substances were then set free from silica gel by elution with dichloromethane.

2.8. Antifungal Activity of Bioactive Molecules Isolated from Z. alatum. Different amounts of Za_1 and Za_2 were loaded onto the TLC plate and bioautography was performed as described earlier using *A. alternata* as test pathogen. Antifungal activity was determined as MIA of active compounds required for the inhibition of fungal growth on TLC plate.

2.9. Identification of Bioactive Compounds. The isolated bioactive compounds corresponding to Za_1 and Za_2 were determined using GC/MS analysis using standard compounds, NIST and Wiley libraries, and those reported in the literature [16].

2.10. DPPH Radical Scavenging Assay for Essential Oil. Radical scavenging activity was determined using DPPH$^\bullet$ method [21]. One mL of different concentrations of the EO or extract was mixed with 1 mL of a 90 μM DPPH$^\bullet$ solution in methanol, and final volume was made to 4 mL with methanol. The mixtures were well shaken and kept at 25°C in the dark for 1 h. The absorbance was measured at 517 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH$^\bullet$ discolouration, using the equation:

$$\%\text{RSA} = \left[\frac{(A_0 - A_s)}{A_0}\right] \times 100, \quad (2)$$

where A_0 is the absorbance of the control (containing all reagents except the test compound) and A_s is the absorbance of test compound. Oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition % against oil concentration. BHT was used as reference.

2.11. DPPH Radical Scavenging Assay for Extracts. In this assay, free radical scavenging activity was determined by measuring the bleaching of purple-coloured methanol solution of DPPH$^\bullet$. The radical scavenging activity was determined according to the method of Abe and others [22] with modifications. One millilitre from a 0.5 mM methanol solution of the DPPH radical was mixed to 2.0 mL sample and to this 2.0 mL of 0.1 M sodium acetate buffer (pH 5.5) was added. The mixtures were well shaken and kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a double beam UV-VIS spectrophotometer. Methanol was used as a negative control. BHT was used as standard antioxidant. The radical scavenging activity (RSA) was calculated as a percentage of DPPH$^\bullet$ discolouration, using the equation

$$\%\text{RSA} = \left[\frac{(A_0 - A_s)}{A_0}\right] \times 100, \quad (3)$$

where A_0 is the absorbance of the control (containing all reagents except the test compound) and A_s is the absorbance of test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition % against extract concentration. BHT was used as reference.

2.12. Reducing Power Assay. The reducing power of EO/extract determined the method as described previously [23]. Different concentrations of EO/extract were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide $[K_3Fe(CN)_6]$(1%). The mixture was incubated at 50°C. for 20 min. Aliquots (2.5 mL) of 10% trichloroacetic acid were added to the mixture. The previously mixture was then centrifuged for 10 min at 1036 g. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 1% ferric chloride solution. The absorbance was measured at 700 nm in a double beam UV-VIS spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. The essential oil/fraction concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against EO/extract concentration and compared with those of standard antioxidant.

2.13. Chelating Capacity Assay. The chelating effect on ferrous ions of *Z. alatum* EO/extract was estimated by the method of Dinis and others [24] with slight modifications. Briefly, 200 μL of different concentrations of EO/extract and 740 μL of methanol were added to 20 μL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 μL of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min before determining the absorbance of the mixture at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation: % inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control)] × 100.

2.14. Estimation of Total Phenolic Content. Total phenolic content was determined according to Folin-Ciocalteu method [25]. Briefly, 0.5 mL of sample was mixed with 0.5 mL of 1 N Folin-Ciocalteu reagent. The mixture was kept for 5 min, followed by the addition of 1 mL of 20% Na_2CO_3. After 10 min of incubation at room temperature the absorbance was measured at 730 nm using UV-VIS spectrophotometer. Gallic acid was used as a standard. The concentration of phenolic compounds was calculated according to the following equation that was obtained from the standard gallic acid (5 to 50 μg) graph:

$$\text{Absorbance} = 0.0271 \text{ gallic acid } (\mu g) - 0.253 \quad \left(R^2 = 0.99\right). \tag{4}$$

2.15. Statistical Analysis. All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation.

Table 1: Percent composition of leaf essential oil from *Zanthoxylum alatum*.

S. No.	Compound	RT [min]	Percent (%)	Mode of identification
1	α-Pinene	10.170	4.11	GC-MS
2	Sabinene	12.063	4.82	GC-MS
3	β-Pinene	12.173	1.31	GC-MS
4	t-Butylbenzene	14.566	2.31	GC-MS
5	β-Phellandrene	14.764	5.99	GC-MS
6	Linalool	18.408	30.58	GC-MS
7	4-Terpineol	22.094	2.36	GC-MS
8	Sabina ketone	22.550	0.94	GC-MS
9	β-Fenchol	22.746	9.43	GC-MS
10	2-Decanone	27.571	20.85	GC-MS
11	Caryophyllene	32.956	2.76	GC-MS
12	2-Tridecanone	36.119	8.86	GC-MS
13	α-Selinene	38.773	0.53	GC-MS
14	Caryophyllene oxide	39.518	2.23	GC-MS

RT: retention time.

Table 2: Relative proportion of solvent fractions obtained during fractionation of crude methanolic leaf extract of *Zanthoxylum alatum* expressed as percent of total amount of crude extract used for the fractionation.

Solvent fractions	Percent yield
Chloroform	31.7 ± 0.98
Ethyl acetate	4.3 ± 0.05
Acetone	6.8 ± 0.09
Methanol	57.2 ± 1.78

Values are given as mean ± SD (n = 3).

3. Results and Discussion

3.1. Chemical Composition of the EO. Fresh leaves of the plant were subjected to hydrodistillation using a Clevenger-type apparatus and the yellow coloured oil was obtained with yield of 0.08% (w/w). Fourteen compounds comprising 98.4% of the EO were identified by GC/MS analysis. The identified compounds are listed in Table 1 according to their elution order in DB-1 column. The major components of the oil were linalool (30.58%), 2-decanone (20.85%), β-fenchol (9.43%), 2-tridecanone (8.86%), β-phellandrene (5.99%), Sabinene (4.82%) and α-pinene (4.11%). It is evident from previous results that *Z. alatum* oil is rich in linalool and previous researchers have also shown linalool as the major component of EOs from *Zanthoxylum* species [26, 27]. Chemical composition of *Z. alatum* seed EO from northern India has been reported consisting of mainly linalool (71%), limonene (8.2%), β-phellandrene (5.7%), and (Z)-methylcinnamate (4.9%) as the major components [28].

3.2. Yield of Crude Methanol Extract and Fractions. Percent yield of the crude methanol extract was 20.14%. Crude methanol extract was further partitioned sequentially in different solvents in the order to increas polarity and the yield of different fractions obtained is shown in Table 2. Highest yield was observed in methanol fraction (57.2%) followed by chloroform (31.7%), acetone (6.8%), and ethyl acetate (4.3%) fractions.

3.3. In Vitro Antibacterial and Antifungal Activities. The results of antibacterial activity assays of the EO and MeOH extract of *Z. alatum* are shown in Table 3. The oil was tested for antibacterial activity against three Gram-negative and two Gram-positive bacteria and was found to be effective against all the tested bacterial strains. The oil strongly inhibited the growth of *M. luteus* (MTCC 4821), *S. aureus* (MTCC 7443), *E. coli* (MTCC 2127), and *B. subtilis* (MTCC 2389) with zones of inhibition ranging from 15–21 mm. Moderate activity was observed against *P. aeruginosa* (MTCC 2642) with the zone of inhibition of 7 mm. All the bacteria were more sensitive to chloramphenicol as compared to the EO except *M. luteus* (MTCC 4821) which was more sensitive to EO. Methanol extract of *Z. alatum* leaf showed no inhibitory effect against both Gram-positive and Gram-negative bacteria under study at tested amount of 2 mg/well. MIC results indicate that out of the five bacterial strains tested *M. luteus* (MTCC 4821) and *S. aureus* (MTCC 7443) were the most sensitive, with the MIC values 62.5 and 125 μg/mL, respectively. *E. coli* (MTCC 2127) and *B. subtilis* (MTCC 2389) were also sensitive to

Table 3: Antibacterial activity of the essential oil and methanol extract of *Zanthoxylum alatum*.

Microorganism	Zone of inhibition (mm)			MIC (μg/mL)
	Essential oil[a]	MeOH extract[b]	CF	Essential oil
B. subtilis MTCC2389	15	—	19	500
M. luteus MTCC4821	21	—	20	62.5
S. aureus MTCC7443	19	—	21	125.5
E. coli MTCC2127	18	—	24	250
P. aeruginosa MTCC2642	7	—	18	>1000

Diameter of inhibition zone includes the diameter of the well (6 mm); [a]essential oil (2 μL well^{-1}); [b]MeOH extract (2 mg well^{-1}); standard antibiotic: CF: chloramphenicol (10 μL well^{-1}); MIC: minimum inhibitory concentration. —: not detected.

the oil (MIC value of 250 and 500 μg/mL, resp.). *P. aeruginosa* (MTCC 2642) was the least sensitive to the oil (MIC value > 2000 μg/mL). Antifungal and antibacterial activities have been reported in *Z. armatum* fruit essential oil [29].

To evaluate the antifungal activity of EO and methanol extract from *Z. alatum* leaves three agriculturally important phytopathogenic fungi, *A. brassicae, A. alternate*, and *C. lunata,* were selected. Figure 1 shows the antifungal activities of the EO and methanol extract against test fungal pathogens. On the basis of the results of the antifungal test, the antifungal indices of *Z. alatum* EO were 14.5%, 35.6%, and 42.0% against *A. brassicae*, *A. alternate*, and *C. lunata*, respectively, and IC_{50} values were 2868, 1623, and 1322 μg/mL, respectively (Table 4). Similarly antifungal indices of methanol extract were 47.4% and 51.4% against *A. alternata* and *C. lunata*, respectively, and IC_{50} values were 1071 and 948 μg/mL, respectively (Table 4). It is clear that in comparison with the IC_{50} values, both EO and methanol extract showed higher antifungal activity against *C. lunata* followed by *A. alternata.* Further antifungal indices showed higher efficacy of methanol extract than EO against all the test fungal pathogens. Antifungal activity of crude methanol extract could be attributed to the presence of phenolic and flavonoid compounds [30, 31]. No antifungal activity was observed in the methanol extract at the highest test concentration of 2 mg/mL against *Alternaria brassicae*. EOs are complex mixtures obtained by distillation of a large number of volatile compounds from the plant. EOs rich in oxygenated monoterpenes have been shown to possess antifungal and antibacterial activities [32, 33]. Many researchers have investigated the antifungal and antibacterial activities of individual chemical constituents of the EOs such as β-caryophyllene, caryophyllene oxide, and linalool [34, 35]. The compounds which are present in small amount such as β-pinene, sabina ketone, and α-selinene in *Z. alatum* EO may also contribute to antimicrobial activity either directly or in synergy with some other bioactive compounds. Here we considered it pertinent to identify for the first time the active compounds present in *Z. alatum* EO and investigate their fungitoxic and antibacterial activity.

Table 4: Percent antifungal index and IC_{50} values of essential oil and methanolic extracts from leaves of *Zanthoxylum alatum* against three crop infecting fungi.

Pathogens	% Antifungal index		Antifungal activity [IC_{50} (μg/mL)]	
	Essential oil	MeOH extract	Essential oil	MeOH extract
Alternaria alternata	35.6 ± 1.49	47.4 ± 1.18	1623 ± 41.5	1071 ± 26.2
Alternaria brassicae	14.5 ± 0.36	—	2868 ± 61.2	—
Curvularia lunata	42.0 ± 1.63	51.2 ± 1.47	1322 ± 24.9	948 ± 21.8

Values are given as mean ± SD (n = 3). —: not active at test concentration.

Table 5: Antifungal activity of essential oil isolated from *Zanthoxylum alatum* leaves against *Alternaria alternata*, *Curvularia lunata,* and *B. subtilis* using bioautography.

Component	R_f value	Diameter of inhibition zone (mm)		
		Alternaria alternata	*Curvularia lunata*	*B. subtilis*
Za_1	0.40	13.0 ± 0.42	11.0 ± 0.30	20.0 ± 0.69
Za_2	0.61	18.0 ± 0.38	22.0 ± 0.72	26.5 ± 0.48

Values are given as mean ± SD (n = 3).

3.4. Antimicrobial Compounds from Z. alatum EO. Antifungal and antibacterial compounds of the EO were observed on TLC plate using direct bioautography. They were further isolated using repeated preparative TLC and identified by GC/MS analysis. Bioautography procedure enables the evaluation of plant extracts and EOs against human and plant pathogens [36]. For isolation of antibacterial compounds bioautography was performed using *B. subtilis* as test organism. Similarly *A. alternata* and *C. lunata* were used as test organisms for isolation of antifungal compounds from *Z. alatum* essential oil. Direct bioautography for observing antibacterial activity showed three inhibition zones at R_f values 0.15, 0.40, and 0.61. Similarly when bioautography was performed for observing antifungal activity two inhibition zones at R_f values 0.40 and 0.61 were observed marked as Za_1 and Za_2 (Figure 1, Table 5). Intriguingly, compounds corresponding to R_f values 0.40 and 0.61 showed both antifungal and antibacterial activities. The EO of *Z. alatum* was further subjected to repeated PTLC for isolation of compounds showing both antifungal and antibacterial activities. PTLC of the essential oil yielded 3.2 mg of Za_1 and 5.7 mg of Za_2. The previous pure compounds were further subjected to bioautography for determination of minimum inhibitory amount (MIA) against *A. alternata* and *C. lunata.* The results of previous investigation showed that Za_1 possessed stronger fungitoxic activity against *A. alternata* (MIA = 25.3 μg) and *C. lunata* (MIA = 10.0 μg) than Za_2 with MIA value of 29.4 μg and 41.5 μg against *A. alternata* and *C. lunata*, respectively (Table 6). Further both the antifungal compounds have different response against both the test organisms. Za_1 was more effective against *C. lunata*, whereas Za_2 was active against *A. alternata*.

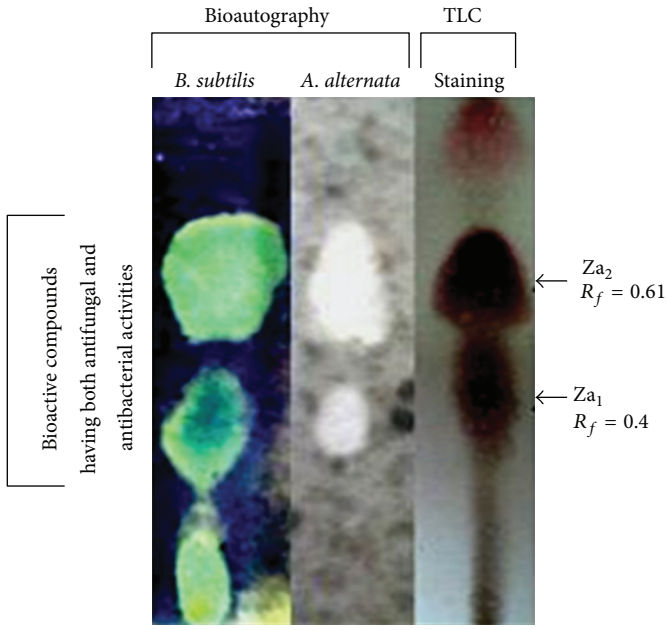
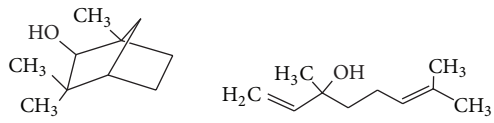


Figure 1: Visualization of antifungal and antibacterial compounds in *Zanthoxylum alatum* leaf essential oil using bioautography.

Table 6: Antifungal activity of compounds isolated from *Zanthoxylum alatum* essential oil against *Alternaria alternata* and *Curvularia lunata*.

Component	Minimum inhibitory amount [μg][a]	
	Alternaria alternata	*Curvularia lunata*
Za_1, β-fenchol	25.3 ± 0.28	10.0 ± 0.09
Za_2, Linalool	29.4 ± 0.37	41.5 ± 0.31

Values are given as mean ± SD ($n = 3$). [a]Minimum inhibitory amount (MIA) of active constituents required for the inhibition of fungal growth on TLC plate in bioautography assay.



β-Fenchol Linalool

Figure 2: Chemical structures of compounds having antifungal and antibacterial activities.

The bioactive constituents of the *Z. alatum* EO corresponding to Za_1 and Za_2 were identified as β-fenchol and linalool using GC-MS analysis (Figure 2). In our previous work, we have reported antifungal activity of β-fenchol isolated from *Eucalyptus tereticornis* EO against *A. alternate* [32].

3.5. Antioxidant Activity of the EO and Extracts of Z. alatum. The results of the antioxidant activity determined by three test assays, namely, DPPH radical scavenging, reducing power, and metal ion chelating activity are shown in Table 7. In the present study, the capacity of the EO and extract samples to scavenge DPPH radical and their reducing power was determined on the basis of their concentration providing 50% inhibition (IC_{50}). Further, their capacity to chelate Fe^{2+} metal ion was determined at 0.5 mg/mL concentration. BHT, BHA, and quercetin were used as controls. Methanol fraction of the plant extract showed the highest radical scavenging activity with an IC_{50} value of 0.044 mg/mL followed by crude methanol extract (IC_{50} = 0.067 mg/mL) and acetone fraction (IC_{50} = 0.086 mg/mL). The EO and nonpolar fractions, namely, chloroform and ethyl acetate showed relatively low antioxidant activity in this assay. The radical scavenging activity of the leaf extract and fractions of *Z. alatum* may be due to its phenolic compounds as their hydroxyl groups confer radical scavenging ability [37].

Reducing power of plant extracts or EOs may serve as indicator of their antioxidant potential [38]. In this test, there is a change in the colour of test solution from yellow to green depending on the reducing power of test sample. The presence of reductants in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to ferrous form. The reducing power of crude extract, its derived fractions, EO, and reference compounds BHT and BHA was dose dependent and increased with increasing concentration. As shown in Table 7, the reducing power is in the order: methanol fraction (IC_{50} = 0.3 mg/mL) > crude extract (IC_{50} = 0.39 mg/mL) > acetone fraction (IC_{50} = 0.96 mg/mL) > ethyl acetate fraction (IC_{50} = 1.15 mg/mL) > chloroform fraction (IC_{50} =1.47 mg/mL) > essential oil (IC_{50} = 11.9 mg/mL). It is interesting and worthy mentioning here that the methanol fraction which had the highest reducing power also possessed the highest radical scavenging activity.

Crude extract, its derived fractions and EO of *Z. alatum* leaves showed metal ion chelating activity. As shown in Table 7 ethyl acetate fraction showed a better metal ion chelating effect with 60.1% chelating capacity at 0.5 mg/mL,

Table 7: Antioxidant activity and total phenolic content of the essential oil and methanol extract of *Zanthoxylum alatum*.

Sample	DPPH radical scavenging IC_{50} (mg/mL)	Reducing power IC_{50} (mg/mL)	% Chelation power at 0.5 mg/mL	TPC (mg g^{-1} GAE)
Essential oil	6.04 ± 0.08	11.9 ± 0.29	42.1 ± 1.03	19.3 ± 0.34
Crude methanolic extract	0.067 ± 0.002	0.39 ± 0.01	43.8 ± 1.92	366.3 ± 15.3
Chloroform fraction	0.155 ± 0.004	1.47 ± 0.04	41.2 ± 0.47	139.5 ± 4.03
Ethyl acetate fraction	0.51 ± 0.01	1.15 ± 0.03	60.1 ± 2.71	155.8 ± 7.62
Acetone fraction	0.086 ± 0.003	0.96 ± 0.02	40.5 ± 0.98	187.6 ± 6.09
Methanolic fraction	0.044 ± 0.001	0.3 ± 0.001	41.2 ± 1.02	383.5 ± 11.4
BHT	0.013 ± 0.000	0.14 ± 0.001	—	—
BHA	0.01 ± 0.000	0.11 ± 0.003	—	—
Quercetin	—	—	42.7 ± 0.80	—

Values are given as mean ± SD ($n = 3$).

followed by crude extract (43.8%), methanol fraction (42.2%), EO (42.1%), chloroform fraction (41.2%), and acetone fraction (40.5%). The results indicate that *Z. alatum* leaf extracts and essential oil showed good chelating activity.

There are very few reports in the literature about antioxidant activity of linalool as the major component of the EO. Recently Ebrahimabadi and others [39] have shown poor antioxidant activity in linalool in β-carotene/linoleic acid test, whereas no radical scavenging activity was reported in DPPH assay. Thus antioxidant activities observed for the *Z. alatum* EO could be attributed to the remaining chemical constituents.

3.6. Total Phenolic Content of Z. alatum Leaf Extract and EO. Total phenolics in EO, crude extract and its derived fractions were determined according to Folin-Ciocalteu method and expressed as GAE. As shown in Table 2. The highest amount of total phenolics was observed in methanolic fraction (383.5 mg of GAE/g) followed by crude extract (366.3 mg of GAE/g), acetone fraction (187.6 mg of GAE/g), ethyl acetate fraction (155 mg of GAE/g), and chloroform fraction (139.5 mg of GAE/g). Least phenols were observed in EO (19.3 mg of GAE/g). Several workers have reported that phenolic content of plants is related to their antioxidant activity [40–42]. Phenolic compounds act as reducing agents, hydrogen donors, and singlet oxygen quenchers due to their redox properties [25]. The aforesaid results demonstrate that the free radical scavenging effect ($1/IC_{50}$) of essential oil, crude extract and its derived fractions of *Z. alatum* correlates closely with their phenolic contents ($r^2 = 0.86$).

4. Conclusions

In conclusion, we investigated the antifungal activities of EO and methanol extract from leaf tissues of *Z. alatum* growing in north-western Himalaya against three phytopathogenic fungi, which had not been reported previously. EO from *Z. alatum* also showed significant antimicrobial activity against Gram-negative bacteria like *M. luteus* and *E. coli* and Gram-positive bacteria, *S. aureus*, and *B. subtilis*. Further, antimicrobial compounds were isolated and identified from the EO as β-fenchol and linalool. The results of antioxidant activity tests show that methanol extract and derived fractions of *Z. alatum* should be treated as efficient radical scavenger and reductant as compared to EO.

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Research Article

Effects of Biochemical Alteration in Animal Model after Short-Term Exposure of *Jatropha curcas* (Linn) Leaf Extract

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This study aims to evaluate potential toxic effect of *Jatropha curcas* leaves methanol extract on laboratory rats as well as determine its LD_{50}. A total of 80 male Wistar rats were used as the experimental animals, 40 for LD_{50} determination and the other 40 for toxicity study. Based on the pretest that was done in order to establish a range of toxicity, 4 dosages (86.00, 58.00, 46.00, and 34.0 kg/body weight) were chosen. The rats were randomly assigned into four groups with 10 rats in each group. Rats in groups 1, 2, 3, and 4 were given 0 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight of *Jatropha curcas* extract, respectively, by oral intubation for 21 days. Thereafter, clinical signs, change in body weight, toxicity symptoms, and biochemical parameters were obtained. The LD_{50} at 95% confidence limits for rats was 46.0 mg/kg body weight (44.95–52.69 mg/kg body mass). There was no clinical and biochemical signs of toxicity when the extract was administered at 500, 1000, and 2000 mg/kg body weight, respectively ($P > 0.05$). Results obtained from this study suggest that liver, kidney, and haematological system of rats tolerated methanolic leave extract of *Jatropha curcas* at a certain concentration.

1. Introduction

The continued interest in the evaluation of natural products as potential chemotherapeutic agents is encouraged by the isolation of phytochemicals in the plants, which could become important drugs in modern medicine. Plants produce bioactive compounds or molecules that act as defence mechanisms against predators and at the same time may be toxic in nature [1, 2]. With the increased interest in medicinal plants, there is a need for thorough scientific investigations of these plants for efficacy and potential toxicity.

Jatropha curcas (Linn) belonging to the family Euphorbiaceae is a shrub that grows 4.5 to 8 meters high. The roots, leaves, and seeds of the plant have been widely used in traditional folk medicine in many parts of West Africa, Central and South America. Previous studies have shown that the plant exhibits bioactive activities for fever, mouth infections, jaundice, and guinea worm sores [3]. Fagbenro-Beyioku et al. [4] reported antiparasitic activity of the sap and crushed leaves of *J. curcas*. The water extract of the branches also strongly inhibited HIV-induced cytopathic effects with low cytotoxicity [5]. Mujumdar et al. [6] also reported that the crude methanol extract from the root of *J. curcas* exhibited antidiarrheal activity in mice through inhibition of prostaglandin biosynthesis and reduction of osmotic pressure. Our biological study on *J. curcas* reported relevant antimicrobial efficacy and antioxidant activities [7, 8]. Balaji et al. [9] reported that methanol extract of *J. curcas* could protect liver against the aflatoxin B1-induced oxidative damage in rats. Despite all beneficial effects of *J. curcas*, some studies have also demonstrated that *J. curcas* exhibited toxicity especially in higher animals. For example, methanol, petroleum

ether, and dichloromethane extracts of *J. curcas* fruit caused fetal resorption indicating pregnancy terminating effect in rats [10]. Methanol fraction from *J. curcas* oil induced tumor promotion upon topical initiation by Makkar et al. [11], dimethylbenz(a)anthracene (DMBA) in mice, with 36% of the animals having skin tumors in 30 weeks [12]. Raw or defatted seeds when administered to fish, chicks, pigs, goats, mice, and rats were associated with toxic symptoms before death [13, 14]. Different aqueous extracts also exhibited different toxic symptoms depending on dose, mode of administration, and sensitivity of the animals that were tested [15, 16].

In recent times, concerns have been raised over the lack of quality control and scientific facts for the efficacy and safety of medical plants [17, 18]. Cautions have been raised regarding the potential adverse effects of herbal remedies including hepatotoxicity and nephrotoxicity [19, 20], even as it is known that medicinal plants typically contain several different pharmacologically active compounds that may act individually, additively, or in synergy to improve health [8, 21, 22]. It has been reported that 80% of the population in the developing world still rely on traditional medicine for primary health care needs. In spite of the diverse uses of plants in folk medicine, there seems to be dearth of information on the possible toxicity of this plant. Therefore, this study evaluates the toxicity risk of the methanol extract of the plant leaves of *J. curcas* using animal model.

2. Results and Discussion

2.1. Clinical Signs and Mortality. Death of rats administered with *J. curcas* extract occurred at a dose-dependent manner with starting dose of 34 mg/kg (Table 1). At the highest dose of 86.0 mg/kg, majority of rats were easily affrighted and stayed crouched together and tend not to eat much. Before rats died, they exhibited signs of depression, closing of eyes, languishment, loss of body mass, and black excreta. Rats began to die on day 2 after administering *J. curcas* extract, continued with a majority of deaths occurring within 7 days. There was no death recorded between observation days, 12 and 21 days.

Table 2 shows the effect of *J. curcas* extract on weight gain, food intake, and fecal output. The result obtained indicates that weight gain, food intake, and fecal output of rat treated with 500, 1000, and 2000 mg/kg body weight of extract were not significantly different ($P > 0.05$). The effects of oral administration of the leaf extract of *J. curcas* at the doses investigated on RBCs and its functional indices in laboratory rats (Wistar) for 21 days are shown in Table 3. The extract did not significantly alter the level of Hg, RBC, PCV, MCHC, and LUC. The administration of the extract effectively reduced the level of WBC and the differentials including basophils, monocytes and platelets throughout the study period (Table 3).

The plant extract showed varied effects on the kidney and its functional indices (Table 3). The levels of sodium, potassium, calcium, urea, and creatinine were not significantly affected when compared with the control animal model. In contrast, however, the level of chlorine ion was decreased as doses dependent factor. The extract did not significantly alter the level of albumin and total bilirubin which is the vital assay in assessing liver damage (Table 3). There was no significant difference in the parameters measured in rats administered with 500, 1000, and 2000 mg/kg body weight of *J. curcas* extract. These parameters include alanine aminotransferases (ALT), aspartate aminotransferases (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and blood urea nitrogen (BUN) (Table 3).

Table 1: Determination of the LD_{50} value.

Dose (mg/kg)	Total dead	Days after administration												
		0	1	2	3	4	5	6	7	8	9	10	11	12–21
86.0	9	0	0	2	0	1	1	1	2	1	0	0	1	0
58.0	7	0	0	0	1	1	2	2	0	0	1	0	0	0
46.0	4	0	0	1	0	1	0	0	1	0	0	0	1	0
34.0	2	0	0	0	0	0	1	0	0	0	0	1	0	0
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2.2. Discussion. *J. curcas* has been a multipurpose perennial plant which has lots of industrial and a long history of various medicinal applications. Antimicrobial and antioxidant activities of *J. curcas* were scientifically established [7, 8]. Since several reports have highlighted toxicity of this plant, this study ascertains safe dose of methanolic leave extract of *J. curcas* using animal model.

There are many methods used in calculating LD_{50} such as the graphical method of Miller and Tainter, arithmetical method of Karber, and statistical approach which include up-and-down procedure, fixed dose procedure, acute toxic class method, Bliss method, and sequential grouping method. Bliss [23] developed the idea of transforming the sigmoid dose-response curve to a straight line. In 1952, Finney popularized Bliss' idea in a book called *Probit Analysis* [24]. Bliss method being classical is still the preferred statistical method. Li et al. [25] in understanding dose-response relationship the procedure was used in our study.

LD_{50} study indicated that *J. curcas* leave extract is toxic to rats at a high dose, and rats develop severe pathological symptoms. The obtained lethal dose as shown in this study may not predict the human lethal dose of a drug or acute poisoning overdose. However, it was used to provide a guideline for selecting doses for subacute dosage of more clinical relevance. Feeding studies on *J. curcas* showed severe clinical and pathological symptoms in a dose-dependent manner [26, 27]; symptoms observed include transient loss of body mass and mild to severe macroscopic and microscopic changes in the kidney, lungs, heart, liver, and spleen. In the present study, the observed dark excreta may be suggestive of gastrointestinal hemorrhage.

The treatment of animals at doses 500, 1000, and 2000 mg/kg for 21 days show selective toxic effect on some biochemical and hematological parameters. These parameters are used to determine the possible alterations in the level of biomolecules such as enzymes, metabolic product, normal functioning and histomorphology of the organs. There were no noticeable hemolytic changes in the plasma of the extract treated rats on RBC, Hg, PCV, MCHC, and LUC. These

Table 2: Weight gain, food consumption, and fecal output of rats administered *J. curcas* extract ($n = 6$, mean ± SD).

Parameter	Dose of extract (mg/kg body weight)			
	Control	500	1000	2000
Weight gain (g/day/rat)	1.52 ± 0.23^{a}	1.64 ± 0.45^{b}	1.57 ± 0.65^{b}	1.2 ± 0.59^{d}
Food intake (g/day/rat)	21.34 ± 1.32^{a}	24.93 ± 0.32^{b}	25.01 ± 1.24^{d}	23.82 ± 1.51^{e}
Dry fecal output (g/day/rat)	1.34 ± 0.09^{a}	1.29 ± 0.05^{c}	1.41 ± 0.06^{c}	2.01 ± 0.03^{e}

Means with the same letter are not significantly different ($P < 0.05$).

Table 3: Effects of methanolic leaf extract of *J. curcas* on plasma biochemical parameters of laboratory rats ($n = 6$, mean ± SD).

Parameter	Doses of extract (mg/kg body weight)			
	Control	500	1000	2000
ALT (U/L)	21.03 ± 0.47^{a}	19.93 ± 0.83^{ab}	22.03 ± 0.93^{ab}	25.07 ± 1.85^{a}
AST (U/L)	19.05 ± 1.03^{a}	24.05 ± 0.93^{a}	25.47 ± 0.46^{ab}	24.93 ± 1.09^{a}
ALP (U/L)	43.03 ± 0.84^{a}	48.03 ± 1.03^{a}	45.04 ± 1.39^{a}	51.09 ± 3.04^{a}
GGT (U/L)	28.72 ± 3.29^{a}	29.54 ± 4.12^{ab}	30.60 ± 2.46^{a}	33.62 ± 3.01^{ab}
Albumin (mmol/L)	19.50 ± 0.85^{a}	19.12 ± 0.15^{a}	18.56 ± 1.74^{a}	17.31 ± 2.10^{a}
Total bilirubin (μmol/L)	12.53 ± 0.71^{a}	11.38 ± 0.21^{a}	11.01 ± 2.08^{a}	9.50 ± 0.53^{a}
Total protein (g/L)	6.07 ± 0.52^{a}	7.99 ± 0.62^{b}	5.80 ± 0.32^{b}	5.94 ± 0.46^{a}
BUN (mg/dL)	8.03 ± 2.01^{a}	10.90 ± 1.84^{ab}	9.91 ± 0.73^{a}	11.05 ± 1.89^{b}
Glucose (mmol/L)	5.59 ± 0.01^{a}	5.78 ± 0.01^{a}	5.71 ± 0.02^{a}	5.40 ± 0.20^{a}
Potassium (mmol/L)	6.95 ± 1.13^{a}	5.52 ± 0.18^{a}	5.94 ± 0.15^{a}	5.96 ± 0.25^{a}
Sodium (mmol/L)	145.33 ± 2.51^{a}	146.52 ± 3.17^{a}	143.76 ± 0.68^{a}	141.98 ± 0.82^{a}
Chloride (mmol/L)	125.5 ± 3.52^{a}	110.45 ± 2.53^{ab}	98.51 ± 1.15^{a}	95.78 ± 1.58^{a}
Calcium (mmol/L)	5.54 ± 0.12^{a}	4.85 ± 0.01^{a}	5.01 ± 0.05^{b}	5.15 ± 0.04^{a}
Urea (mmol/L)	6.08 ± 0.21^{a}	7.89 ± 1.50^{a}	7.45 ± 0.58^{a}	6.50 ± 0.15^{a}
Creatinine (mmol/L)	0.56 ± 0.23^{ab}	0.73 ± 0.23^{b}	0.48 ± 0.35^{a}	0.66 ± 0.34^{b}
WBC count ($\times 10^{9}$/L)	16.20 ± 2.5^{a}	7.53 ± 1.60^{b}	9.02 ± 1.5^{b}	8.9 ± 2.4^{b}
Platelets ($\times 10^{9}$/L)	789.45 ± 5.84^{a}	734.01 ± 2.52^{c}	650.53 ± 7.89^{c}	645.32 ± 3.50^{b}
Basophils (%)	0.85 ± 0.51^{a}	0.55 ± 0.01^{a}	0.53 ± 0.21^{a}	0.50 ± 0.35^{a}
Monocytes (%)	27.56 ± 3.21^{a}	15.65 ± 4.50^{c}	12.89 ± 5.01^{c}	18.98 ± 2.51^{b}
Hg (g/L)	12.80 ± 1.6^{ab}	10.04 ± 2.04^{a}	9.03 ± 2.09^{ac}	8.9 ± 1.76^{ab}
RBC ($\times 10^{9}$/L)	8.75 ± 0.05^{a}	9.84 ± 0.51^{a}	8.70 ± 0.53^{a}	8.45 ± 0.15^{a}
PCV (L/L)	0.50 ± 0.03^{a}	0.52 ± 0.2^{bc}	0.48 ± 0.02^{ab}	0.47 ± 0.04^{ac}
MCHC (g/dL)	29.50 ± 1.52^{a}	32.55 ± 0.52^{a}	31.81 ± 1.20^{a}	31.50 ± 0.51^{a}
LUC (%)	8.95 ± 1.05^{a}	10.25 ± 0.78^{a}	9.85 ± 1.07^{a}	8.45 ± 0.36^{a}

Means with the same letter are not significantly different ($P < 0.05$).

Legend: alanine transaminase (ALT); aspartate transaminase (AST); alkaline phosphatase (ALP); gamma glutamyl transferase (GGT); blood urea nitrogen (BUN); haemoglobin (Hg); pack cell volume (PCV); white blood cell (WBC); red blood cell (RBC); mean corpuscular haemoglobin concentration (MCHC); large unstained cell (LUC).

indices are well known to determine the hemolytic damage on RBCs. The absence of changes on these functional properties suggests that the extract does not possess toxic substances that can cause anemic condition in rats. A decreased hemoglobin and hematocrit levels, indicative of anemia, is associated with hemolysis from antigen-antibody response. Rise in white blood cell (WBC) is generally considered to be a marker of stress and a defence mechanism triggered by immune system.

In this study, there was no significant difference in WBC and hemoglobin in all observed groups. The blood creatinine or urea nitrogen levels are indicative of renal function [28]. Awasthy et al. [29] reported significant increase in creatinine of rats when basal feed was supplemented with 25% and 50% Jatropha seed protein. However, administering *J. curcas* at doses far below lD_{50} (500, 1000, and 2000 mg/kg body) in this study was not associated with significant change in weight, liver chemistry, and hematologic profile when compared with control.

Elevated serum transaminase activity is highly suggestive of hepatic impairment in animals [30]. Serum transaminases was reported to be significantly elevated in goats fed with *J. curcas* seeds at 0.25 to 10 g/kg/day up to 21 days; 23 in calves orally administered *J. curcas* seeds in suspension at 0.25, 1.0, and 2.5 g/kg within 14 days [31]. Also desert sheeps were fed with the seeds at 0.5 and 1.0 g/kg/day [32]. The common denominator in the above toxicity studies is that they were all administered seeds. Our current study on *J. curcas* leaves

did not show any significant increase in serum transaminase. This observation may be due to lower concentration phorbol esters in *J. curcas* leaves, exposure time, dose, and extraction type (methanolic extracts). Several compounds have been isolated from *J. curcas* seeds, it is reasonable to extrapolate that these compounds are present at lower concentration in the leaves as well. The compounds include saponins, lectins (curcin), phytates, protease inhibitors, curcalonic acid, and phorbol esters, but studies that isolate phorbol esters in toxic and non toxic strains have determined that phorbol esters as compounds of concern [28]. Phorbol esters are present in leaves, stems, flowers, and roots of *J. curcas* [11].

3. Experimental Section

3.1. Collection and Identification of Plant Materials. Fresh leaves of *J. curcas* were collected from a local farm in Benin City, Edo State, Nigeria, in the month of June 2010 and were identified by the Botany Department of Ambrose Alli University, Ekpoma, Nigeria.

3.2. Extraction of Plant Materials. Powdered plant materials (100 g of each) were extracted with 1000 mL methanol in a Soxhlet apparatus for 8 hours. The obtained methanolic extracts were filtered and evaporated by using a rotary evaporator and freeze dryer to give the crude dried extract.

3.3. Experimental Animals. Total of 80 male Wistar (8 weeks old) rats were used as the experimental animals; 40 for LD_{50} determination and the other 40 for toxicity study. The animals were maintained in a room with controlled temperature (35 ± 2°C) for 12 h light/12 h dark cycle with food (standard pellet diet) and sterile water provided *ad librium*. The animals were habituated to the experimental room for at least 24 h before the experiments. After acclimatization, the mice were weighed and numbered. The experiment was conducted in a barrier system with an experimental facility. Animal studies were in compliance to the ethical procedure for the care and use of laboratory animals that corresponds with NIH guidelines.

3.4. Assay Kits. The assay kits for total protein, creatinine, urea, calcium, sodium, potassium, chloride, albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), alanine aminotransferases (ALT), and aspartate aminotransferases (AST) were obtained from Bayer's Diagnostics, Baroda, India.

3.5. Determination of LD_{50} of the Extract. Rats weighing 102 ± 4.8 g were used for the determination of LD_{50} of the extract. Based on dose levels that decrease in geometrical progression, the regression equation between the probits of mortalities (Y) and the log of doses (D) was also derived: Y (probit) = −9.67 + 10.21 log (D). Four (4) doses (86.00, 58.00, 46.00, and 34.0 kg/body mass) were ultimately required for establishing the LD_{50}. Calculation of LD_{50}, was based on 95% confidence limits [33]. The Bliss was calculated by using the NDST Software Version 8.0 [34].

3.6. Toxicity Study Design. A pretest was conducted to observe the range of toxicity in others to establish dose range for LD_{50} determination. Three dose levels (6, 12, and 18 mg/kg body mass) of methanoic leave extract of *J. curcas* were used for the pretesting. Based on the pretest results, 4 dosages (86.00, 58.00, 46.00, and 34.0 kg/body mass) were established with each group comprised of 10 rats using random block design.

The rats were randomly assigned into four groups with 10 rats in each group. Rats in groups 2, 3, and 4 were given 500, 1000, and 2000 mg/kg body weight, respectively, orally by intubation. Control rats (group 1) were administered the same volume of deionized water. The doses administered were below the LD_{50} of the extract which was found to be 46.0 g/kg body weight of rat.

The rats were fed daily for two weeks. At the end of study period after fasting for 3 hours, each rat was anaesthetized in chloroform-saturated chamber. Under anesthesia, the abdominal and thoracic region of each rat was opened to assess the heart. The blood samples were collected through cardiac puncture in properly heparinized vials. The tube was swirled and placed on ice. Plasma was obtained from blood by centrifugation at 3000 rpm for 5 min. The different biochemical parameters were analyzed from blood plasma by using the diagnostic kits (Bayer's Diagnostics, Baroda, India) with the help of a semiautomated analyzer (RA-50 chemistry). Clinical signs, change in body mass, and toxicity symptoms were observed daily for 21 days.

3.7. Statistical Analysis. Experimental data were expressed as mean of six replicates and subjected to one way analysis of variance (ANOVA). Means were separated by Duncan multiple range test using the Statistical Analysis System (SAS version 8, SAS Institute, Cary, NC, USA). Values were considered statistically significant level at $P < 0.05$.

4. Conclusions

In conclusion, although direct extrapolation of results from animal models cannot be applied to humans, results obtained from this study suggest that the liver, kidney, and hematological system of rats tolerated leave extract of *J. curcas* at a certain concentration. However, histopathology study is desirable to confirm these findings. One issue that remains unsolved is the efficacy of this plant under investigation at nontoxic doses.

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Review Article

Alternative Splicing for Diseases, Cancers, Drugs, and Databases

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Alternative splicing is a major diversification mechanism in the human transcriptome and proteome. Several diseases, including cancers, have been associated with dysregulation of alternative splicing. Thus, correcting alternative splicing may restore normal cell physiology in patients with these diseases. This paper summarizes several alternative splicing-related diseases, including cancers and their target genes. Since new cancer drugs often target spliceosomes, several clinical drugs and natural products or their synthesized derivatives were analyzed to determine their effects on alternative splicing. Other agents known to have modulating effects on alternative splicing during therapeutic treatment of cancer are also discussed. Several commonly used bioinformatics resources are also summarized.

1. Introduction to Alternative Splicing

Alternative splicing of RNA is a key mechanism of increasing complexity in mRNA and proteins [1]. Since alternative splicing apparently controls almost all human gene activities, imbalances in the this splicing process may affect the progression of various human diseases and cancers [2]. Varying alternations in excision and/or inclusion of exons may generate different mRNA transcripts and corresponding proteins. Therefore, in addition to mediating changes in protein structure, function, and localization [3], alternative splicing in higher eukaryotes affects the differentiation and development of cancer and other diseases [4].

2. Alternative Splicing and Diseases

Alternative RNA splicing is commonly reported in neurological and muscle diseases [5–7]. Studies show that these diseases at least partly result from alternative splicing, which regulates the complexity of integral membrane proteins, including changes in their topology, solubility, and signal peptides [3]. For example, aberrant alternative splicing has shown associations with Parkinson disease [3, 8]. For spinal muscular atrophy (SMA), the level of survival motor neuron (SMN) protein was downregulated by its alternative splicing [9]. Therapies for SMA have recently improved by targeting RNA splicing for inclusion of exon 7 into SMN mRNA [10].

Phorbol 12-myristate 13-acetate was reported to modulate the alternative splicing of sarcoplasmic reticulum Ca^{2+}-ATPase1 (SERCA1) which is dysregulated in myotonic dystrophy type 1 disease [11].

Additionally, alternative splicing reportedly regulates heart development [12], cardiovascular disease [13], blood coagulation [14], cholesterol homeostasis [15], cellular proliferation, apoptosis, immunity [16], and systemic sclerosis [17]. For example, heart-specific knockout of the serine/arginine- (SR-) rich family of splicing factors, ASF/SF2, produces cardiomyopathy and affects splicing of cardiac troponin T and LIM domain-binding protein [18]. Specific CLK inhibitors (dichloroindolyl enaminonitrile KH-CB19) of CDC2-like kinase isoforms 1 and 4 (CLK1/CLK4) can inhibit phosphorylation of cellular SR splicing factors and affect the splicing of tissue factor isoforms flTF (full-length TF) and asHTF (alternatively spliced human TF) [19].

Using exon array, the global mRNA splicing profile of ischemic cardiomyopathy has been investigated, and the alternative splicing of four main sarcomere genes, such as cardiac troponin T (TNNT2), cardiac troponin I (TNNI3), myosin heavy chain 7 (MYH7), and filamin C, gamma (FLNC), was dysregulated [20]. The alternative splicing of blood coagulation-related genes including tissue factor (coagulation factor III), tissue factor pathway inhibitor (TFPI), and coagulation factor XI has been well reviewed [14]. For cholesterol production and uptake, alternative splicing of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and LDL receptor (LDLR) can suppress their protein activities [21, 22]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) [23], HMG-CoA synthase (HMGCS1) [24], and mevalonate kinase [25] also reported to be involved in cholesterol biosynthesis and receptor-mediated uptake through alternative splicing.

3. Alternative Splicing and Cancer

In cancer-associated genes, splicing has important roles in oncogenesis, tumor suppression [26], and metastasis [27]. Alterations in alternative splicing are commonly reported in various cancers [27–29]. Reported examples include p53 and PTEN [30], kallikrein-related peptidase 12 (KLK12) [31], breast cancer early-onset 1 (BRCA1) [32], protein N-arginine methyltransferases 2 (PRMT2) [33], and CDC25 phosphatases [34] in breast cancer; lysyl oxidase-like 4 (LOXL4) [35] and growth factor receptor-bound protein 7 (GRB7) in ovarian cancer [36]; androgen receptor in prostate cancer [37]; tissue inhibitor of metalloproteinases-1 (TIMP1) and the cell adhesion molecule CD44 in colon cancer [38, 39]; Bcl-xL, CD44, and others in lung cancer [40]; calpain 3 in melanoma [41]; and Krüppel-like factor 6 (KLF6) in liver cancer [42]. Therefore, alternative spliced variants are potential biomarkers [43, 44] for the cancer diagnosis/prognosis and may be the targets for cancer therapy based on specific splicing correction treatments.

Single nucleotide polymorphisms (SNPs) affecting exon skipping has reviewed to confer to complex diseases [45]. The improvement of high-throughput technologies such as RNA-Seq [46, 47] and exon arrays [48, 49] is helpful to identify the genome-wide cancer-associated splicing variants. Splicing changes may associate with lung and prostate cancer risk in terms of some SNPs. For example, a coding synonymous SNP G870A of cyclin D1 (CCND1) with a modulating ability to its splice pattern was reported to be associated with lung cancer susceptibility [50]. Similarly, some coding synonymous SNPs may generate new splicing sites in the middle of an exon of p53 gene to change splicing [51]. Mutations in the adenomatous polyposis coli (APC) [52] and BRCA1 [53, 54] genes have reported to skip exon by altering splicing. Furthermore, an intronic SNP, IVS −27 G>A/IVSΔA, creates a new splicing factor SR-binding site and deletes two other overlapping SR-binding sites, generating three alternative splicing forms of KLF6 (KLF6 SV1-3) [55]. This SNP was found to be associated with prostate cancer [56]. For lung cancer study, the tumor patients with overexpression of KLF6 SV1 have lower survival [56, 57].

Actually, the information of many SNPs located in 3′ and 5′ splicing sites is searchable in the dbSNP in NCBI website (http://www.ncbi.nlm.nih.gov/snp) when the "limit" function is chosen. Researchers may choose the SNPs of splicing sites located in interested genes to identify their association relationships to diseases and cancers. A database consisting of genome-wide SNP and splicing sites, namely, ssSNPTarget [58] was designed to search the splice site SNPs (ssSNPs) by input of gene symbol, SNP rs number, transcript ID, or genomic position (http://variome.kobic.re.kr/ssSNPTarget/).

4. Alternative Splicing-Related Drugs and Natural Products

Splice modulating therapies have been developed for human disease [59–61] and cancer therapy [62, 63]. Antitumor drugs have been developed to target alternative splicing [64], splice variants [65], and spliceosomes [66, 67]. For example, pharmacological interventions may be affected by mRNA transcript diversity [68]. To correct aberrant splicing, specific mRNA transcripts have been targeted in genetic disorders such as Duchenne muscular dystrophy. Since mutations of splicing factor 3B subunit 1 (SF3B1) are common in several haematological malignancies, the use of various natural products and their synthetic derivatives in therapies targeting SF3B has proven highly effective [67].

For drug discovery for SMA, several small molecules including sodium vanadate [69], aclarubicin [70], and indoprofen [71], hydroxyurea [72], valproate [73], 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) [74], and phenylbutyrate [75] that increases inclusion of exon 7 of SMN2 gene have been identified, although some of them may have side effects. Recently, novel phosphatase modulators, namely, pseudocantharidins have been discovered with the similar regulating function to SMN splicing [76]. Valproic acid was found to enhance SMN2 expression in SMA cell model involving the SF2/ASF and hnRNPA1 [77].

Clinical drugs such as novantrone (mitoxantrone) can enhance the effectiveness of therapeutic treatments for familial neurodegenerative diseases by stabilizing the tau pre-mRNA splicing regulatory element [78]. Tamoxifen has proven effective for clinical treatment of estrogen receptor-

(ER-) positive breast cancer [79]. In endometrial cancer cells, alternative splicing of ER involving ER-alpha36 is also known to enhance the agonist activity of tamoxifen [80].

Natural products, including many xenobiotics, are also known to impair alternative splicing [81]. For example, natural products such as pladienolide B and FR901464 [82, 83] are known to affect spliceosome function. However, the synthesis of these compounds is complicated by their multiple stereocenters. A recent study synthesized Sudemycins, which are novel analogues of FR901464. By inducing alternative gene splicing, the Sudemycins conferred both *in vitro* and *in vivo* antitumor effects [84]. Alternative splicing has also shown regulating effects on the antitumor drug Spliceostatin A, a stabilized derivative of a *Pseudomonas* bacterial fermentation product [85] which specifically targets the SF3b spliceosome subcomplex to inhibit pre-mRNA splicing [86]. Meayamycin, an analogue of the natural antitumor product FR901464 [87], inhibits RNA splicing against multidrug-resistant cells and performs antiproliferative effect against human breast cancer MCF-7 cells by suppression of alternative splicing [88]. These results suggest that, because of their modulating effects on RNA splicing, xenobiotic analogs have potential use as chemical probes and as anticancer agents. Similarly, the polyketide natural product borrelidin inhibits cancer metastasis by modulating alternative splicing in VEGF [89]. Antitumor effects involving alternative splicing [90, 91] have also been reported in natural dietary products. For example, resveratrol can modulate exon inclusion of SRp20 and SMN2 pre-mRNAs and induce the expression of processing factors of alternative splicing such as ASF/SF2, hnRNPA1, and HuR [92].

Recent studies have investigated the role of splice variants in apoptotic pathways [93, 94]. Regulation of alternative splicing genes may have anticancer effects. For example, BCL-X_s and BCL-X_L have been associated with proapoptotic and antiapoptotic effects, respectively, during the progression of cancer [67, 95, 96]. The ratio of BCL-X_s/BCL-X_L can be decreased by the treatment of protein kinase C (PKC) inhibitor and apoptotic inducer staurosporine in 293 cells [97]. Soluble and membrane-bound forms of TNF receptor superfamily, member 6 (FAS) containing exons 5/7 and 5/6/7 also display proapoptotic and antiapoptotic effects, respectively [67]. The caspase 9 (CASP9) gene has two antagonistic isoforms, proapoptotic Casp9a and prosurvival Casp9b, and its splicing is dysregulated in NSCLC lung cancer cell lines [98].

Alternative splicing is regulated by chromatin structure and histone modifications [4]. In thyroid tumor cells, for example, histone deacetylase inhibitors such as butyrate modulate transcription and alternative splicing of prohibitin [99]. A study of bovine epithelial cells showed that butyrate, a major metabolite generated by bacterial fermentation of dietary fiber in colon cells, has regulating effects on apoptosis and cell proliferation through alternative splicing [100]. Since histone deacetylase inhibitor may have antitumor effects, the identification of this kind of inhibitor in natural products can improve drug development for tumor therapy.

5. Alternative Splicing-Related Bioinformatics Resources

Several bioinformatics analyses for the detection and regulation of alternative splicing have been well reviewed [101–103]. However, these literatures mainly focused on the methodology for detection of alternative splicing, and the databases of alternative splicing are less addressed and summarized. Here, we collect several helpful bioinformatics resources related to alternative splicing as shown in Table 1.

For example, AsMamDB [104] is one of the early established alternative splice databases of mammals, although their websites are not functional currently. PALS db [105] provides the putative alternative splicing database based on UniGene clusters of human and mouse sources which mainly consist of EST data. Similarly, some databases such as EASED [107] and AVATAR [108] are constructed by datasets of EST and mRNAs. ASAP [106] provides the detail annotation for exon-intron boundary, alternative splicing, and its tissue specificity for the user to design probes for distinguishing different splicing isoforms. MAASE [109] is also specifically designed to apply in splicing microarray experiments. In contrast, Splicy [115] provide the web-based tool to predict possible alternative splicing events from Affymetrix probe set inputs. ASTALAVISTA [116] provides alternative splicing prediction for transcriptome data from GENCODE, REFSEQ, and ENSEMBL as well as from custom gene datasets. Furthermore, SpliceCenter [120] is a web server for predicting the influence of alternative splicing on RT-PCR, RNAi, microarray, and peptide-based data.

Both PolyA_DB [110] and AltTrans provide the information for alternative polyadenylation [111]. For AltTrans, the AltSplice pipeline on splicing and the AltPAS pipeline on polyadenylation were implemented. ASTD [126] also provides the variants for splicing, transcription initiation, and polyadenylation. Of note, the dataset of transcriptomics for alternative splicing is larger than for alternative polyadenylation. GRSDB [112] is a mammalian database of alternative splicing based on quadruplex forming G-rich sequences which modulate the 3′ end processing of pre-mRNAs.

Additionally, several comprehensive databases for alternative splicing have been developed such as HOLLYWOOD [113], ASD [114], BIPASS [117], ECgene [118], ASPicDB [121], AspAlt [125], H-DBAS [128], SPLOOCE [129], and APPRIS [130]. For example, the ECgene provides EST and serial analysis of gene expression (SAGE) data-based annotation and visualization for alternative splicing (AS). The ASPicDB provides EST-based tissue-specific splicing information of normal and cancer cells. The H-DBAS provides alternative splicing annotation based on RNA-Seq transcriptomics data. The APPRIS provides the annotation for principal isoform as the standard reference sequence for each gene.

Some resources of alternative splicing have special features such as splice signals in EuSplice [119], tandem splice sites in TassDB2 [127], mutational evidence-based analysis in Alternative Splicing Mutation Database [122], splicing proteins in SpliceAid 2 [131], and transcription factors in TFClass [132]. However, the impacts of alternative splicing on the spliced transcripts encoded protein structure are less

Table 1: The bioinformatics resources related to alternative splicing (yrs 2001–2013)*.

AsMamDB [104]	An alternative splice database of mammals (website is unavailable)
PALS db [105]	Putative alternative splicing database
ASAP [106]	Alternative splicing annotation project (http://www.bioinformatics.ucla.edu/ASAP/)
EASED [107]	Extended alternatively spliced EST database
AVATAR [108]	Database for EST and mRNA
MAASE [109]	Alternative splicing database designed for splicing microarray (http://maase.genomics.purdue.edu/)
PolyA_DB [110]	Database for mRNA polyadenylation in mammalian
AltTrans [111]	Annotation for both alternative splicing and alternative polyadenylation
GRSDB [112]	Database of quadruplex forming G-rich sequences in alternative splicing sequences (http://bioinformatics.ramapo.edu/grsdb/)
HOLLYWOOD [113]	A comparative relational database of alternative splicing (http://hollywood.mit.edu/)
ASD [114]	A bioinformatics resource on alternative splicing
Splicy [115]	Prediction of alternative splicing from Affymetrix data
ASTALAVISTA [116]	Analysis of alternative splicing for custom datasets (http://genome.imim.es/astalavista/)
BIPASS [117]	Bioinformatics pipeline alternative splicing services
ECgene [118]	Alternative splicing database update (http://genome.ewha.ac.kr/ECgene/)
EuSplice [119]	A resource for splice signals and alternative splicing in eukaryotic genes (http://www.genome.com/products-1/integrated-genomics-resources/eusplice)
SpliceCenter [120]	A server for analysis of alternative splicing on RT-PCR, RNAi, microarray, and peptide-based studies (http://discover.nci.nih.gov/splicecenter/)
ASPicDB [121]	Database for alternative splicing analysis (http://www.caspur.it/ASPicDB/)
The alternative splicing mutation database [122]	A hub for analyzing alternative splicing from mutational evidence
ProSAS [123]	Database for analyzing alternative splicing in the context of protein structures (http://www.bio.ifi.lmu.de/ProSAS/)
Splice-mediated Variants of Proteins (SpliVaP) [124]	Signatures for protein isoforms due to alternative splicing (http://www.bioinformatica.crs4.org/tools/dbs/splivap/)
AspAlt [125]	A interdatabase for comparative analysis of alternative transcription and splicing (http://www.genome.com/products-1/integrated-genomics-resources/products-integrated-genomics-resources-igr-aspalt)
ASTD [126]	Alternative splicing and transcript diversity database
TassDB2 [127]	A comprehensive database of subtle alternative splicing (http://www.tassdb.info/)
H-DBAS [128]	Human-transcriptome database for alternative splicing (http://h-invitational.jp/h-dbas/)
SPLOOCE [129]	Analysis server of human splicing variants (http://www.bioinformatics-brazil.org/splooce/)
APPRIS [130]	Annotation of human alternative splice isoforms (http://appris.bioinfo.cnio.es/)
SpliceAid 2 [131]	Database of human splicing factors expression data and RNA target motifs (http://www.introni.it/spliceaid.html)
TFClass [132]	Classification of human transcription factors (http://tfclass.bioinf.med.uni-goettingen.de/)

*The websites for some resources without function currently are not provided.

addressed. Some databases such as ProSAS [123] and SpliVaP [124] also provide the protein isoforms from the alternative splicing effects. In ProSAS, the protein isoforms of splicing transcripts are annotated in Ensembl or SwissProt. In SpliVap, protein signatures of alternative forms are annotated in terms of Pfam domains and PRINTS fingerprints.

6. Conclusion

Accumulating evidence shows that alternative splicing can be selectively targeted in several genes of cancer cells. An exciting possibility raised by this study is that the effectiveness of anticancer therapies may be enhanced by clinical drugs, natural products, and their synthesized analogs that target alternative splicing machinery. Some alternative splicing-related databases and web servers may also helpful to improve the alternative splicing therapy for treating cancer and other diseases.

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Research Article

TIPdb: A Database of Anticancer, Antiplatelet, and Antituberculosis Phytochemicals from Indigenous Plants in Taiwan

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The unique geographic features of Taiwan are attributed to the rich indigenous and endemic plant species in Taiwan. These plants serve as resourceful bank for biologically active phytochemicals. Given that these plant-derived chemicals are prototypes of potential drugs for diseases, databases connecting the chemical structures and pharmacological activities may facilitate drug development. To enhance the utility of the data, it is desirable to develop a database of chemical compounds and corresponding activities from indigenous plants in Taiwan. A database of anticancer, antiplatelet, and antituberculosis phytochemicals from indigenous plants in Taiwan was constructed. The database, TIPdb, is composed of a standardized format of published anticancer, antiplatelet, and antituberculosis phytochemicals from indigenous plants in Taiwan. A browse function was implemented for users to browse the database in a taxonomy-based manner. Search functions can be utilized to filter records of interest by botanical name, part, chemical class, or compound name. The structured and searchable database TIPdb was constructed to serve as a comprehensive and standardized resource for anticancer, antiplatelet, and antituberculosis compounds search. The manually curated chemical structures and activities provide a great opportunity to develop quantitative structure-activity relationship models for the high-throughput screening of potential anticancer, antiplatelet, and antituberculosis drugs.

1. Introduction

Plants have been valuable resources of traditional remedies since ancient times and continue to be major sources and inspirations for the development of therapeutic agents [1]. It was estimated that current global market for plant-derived drugs is worth more than 20 billion and the market continues growing [2]. Many clinically important drugs, from the oldest drugs on the market to recent approved drugs, are originated from plants. As an example, anti-inflammatory and antiplatelet drug aspirin, one of the most widely prescribed drugs on the market, is a plant-derived compound originally from willow and other salicylate-rich plants. Clinically important anticancer agents, such as paclitaxel, camptothecin, and vinblastine, and many promising anticancer agents currently under clinical trials are also plant-derived compounds [3, 4]. Yet, these clinically important drugs are only from 10–15% of plant species that have been explored for pharmaceutical purpose [2]. Taiwan, including the island of Taiwan and adjacent islets, is located at the boundary of tropical and subtropical areas, with the Tropic of Cancer passing through the middle. The island of Taiwan is mountainous, with a broad range of altitude, from sea level to the highest altitude of 3900 km. Owing to the unique geographical features and location, Taiwan is rich in diversity of plants [5]. The isolation of the islands from continent further contributes to the abundance of endemic species in Taiwan. As phytochemicals are evolved as part of the plant defense system in response to environmental stress, major phytochemical compositions between species may

diverse and largely differ in their relative abundance [6, 7]. Indigenous plants in Taiwan, especially endemic species, are therefore precious sources of novel pharmacologically active compounds.

Modern chemical purification and identification technologies have allowed the identification of many novel phytochemicals from plants. In the past few decades, efforts have been made to explore novel phytochemicals from indigenous plants in Taiwan. A few of them were further examined for their biological activities, including cytotoxic activity against cancer cells, antiplatelet activity, and antituberculosis activity. With the accumulating information on the biological activities of these plant-derived compounds from indigenous plants in Taiwan, there is still no comprehensive database which links the novel chemical structures and their known pharmacological activities in a quantitative manner. Such information may not only improve our understanding to indigenous plants in Taiwan, but also facilitate the development of new therapeutic agents. An integrated platform with convenient search function for chemical structures and known biological activities is of great value. As a comparison, databases of Indonesian herbal constituents and aldose reductase inhibitors have been constructed and have enabled the building of quantitative-structure activity relationship (QSAR) models for virtual screening of novel aldose reductase inhibitors, potential therapeutic agents for diabetic retinopathy, and related neuropathy diseases [8].

The presented database is designed to center with compounds identified from indigenous plants in Taiwan which have been examined for their anticancer, antiplatelet, or antituberculosis potential. The biological activities of a total of 99 indigenous species, including 29 endemic and 3 naturalized species, are curated into the database, with 5243 records of anticancer, antiplatelet, and antituberculosis activities, respectively. The curated data includes the recent isolated antitubercular flavonoids with promising minimal inhibitory concentration (MIC) [9, 10]. With the emerging prevalence of multidrug-resistant tuberculosis and lack of effective treatments [11, 12], these phytochemicals are especially valuable sources serving as promising candidates and/or QSAR skeletons for novel antituberculosis drugs development. Similar strategies are also applicable for anticancer and antiplatelet drug development. To our knowledge, this is the first publicly available database containing quantitative pharmacological data of phytochemicals from indigenous plants in Taiwan.

2. Construction and Content

The TIPdb database is implemented using MySQL Server Edition 5.1. The TIPdb website is publicly available at http://cwtung.kmu.edu.tw/tipdb. The web interface and all functions are implemented using PHP and JavaScript languages. The software libraries of jQuery UI [13] and Google Chart Tools [14] are utilized to make menus and sortable tables.

2.1. Database Content. Indigenous plants and corresponding chemical compositions were extracted and manually curated from the published literature. Taxonomy classifications were manually assigned to the indigenous plants. Experimental information of cytotoxicity against cancer cells, antiplatelet activity, and antituberculosis activity of the chemical compounds was also manually curated from the published literatures (Figure 1).

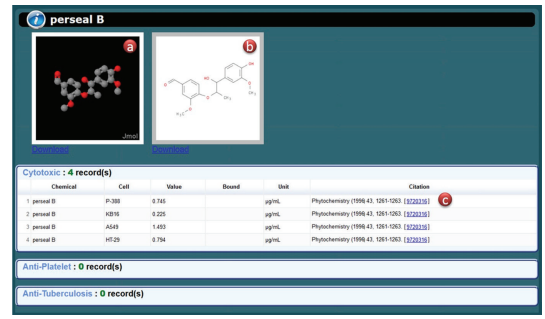

Figure 1: Content of a typical TIPdb entry. (a) 3D structure, (b) 2D structure, and (c) activity data and citation.

For cytotoxicity data, the curated information includes cell type, assay condition (effective concentration (EC) or test concentration measured by MTT assay), activity value, and assay bound. For antiplatelet compounds, both chemical and inducer names with their concentrations are curated in addition to activity value. The information curated for antituberculosis compounds includes testing strain, activity value (MIC), and assay bound. Plants which were published in the literature with synonym names were manually changed to accepted names, following classification in the Flora of Taiwan, 2nd edition [5]. Such examples include *Persea obovatifolia* Kost. (now classified as *Machilus* genus) [15] and *Hernandia sonora* (now as *Hernandia nymphaeifolia*) [16].

Chemical structure files were either manually created or downloaded from PubChem database [17]. MySQL Server Edition 5.1 was utilized to implement databases. Web user interface and functions were implemented using PHP and JavaScript languages. The java applet-based program Jmol [18] has been extensively used in many websites for interactive displays of structure of biomolecules and chemicals. For example, Protein Data Bank (PDB) [19] and PupDB [20] utilize Jmol to display protein structures with ligands and pupylation sites, respectively. The chemical information resource ChemSpider also displays chemical structures using Jmol [21, 22]. In this study, TIPdb integrates Jmol for interactive displays of chemical structures.

3. Utility and Discussion

TIPdb is a database of phytochemicals from Formosan indigenous plants aiming to provide an easily accessible web service for data management, analysis, and novel therapeutic agent screening. Citation information is provided for accessing the original article source. For articles indexed in PubMed databases, hyperlinks to PubMed citations are available.

The analysis of the bioactive chemical structures in TIPdb may provide insights into structure diversity and functional groups and substructures of the chemicals. For example, quantitative structure-activity relationship (QSAR) models can be developed based on the collected chemicals and

Figure 2: The taxonomy toolbar of browse tool. Users can browse plants by sequentially select (a) order, (b) family, (c) genus, and (d) species.

Figure 3: The search tools. Five search tools are available, including (a) the part of the plants, (b) the class of the chemicals, (c) botanical name, and (d) chemical activity.

activities. To facilitate such applications, several tools are constructed and integrated into TIPdb to provide useful functions of browsing, searching, and interactive displays of chemical structures. The functions of the integrated tools are introduced in what follows.

3.1. Browse Tool. Users can browse TIPdb by selecting the "Browse" option. The left panel will show a taxonomy toolbar. The Taiwan indigenous plants in TIPdb are categorized into a taxonomy tree. Users can browse the taxonomy tree in the sequence of order, family, genus, and species (Figure 2). Corresponding chemical compounds from the plants will be shown in the right panel as a sortable table. By clicking the caption of a specific column in the sortable table, data of the selected column will be sorted in the output table. Users are allowed to specify the number of rows shown per page.

To browse details of a specific chemical compound, users can click the chemical name to show its corresponding activities of cytotoxicity against cancer cells, antiplatelet activity, and antituberculosis activity in the bottom panel. The browse results are presented as a sortable table. Chemical structures of the compounds are shown as an interactive program by using Jmol.

3.2. Search Tools. For retrieving entries of interest, TIPdb provides a search tool using keywords of botanical name, part, chemical name, chemical class, and activity (Figure 3). The search fields can be individually or simultaneously utilized to filter entries. The research results will be shown in the right panel with links to detailed information of chemical structure and activity.

3.3. Interactive Tool for Protein Structure. TIPdb incorporates the Jmol applet of latest version 13.0 for interactive displays of chemical structures. Users can either use the user interface or scripting console to manipulate chemical structures. There is also a link for downloading chemical structure file.

4. Conclusions

Phytochemicals from indigenous plants in Taiwan could be potential drugs. A structured and searchable database, TIPdb, was constructed to serve as a comprehensive and standardized searching resource for anticancer, antiplatelet, and antituberculosis activities. The chemical structures of these phytochemicals are also curated in the database to provide a great opportunity to develop quantitative structure-activity relationship models for high-throughput screening of potential anticancer, antiplatelet, and antituberculosis drugs. The unique content of TIPdb is expected to be a useful resource for drug discovery.

Availability and Requirements

The TIPdb is freely available at http://cwtung.kmu.edu.tw/tipdb. The website has been tested with the browsers Safari, Opera, Internet Explorer 7 or later, Firefox, and Google Chrome. The Java Runtime Environment (JRE) is required for interactive displays of pro-tein 3D structures by Jmol.

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Review Article

RNA Editing and Drug Discovery for Cancer Therapy

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RNA editing is vital to provide the RNA and protein complexity to regulate the gene expression. Correct RNA editing maintains the cell function and organism development. Imbalance of the RNA editing machinery may lead to diseases and cancers. Recently, RNA editing has been recognized as a target for drug discovery although few studies targeting RNA editing for disease and cancer therapy were reported in the field of natural products. Therefore, RNA editing may be a potential target for therapeutic natural products. In this review, we provide a literature overview of the biological functions of RNA editing on gene expression, diseases, cancers, and drugs. The bioinformatics resources of RNA editing were also summarized.

1. Introduction

RNA editing is the change of nucleotide sequence of RNA transcripts relative to that of the encoding DNA [1]. RNA editing can enhance the RNA and protein diversity [2]. Although five types of RNA editing have been discovered [3], the adenosine-to-inosine (A-to-I) editing is the most common type in higher eukaryotes [4–6]. The A-to-I editing may lead to changes in amino acid type and alternative splicing [7], thereby increasing the complexity of gene expression [8].

2. RNA Editing and Gene Expression

The A-to-I editing is an enzymatic process mediated by proteins of the family of adenosine deaminase acting on RNA (ADAR). Two types of A-to-I RNA editing such as site selective and hyper-editing have been summarized [9]. The site selective way usually converses one or a few A-to-I sites but the hyperediting way causes adenine deamination of long stretches of double-strand RNA [9]. Accordingly, the A-to-I RNA editing contributes a global posttranscriptional modification to the transcriptome diversity [10, 11]. A-to-I RNA editing is a common event that can lead to amino acid changes in translated exons and RNA folding or may edit in noncoding exons or introns [12, 13]. Additional gene products and functions are further generated than the original encoded genes to improve the complexity of gene expression.

RNA editing is essential in many organisms. Correct RNA editing is important in organism's development [11]. For example, RNA editing deficiency may display the deleterious phenotypes in plant and in mammals. For example, RNA editing mutant was reported with strong defects in organelle development [14] and with pollen abortion in male sterility [15]. A RNA editing deficiency of glutamate receptor subunit

GluR2 was reported in motor neurons of amyotrophic lateral sclerosis [16, 17]. The deficiency or misregulation of RNA editing may result in the development of diseases and cancers [17] as described later.

3. RNA Editing and Diseases

Since the RNA editing is essential in regulating gene expression of organisms, imbalance of RNA editing may lead to dysfunction of some proteins involved in normal physiology such as neural and immune functions. A large number of nervous system targets such as neurotransmitter receptors and ion channels [18, 19] undergo A-to-I RNA editing by ADARs [20]. For example, the RNA editing of serotonin (5-hydroxytryptamine (5-HT)) 2C receptor (HTR2CR) was altered in a depression animal model and antidepressants commonly reduced its RNA editing efficiency [21, 22]. Furthermore, the ADAR-mediated RNA editing in nervous system tissues may occur in both coding and noncoding transcriptomes [5, 13, 23, 24]. Particularly in the nervous system, editing in non-coding regions such as microRNA and 3′ untranslated regions (UTR) of mRNAs is more frequent than in coding regions [4, 23]. Therefore, correct and regulated RNA editing is important for marinating functional nervous system and avoiding neurological diseases [8, 25].

ADARs can regulate the innate immune system by editing the RNA transcripts of immune-related genes [26]. ADAR (ADAR1) may be involved in regulating the RNA editing and the replication of hepatitis delta virus (HDV). For example, the two forms of ADAR1 (ADAR1-S and ADAR1-L) are involved in HDV editing, where the ADAR1-S functions in unstimulated cells and ADAR1-L functions in IFN-alpha-stimulated cells [27]. RNA editing exhibits interactions between the host ADAR1, and structural motifs in the HDV RNA may play important roles in the HDV replication cycle [28].

4. RNA Editing and Cancers

The regulation of ADAR was found to depend on the differentiation status of pluripotent human embryonic stem cells [29], suggesting that A-to-I RNA editing is involved in human embryogenesis. Interfering the regulation of differentiation and apoptosis may promote carcinogenesis [30]. RNA regulation can modulate the expression of oncogenes or tumor suppressor genes [31]. A-to-I editing is responsible for structure change and base pairing features of the RNA molecule [32] and is involved in cell differentiation [33]. Accordingly, A-to-I RNA editing may contribute to cancer development and progression [34, 35].

For example, ADAR1 was downregulated when growth rates of HeLa-cell-derived tumors in xenograft model were inhibited [45]. ADAR1 deletion leads to regression of established chronic myelogenous leukemia in mice [46]. ADARB2 (ADAR3) mRNA was decreased in glioblastoma multiforme [34], suggesting that reduced A-to-I editing is involved in brain carcinogenesis. However, downregulation of ADARB1 (ADAR2) inhibited cellular proliferation of pediatric astrocytoma [47] and glioblastoma [48]. Additionally, RNA editing may not be involved in the carcinogenesis of urinary bladder cancer [49].

Recently, some cancer-related RNA editing targets were discovered such as antizyme inhibitor 1 (AZIN1) and glioma-associated oncogene 1 (GLI1). A-to-I RNA editing of AZIN1 is increased in hepatocellular carcinoma [50]. RNA editing of GLI1 transcription factor involved in Hedgehog signaling is decreased in basal cell carcinoma tumor [51]. Therefore, the imbalance in expression of ADAR enzymes is highly correlated with cancer development and progression [52, 53].

5. RNA Editing and Drugs

mRNA transcript diversity such as RNA editing has profound impact on drug discovery [2, 54]. In addition to main gene products, isoforms generated by RNA editing may provide additional drug targets that have preferential physiological effects. Accordingly, transcript diversity creates potentially new opportunities for drug design, development, and therapy [54].

RNA editing has been suggested to be a therapeutic target for CNS disorders [55]. For example, RNA editing of the 5HT2C receptor may affect cell signaling, drug response, and brain function [56]. A-to-I RNA editing can also modulate the drug response of some channels, such as Kv1.1 channel [2, 57]. Therefore, RNA editing of these receptor and channels may change their protein functions and become a target for disease therapy [57].

Recently, some drugs for inhibiting RNA editing enzymes were discovered. For example, novel inhibitors of *Trypanosoma brucei* RNA editing ligase 1 were reported to be potential therapeutic drugs [58, 59].

6. RNA Editing and Bioinformatics Resources

Although there is a high chance of finding natural products that could target the RNA editing enzymes or lead to the RNA editing of some target genes, such investigations are still rare. It is possible that certain natural products have the potential to be the inhibitors or modulators for RNA editing and may have impacts on the disease and cancer therapy. Therefore, we collected the bioinformatics resources of RNA editing (Table 1) to help the researchers of natural products to investigate the effect of natural products on RNA editing.

In brief, dbRES [36] contains known RNA editing sites curated from the literature and GenBank. DARNED [37, 38] contains region-, gene-, and sequence-based inputs for RNA editing data retrieval from human and model organisms. miR-EdiTar [39] contains predicted miRNA binding sites that could be modified by A-to-I-editing, as well as A-to-I editing-induced miRNA binding sites. Both ExpEdit [40] and RNA-eXpress [41] are the annotation tools of RNA editing prediction for RNA-Seq data. For organellar RNA editing resources, GOBASE [42], REDIdb [43], and PREPACT 2.0 [44] contain the interface for RNA editing data of mitochondrion- and chloroplast-encoded sequences. Some bioinformatics

Table 1: RNA editing bioinformatics resources.

Tools	Functions (Web sites)
dbRES [36]	A database for annotated RNA editing sites. (http://bioinfo.au.tsinghua.edu.cn/dbRES)
DARNED [37, 38]	A database of RNA editing in humans and model organisms with Wikipedia. (http://darned.ucc.ie)
miR-EdiTar [39]	A database of predicted A-to-I edited miRNA target sites. (http://microrna.osumc.edu/mireditar)
ExpEdit [40]	A webserver for human RNA editing in RNA-Seq experiments. (http://www.caspur.it/ExpEdit/)
RNA-eXpress [41]	An annotation tool for novel transcript features in RNA-Seq data including RNA editing and others. (http://www.rnaexpress.org)
GOBASE [42]	An organelle genome database with an interface for RNA editing data and others using multiple alignments. (http://gobase.bcm.umontreal.ca/)
REDIdb [43]	A database for organellar RNA editing sites. (http://biologia.unical.it/py_script/REDIdb)
PREPACT 2.0 [44]	Predicting RNA editing in organelle genome sequences with multiple references and curated RNA editing annotation. (http://www.prepact.de)

resources of RNA editing in plants [60–63] were not included in Table 1 because they have little relationships to drug discovery.

7. Conclusion

In the future, we expect that RNA editing studies related to natural products may be accumulating. We hope that this concept can inspire the scientific idea to connect the fields of RNA editing research and natural products for drug discovery in cancer therapy.

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Research Article

Cardiotoxin III Inhibits Proliferation and Migration of Oral Cancer Cells through MAPK and MMP Signaling

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Cardiotoxin III (CTXIII), isolated from the snake venom of Formosan cobra *Naja naja atra*, has previously been found to induce apoptosis in many types of cancer. Early metastasis is typical for the progression of oral cancer. To modulate the cell migration behavior of oral cancer is one of the oral cancer therapies. In this study, the possible modulating effect of CTXIII on oral cancer migration is addressed. In the example of oral squamous carcinoma Ca9-22 cells, the cell viability was decreased by CTXIII treatment in a dose-responsive manner. In wound-healing assay, the cell migration of Ca9-22 cells was attenuated by CTXIII in a dose- and time-responsive manner. After CTXIII treatment, the MMP-2 and MMP-9 protein expressions were downregulated, and the phosphorylation of JNK and p38-MAPK was increased independent of ERK phosphorylation. In conclusion, CTXIII has antiproliferative and -migrating effects on oral cancer cells involving the p38-MAPK and MMP-2/-9 pathways.

1. Introduction

Oral squamous cell carcinoma (OSCC), the sixth most common form of cancer worldwide [1, 2], especially occurs in India, Taiwan, and Southeast Asia [3, 4]. Although many antioral cancer drugs were reported [5–9], the drug discovery against oral cancer remains a challenge.

Cardiotoxin III (CTXIII), composed of 60 basic amino acid residues, is isolated from the snake venom of Formosan cobra *Naja naja atra*. Although some anticancer drugs such as doxorubicin, anthracyclines, and trastuzumab have the well-known cardiotoxicity [10, 11], CTXIII was found to exhibit a variety of bioactivities with anticancer potential. For example, we previously found that CTXIII inhibits the cellular proliferation and induces apoptosis of various cancer cells, including breast cancer [12], leukemia cells [13], colorectal cancer [14], and oral cancer [15, 16].

The metastasis plays an important role in oral carcinogenesis [17]. However, little is known about the antimigration effect of CTXIII on oral cancer cells. In this study, we evaluated the role of CTXIII on cellular proliferation and migration of oral cancer cells Ca9-22. The role of mitogen-activated protein kinase (MAPK) family in CTXIII-induced antimigration in oral cancer cells was also investigated.

2. Methods

2.1. CTXIII Isolation. The isolation procedure of CTXIII was described previously [18]. Briefly, CTX III was purified from the venom of *Naja naja atra,* the Formosan cobrausing a chromatography on Sephadex G-50 and SP-Sephadex C-25. CTX III was dissolved in phosphate buffered saline (PBS) and filter sterilized through 0.2 μm pore-size membrane filter (Millipore Corp, Bedford, MA, USA).

2.2. Cell Cultures. Human gingival carcinoma Ca9-22 cells [5] were cultured in DMEM-F12 medium (Gibco, Grand Island, NY). Cells were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.03% glutamine, and 1 mM sodium pyruvate. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Growth Inhibition Test. The growth inhibition was determined by trypan blue dye exclusion assay combined with the Countess automated cell counter (Invitrogen, Carlsbad, CA, USA) as described previously [19, 20]. In brief, 1×10^5 Ca9-22 cells were seeded on a 12-well plate. Cells were treated with PBS as vehicle or indicated concentrations for 24 h, respectively. After incubation, cells harvested and exposed to 0.2% Trypan blue were counted.

2.4. Wound-Healing Assay. Cell migration was examined by wound-healing assay as described [21]. Briefly, a total of 3×10^5 Ca9-22 cells were seeded onto 12-well plates and then grown to complete confluence. A 200 μL plastic pipette tip was used to scratch the culture monolayer and create a clean 1 mm wide wound area. Cells were treated with PBS (as vehicle control) or indicated concentrations of CTXIII (from 1, 3, and 5 μg/mL). After incubation for 8 h, wound gaps were photographed at each time interval. The wound areas were then analyzed and calculated using the online software Wimasis (http://www.wimasis.com/; Wimasis GmbH, Munich, Germany).

2.5. Western Blotting. Western blot assay was described previously [22]. Briefly, cells were harvested and lysed. Lysates were centrifuged, and the protein concentration was determined. A total of 40 μg protein lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to 0.22 μm pore-size nitrocellulose membranes (Pall Life Sciences, Ann Arbor, MI). Membranes were blocked with 5% nonfat milk. Afterwards, the membranes were incubated with primary antibodies against MMP-2 (AnaSpec, no.29575), MMP-9 (AnaSpec, no.53678), phospho-JNK (Thr183/Tyr185, Upstate, no.07-175), phospho-p38 (Tyr182, Santa Cruz Biotech., sc-7973), phospho-ERK (Tyr204, Santa Cruz Biotech., sc-7976), and β-actin (Santa Cruz, sc-7963), their corresponding secondary antibodies, respectively. The signals were detected using a chemiluminescence detection kit ECL (Amersham Piscataway, NJ, USA).

2.6. Statistical Analysis. All data are presented as the means ± SD. All data were analyzed by Student's *t*-test.

3. Results

3.1. The Effect of CTXIII on Cellular Growth of Ca9-22 Cells. To examine the effect of CTXIII on cell growth, Ca9-22 cells were treated with PBS as vehicle control or indicated concentrations of CTXIII (1, 3, and 5 μg/mL) for 24 h, respectively. As shown in Figure 1, the cell viability was assessed by trypan blue exclusion assay, and CTXIII exerted a moderate cytotoxic effect on cell proliferation in a dose-responsive manner.

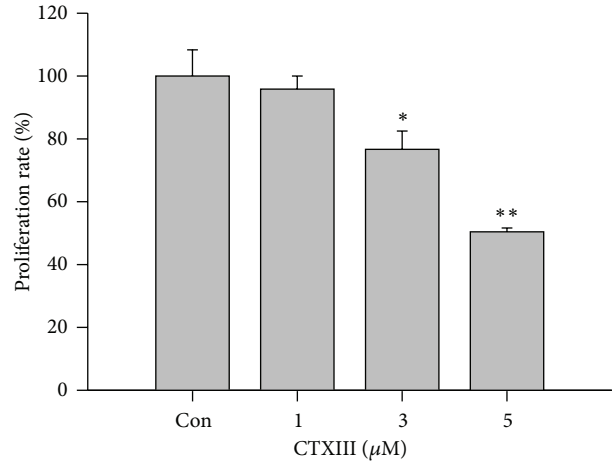

Figure 1: Inhibitory effect of CTXIII on proliferation of Ca9-22 cells. Cells were incubated with indicated concentrations of CTXIII (from 0, 1, 3, and 5 μg/mL) for 24 h. The proliferation inhibition was determined by trypan blue exclusion assay. Data, means ± SD ($n = 3$). $^*P < 0.05$ and $^{**}P < 0.001$ for control versus CTXIII-treated, respectively.

3.2. CTXIII Attenuates the Migration of Ca9-22 Oral Cancer Cells. Figure 2 showed that the migration of Ca9-22 oral cancer cells was significantly inhibited by CTXIII at concentrations of 2, 3, and 5 μM. Additionally, the cell motility of CTXIII-treated Ca9-22 cells was inhibited in a dose-responsive and time-dependent manner.

3.3. Assessment of the MMP-2 and MMP-9 Expressions. To examine whether CTXIII-induced anticellular migration involves the regulation of the expression of MMPs, Ca9-22 cells treated with indicated concentrations of CTXIII (vehicle control, 3 and 5 μg/mL) were subjected to the Western blotting assay. As shown in Figure 3, both MMP-2 and MMP-9 expressions were downregulated in CTXIII-treated Ca9-22 cells.

3.4. Assessment of the Mitogen-Activated Protein Kinase (MAPK) Signaling. To examine whether p38-MAPK involves CTXIII-induced migration-inhibitory effect, Ca9-22 cells treated at concentrations of CTXIII (vehicle control, 3 and 5 μg/mL) were subjected to the Western blotting assay. In CTXIII-treated Ca9-22 cells (Figure 4), the phosphorylation of JNK and p38-MAPK was increase, but the phosphorylation of ERK was not affected.

4. Discussion

In this study, the antiproliferation effect was found in CTXIII-treated oral cancer Ca9-22 cells. The inactivation of epidermal growth factor receptor (EGFR) and downstream pathways [15] and Src kinase were found to involve apoptosis

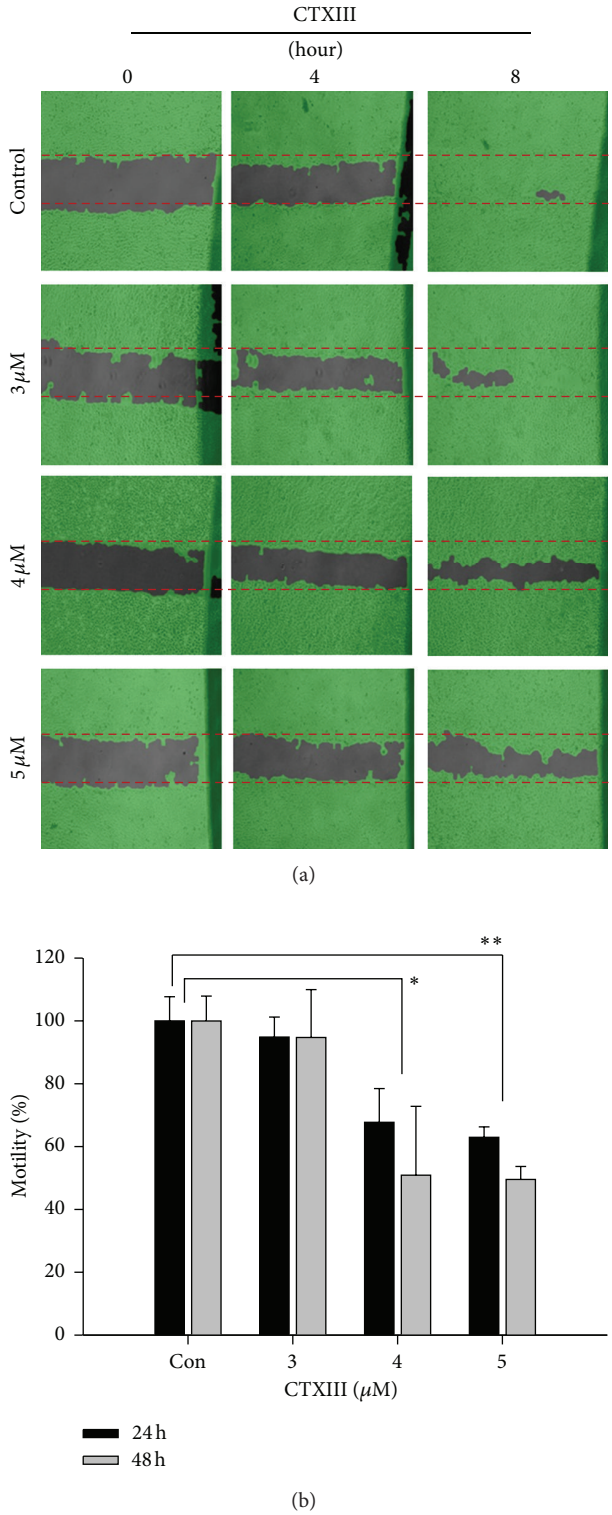

Figure 2: CTXIII inhibits cellular migration of Ca9-22 oral cancer cells. (a) 5×10^5 cells were seeded onto a 12-well plate, and cells were scraped to create a clean 1 mm wide wound area. Cells then were treated with the indicated doses of vehicle control, 3, 4, and 5 μg/mL of CTXIII for 8 hours. The wound areas were then analyzed and calculated using an online image analysis software Wimasis. (b) The quantitative results. Data, means ± SD ($n = 3$). $^*P < 0.05$ and $^{**}P < 0.001$ for control versus CTXIII treated, respectively.

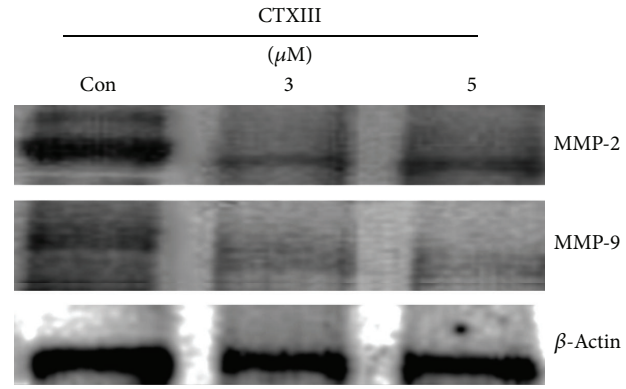

Figure 3: The regulation of MMP-2 and -9 expressions by CTXIII. Ca9-22 cells were treated with vehicle control, 3 and 5 μg/mL of CTXIII for 24 h, respectively. Two major prometastasis associated extracellular matrix metalloproteinases MMP-2 and MMP-9 were examined using the Western blot assay. β-Actin was used as an internal control. Each representative blot was performed in at least triplication.

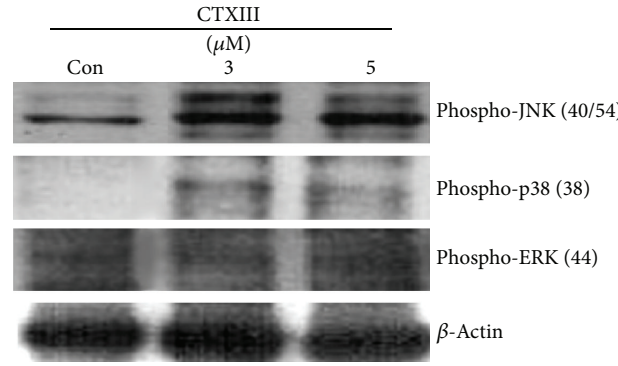

Figure 4: The regulation of MAPK signaling by CTXIII. Ca9-22 cells were treated with vehicle control, 3 and 5 μg/mL of CTXIII for 24 h, respectively. The phosphorylation levels of three major MAPK members JNK, p38, and ERK were examined using the Western blot assay. β-Actin was used as an internal control. Each representative blot was performed in at least triplication.

and cell cycle arrest of Ca9-22 cells after CTXIII treatment [16].

For other types of cancer cells, the antiproliferation and apoptosis-inducible effects of CTXIII have been reported [12–16, 23, 24]. The detailed mechanism of CTXIII-induced apoptosis have well demonstrated, such as mitochondrial alteration, reactive oxygen species generation of neuroblastoma SK-N-SH cells [23], NF-κB inactivation in breast MCF-7 cancer cells [12], and downregulation of the JAK2/PI3K signaling in breast MDA-MB-231 cancer cells [24].

In addition to the antiproliferation and apoptosis-inducible effects, we found that CTXIII can inhibit the migration of oral cancer cells. Early metastasis is a critical step for oral carcinogenesis and the overexpression of MMP-9, and extracellular matrix metalloproteinase leads to a poor prognosis of oral cancer [25]. Therefore, we found that downregulation of MMP-9 in oral cancer cells by CTXIII

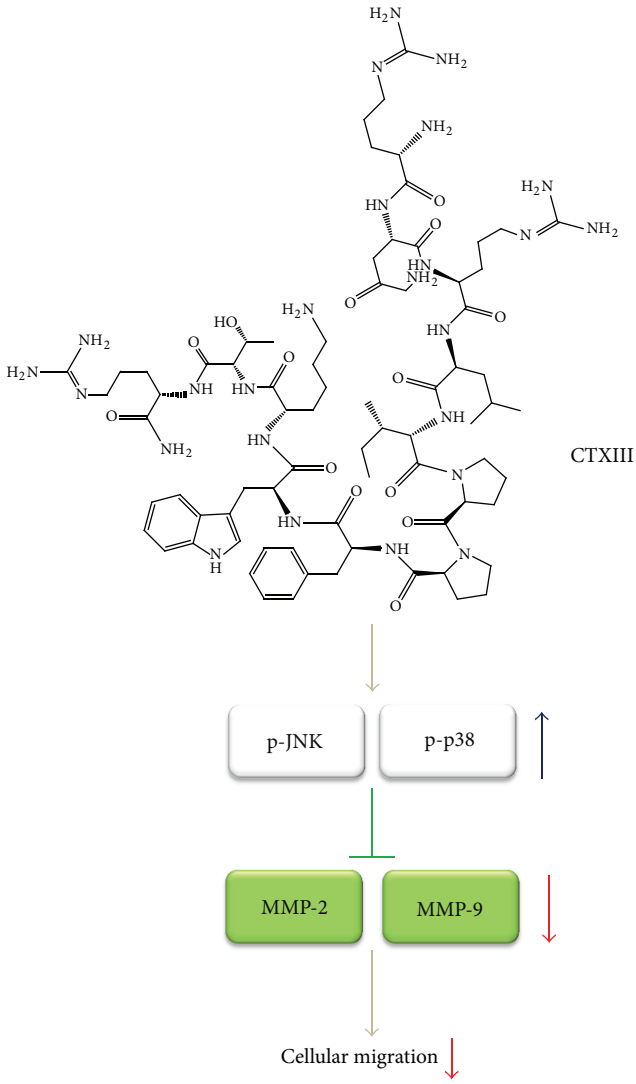

Figure 5: Proposed schematic mechanism of CTXIII-induced antimigration in human oral cancer cells. In current study, CTXIII exerts the antimigration potential against Ca9-22 cells in a dose-responsive manner. CTXIII causes the activation of MAPK member JNK and p38 without affecting ERK signaling. This may downregulate the expression levels of MMP-2 and MMP-9, the two major migration-associated extracellular matrix proteinases. Finally, the downregulated MMP-2 and MMP-9 expressions result in attenuating the migration potential of Ca9-22 cells.

treatment was helpful to therapeutic effect of oral cancer. Because MMPs are highly expressed in invasive tumors, it may play a vital role in tumor invasion and metastasis [26]. For example, the levels of MMP-2 and MMP-9 proteins were related to invasion of oral cancer [27]. Several drugs such as goniothalamin [21] and 17β-estradiol [28] were reported to inhibit the migration of lung and colon cancer cells by attenuating MMP-2 and MMP-9 activities, respectively. Consistently, the CTXIII mediated MMP2/9 to inhibit the migration of oral cancer cells in current study.

p38-MAPK can regulate invasion by modulation of MMP-2/-9 mRNA level and zymographic activity in bladder cancer model [29]. p38-MAPK also modulated the inhibition of migration in 17β-estradiol-treated human colon cancer cells by inhibition of MMP-2/-9 expression [28]. Similar to the current study, CTXIII-induced inhibition of migration downregulated the p38-MAPK phosphorylation and MMP2/9 protein expression. Consistent with these findings, both MMP-2/-9 and invasive activities were enhanced by exogenous expression of wild-type MAPK-activated protein kinase 2 and inhibited by p38-MAPK inhibitor [29].

Although the p38-MAPK and MMP-2/-9 were mediated in CTXIII-induced inhibition of migration in oral cancer cells, the phosphorylation of ERK was not involved in current study. Similarly, TGF-β-induced overexpression of MMP-2 and MMP-9 was mediated by p38 MAPK but not by ERK signaling in breast cancer cells [30]. Other mechanisms also reported the antimetastatic potential of CTXIII in breast cancer, such as EGFR signaling [18] and PI3K/Akt and p38 MAPK signaling [31].

5. Conclusions

This study demonstrates the roles of p38-MAPK and MMP-2/-9 pathways involved in the inhibition effect of proliferation and migration under CTXIII treatment in human oral cancer cells (Figure 5), and it may provide a potential oral cancer therapy.

Conflict of Interests

The authors declare no conflict of interests in the study.

Acknowledgments

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Research Article

Chemical Composition and Fatty Acid Content of Some Spices and Herbs under Saudi Arabia Conditions

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Some Saudi herbs and spices were analyzed. The results indicated that mustard, black cumin, and cress seeds contain high amount of fat 38.45%, 31.95% and 23.19%, respectively, as compared to clove (16.63%), black pepper (5.34%) and fenugreek (4.51%) seeds. Cress, mustard, black cumin and black pepper contain higher protein contents ranging from 26.61 to 25.45%, as compared to fenugreek (12.91%) and clove (6.9%). Crude fiber and ash content ranged from 6.36 to 23.6% and from 3.57 to 7.1%, respectively. All seeds contain high levels of potassium (ranging from 383 to 823 mg/100g), followed by calcium (ranging from 75 to 270 mg/100g), Magnesium (ranged from 42 to 102 mg/100g) and iron (ranged from 20.5 to 65 mg/100g). However, zinc, manganese and copper were found at low levels. The major fatty acids in cress and mustard were linolenic acid (48.43%) and erucic acid (29.81%), respectively. The lenoleic acid was the major fatty acid in black cumin, fenugreek, black pepper and clove oils being 68.07%, 34.85%, 33.03% and 44.73%, respectively. Total unsaturated fatty acids were 83.24, 95.62, 86.46, 92.99, 81.34 and 87.82% for cress, mustard, black cumin, fenugreek, black pepper and clove, respectively. The differences in the results obtained are due to environmental factors, production areas, cultivars used to produce seeds and also due to the different methods used to prepare these local spices.

1. Introduction

Some seeds are grown primarily for their use as condiments or for herbal medicine such as fenugreek (*Trigonella foenum-gracecum* L.), cress (*Lepidium sativum* L.), mustard (*Sinapis alba* L.), black cumin (*Nigella sativa* L.), black pepper (*Piper nigrum* L.), and clove (*Dianthus* sp.). Fenugreek is an annual crop belonging to the Fabaceae family. This crop is native to an area extending from Iran to northern India, but is now widely cultivated in China, north and east Africa, Ukraine, and Greece [1]. In parts of Asia, the young plants are used as pot herbs and the seeds as a spice or as herbal medicine [2]. Medically, fenugreek is reported to have antidiabetic, antifertility, anticancer, antimicrobial, and antiparasitic, and hypocholesterolaemic effects [3]. Seeds contain 26% mucilage, 22% protein comprising of globulin, histidine, and albumin with a good amount of phosphorus, sulphur, and also lecithin, it contains also 50% of soluble and insoluble fiber found essential for good health [4]. Fenugreek oil contains ω-3, ω-6, and ω-9 fatty acids along with many saponins, alkaloids, and sterols that serve as a source of proestrogens and inhibit intestinal cholesterol absorption [5]. Kochhar et al. [6] reported that fenugreek seeds contain 11.8% moisture, 25.8% crude protein, 6.53% oil, 3.26% ash, and 6.28% crude fiber and 58.13% total carbohydrates on dry basis. However, El-Nasri and El-Tinay [7] found that protein content of fenugreek was found to be 28.4%, crude fiber content was 9.3%, and crude fat was 7.1%. The fatty acid profile was dominated by unsaturated acids, namely, oleic, linoleic, and linolenic acids accounting for 16.3%, 50% and 24.4%, respectively of the total fatty acids. However, El-Sebaiy and El-Mahdy [8] reported that the fatty acids $C_{18:2}$ and $C_{18:3}$ were the most abundant fatty acids in the lipids of the fenugreek seeds. Thus fenugreek seeds may serve to be a beneficial health food if consumed regularly.

Cress is known as garden cress or garden cress pepper weed, and it is a fast growing annual herb. It belongs to the Brassicaceae family that is native to Egypt and west Asia but is widely cultivated in temperate climates throughout the world for various culinary and medicinal uses [9]. It was also reported cress seeds contain 22.5% protein, 27.5% fat, 30% dietary fiber, and 1193 mg/100 g potassium. Hence, it was assumed that these seeds can be used as a functional food. Moreover, Moser et al. [10] found that the oil content of dried cress seeds was 22.7% and the primary fatty acids found in cress oil were oleic (30.6%) and linolenic acids (29.3%). Cress oil contained high concentrations of γ- (1422 ppm) and (356 ppm) tocopherols. However, Gokavi et al. [9] reported that the primary fatty acids found in cress oil were oleic ($C_{18:1}$; 30.6%), linolenic ($C_{18:3}$; 29.3%), palmitic ($C_{16:0}$; 9.4%), linoleic ($C_{18:2}$; 7.6%), erucic ($C_{22:1}$; 3.0%), stearic ($C_{18:0}$; 2.8%), and arachidic ($C_{20:0}$; 2.3%) acids among the minor fatty acids found in cress oil.

Mustard is a herb belonging to the Brassicaceae family and the dry seeds are the only part used. It stimulates digestion and salivary secretion [11]. Mustard seeds have an advantageous chemical composition such as its protein content and fairly well-balanced amino acid composition, rich in dietary fiber and natural antioxidants. In addition to its nutritional value, mustard seed flour offers rather unique functional properties; therefore, it could be taken into consideration as potential component of many food products [12]. White mustard has been used effectively for food and medical applications, one of the limiting factors for human use of mustard products is the spicy flavor produced by myrosinase enzyme activities. Mustard seeds have high-energy content, having 28–32% oil with relatively high protein content (28–36%). The amino acid composition of mustard protein is well balanced; it is rich in essential amino acids. Mustard seeds until now have been used mainly for condiment production, however, this advantageous chemical composition and its relatively low price offer wide possibilities for utilization of this valuable seed, for example, in human foods as additive and to feed animals. Mustard oil has a special fatty acid composition, it contains about 20–28% oleic acid, 10–12% linoleic, 9.0–9.5% linolenic acid, and 30–40% erucic acid, which is indigestible for human and animal organisms. The high erucic acid content of mustard seed could be reduced by breeding, some low erucic acid content genotypes are in cultivation in several countries. Mustard oil is rich in tocopherols, as a consequence of their antioxidant characteristic, they act as a preservative against rancidity [10].

Black cumin is a herb belonging to the Ranunculaceae family and it is widely distributed in countries bordering the Mediterranean Sea, middle Europe, and western Asia [13]. The seeds of N. sativa have been known also as black cumin or black caraway in English and corek out in Turkish, and used as spice and culinary purposes [14]. Black cumin contains 30 to 40% oil and 20 to 30% protein, 3.7–4.7% ash and 25–40% total carbohydrates with antioxidants lignans such as saponin, melantin [15]. Fatty acid compositions of black cumin were $C_{14:0}$ (12.97–13.23%), $C_{16:0}$ (9.47–13.34%), $C_{18:1}$ (15.17–24.15%), and $C_{18:2}$ (54.32–70.81%) as reported by Cheikh-Rouhou et al. [13] and Tulukcu [16]. On the other hand, black cumin oil is considered as one among the newer sources of edible oils. Linoleic acid, undoubtedly one of the most important polyunsaturated fatty acids in human food because of its prevention of distinct heart vascular diseases is present in all the seed oils [17]. It was revealed that the oleic and linoleic acids are the most abundant monounsaturated and polyunsaturated fatty acids in all samples, respectively. The total MUFA composition of the studied species is assigned between 15.17 and 24.15%.

Pepper is a flowering vine belonging to the Piperaceae family and is the most widely used of all condiments. The components contributing to its value are the alkaloids, of which piperine is the most important, for pregnancy, and the volatile (essential) oil for odor and flavor as well as for massage [18]. Black pepper contains (11–14%) protein, (47–53%) fiber, and (10–13.5%) starch [19]. The content of piperine, volatile oil, starch, and fiber can vary markedly in different pepper varieties and is indicative of the quality of black pepper [20]. Black pepper contains about 5–9% of the alkaloids piperine and piperettine and about 1.2–5% of volatile oil [21]. Essential oil is a small portion of a plant material, which consists mainly of terpenes, sequiterpenes, and their derivatives that are responsible for the characteristic aroma, and imparts the identifying flavor and odor most closely associated with the plant itself [22].

Clove is the dried unopened flower bunds of *D. caryophyllus* L. that belong to the Caryophyllaceae family. The dried bulbs resemble a round-headed nail, are dark reddish-brown in color, have a strong aromatic odor and a hot pungent taste. Cloves are now cultivated in many parts of the tropics, particularly Tanzania, Madagascar, Malaysia, India, Srilanka, Jamaica, and French Guiana [23]. Oil of cloves has some antiseptic qualities and is recommended by some dentists as a flavoring aid. Milind and Deepa [24] revealed that clove seeds contains 5.98% protein, 20% total fat, 61.21% carbohydrates, 34.2 fibers, and 5.88% ash. However, it contains also high levels of potassium (1102 mg/100 g) and magnesium (268 mg/100 g). Milind and Deepa [24] also found total saturated fatty acids (5.38%), total monounsaturated fatty acids (1.47%), and total polyunsaturated acids (7.09%).

Although numerous studies on the effects of cultural practices on spices were conducted, changes in their chemical compositions are still far from being finalized specially in Saudi Arabia. Hence, the aim of this study was to investigate the chemical composition, mineral content, and fatty acid profiles of some locally produced spicesand herbs obtained from markets in Saudi Arabia.

2. Material and Methods

2.1. Materials. Fenugreek, cress, mustard, black cumin, black pepper, and clove were *grown in Saudi Arabia.*

2.2. Chemical Composition. Moisture, crude protein, crude fat, crude fiber, and ash were determined according to the AOAC [25] in 2 g, 2 g, 5 g, 2 g, and 5 g sample of each spice.

Table 1: Chemical composition (%) of cress, mustard, black cumin, fenugreek, black pepper, and clove seeds*.

Analysis	Cress	Mustard	Black cumin	Fenugreek	Black pepper	Clove
Moisture	2.88 ± 0.1	4.36 ± 0.1	2.55 ± 0.2	7.71 ± 0.2	4.68 ± 0.3	7.74 ± 0.2
Crude fat	23.19 ± 0.2	38.45 ± 0.5	31.95 ± 0.3	4.51 ± 0.2	5.34 ± 0.6	16.63 ± 0.3
Crude protein	24.19 ± 0.5	25.39 ± 0.3	20.61 ± 0.3	12.91 ± 0.4	25.45 ± 0.4	6.9 ± 0.4
Crude fiber	11.9 ± 0.4	6.36 ± 0.1	10.37 ± 0.1	13.14 ± 0.3	23.6 ± 0.3	11.47 ± 0.5
Ash	7.1 ± 0.1	4.25 ± 0.1	4.51 ± 0.1	4.23 ± 0.05	3.57 ± 0.1	5.96 ± 0.1
Total carbohydrate**	30.74 ± 1.2	21.19 ± 0.9	30.0 ± 1.2	57.5 ± 2.2	37.36 ± 1.4	51.3 ± 2.7

*Means ($n = 3$) ± SD.
**Calculated by difference.

Table 2: Mineral content (mg/100g) of cress, mustard black cumin, fenugreek, black pepper, and clove seeds*.

Mineral	Cress	Mustard	Black cumin	Fenugreek	Black pepper	Clove
K	663 ± 20.0	383.0 ± 10.0	823.0 ± 30.0	603.0 ± 15.0	663 ± 25.0	650.0 ± 30.0
Mg	102.0 ± 10.0	100.0 ± 8.0	80.0 ± 10.0	42.0 ± 5.0	52.0 ± 8.0	97.0 ± 10.0
Ca	105.0 ± 10.0	159.0 ± 15.0	160.0 ± 10.0	75.0 ± 9.0	195.0 ± 15.0	270.0 ± 20
Zn	3.7 + 0.3	3.4 + 0.3	2.5 ± 0.2	2.4 ± 0.2	0.9 ± 0.1	0.7 + 0.1
Mn	1.1 ± 0.1	1.8 ± 0.2	1.5 ± 0.1	0.9 ± 0.1	3.5 ± 0.2	43.8 ± 1.5
Cu	4.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	0.8 ± 0.1
Fe	21.0 ± 1.1	25.5 ± 1.5	65.0 ± 2.5	25.8 ± 1.2	20.5 ± 0.5	36.0 ± 0.8

*Means ($n = 3$) ± SD.

Potassium, magnesium, calcium, zinc, manganese, copper, and iron were extracted with acids from 5 g samples according to McGrath and Cubliffe [26] and their concentrations were detected using a PE model 2380 atomic absorption spectrophotometer.

2.3. Fatty Acids Analysis. Oil samples extracted from 5 g seed samples were methylated with 14% boron trifluoride (BF_3, BDH-Company) in methanol [27]. Analysis of the fatty acids was carried out with a GLC-Varian 6000 gas chromatograph with Flame Ionization Detector (FID), 2 m length, 0.32 cm internal diameter stainless steel column, packed with 15% OV-275, chrome P/AW/80-100 mesh stationary phase which operated at 180°C, injection temperature 230°C, detector temperature 250°C with carrier-gas (Helium) at a flow rate of 25 mL/min, hydrogen flow 30 mL/min, and air flow 300 mL/min. Identification of the fatty acid methyl esters was carried out by comparison of their retention times with that of the standards (Polyscience Corporation, Kit number 61 CX) and the quantities were calculated from the area obtained by the LKB 2200 recorded integrator.

3. Statistical Analyses

The factorial experiment in the completely randomized design was done with three replicates. Then all data were statistically analyzed using analysis of variance.

4. Results and Discussion

Chemical composition of spices is given in Table 1. The results indicated that mustard, black cumin, and cress seeds had higher fat content of (38.45%), (31.95%), and (23.19%), respectively, as compared to clove (16.63%), black pepper (5.34%), and fenugreek (4.51%) seeds. It could be noticed that cress, mustard, black cumin, and black pepper had higher protein content that ranged from 20.61% to 25.45%, as compared to fenugreek (12.91%) and clove (6.9%). Crude fiber and ash contents ranged from 6.36 to 23.6% and from 3.57 to 7.1%, respectively. Table 1 shows the obtained results. Kochhar et al. [6] and El Nasri and El Tinay [7] reported that fat, protein, and fiber content of fenugreek seeds ranged from 6.53% to 7.1%, 24.4% to 25.8%, and 6.28% to 9.3%, respectively. On the other hand, Ildikó et al. [12] found that mustard seeds contain 28–32% fat and 28–36% proteins. Gokavi et al. [9], mentioned that cress seeds contain fat, protein and fiber of 27.5, 22.5, and 30%, respectively. It has also been reported that black cumin had 30–40% fat, 20–30% protein, 3.7–4.7% ash, while the protein and fat contents of black pepper ranged from 11–14 and 47–53%, respectively, as reported by Jayashree et al. [19]. Moreover, clove contains 20% fat, 5.98% protein, 34.2% fiber, and 5.88% ash [10].

The low percentage of moisture in cress and black cumin as compared to the others may increase the shelf life of these spices during packaging and storage. They also limit fungal and contamination effects. Data presented in Table 2 shows the mineral contents of the different seeds under investigation. It could be noticed that all seeds contains higher levels of potassium (ranged from 383 to 823 mg/100 g) followed by calcium (ranged from 75 to 270 mg/100 g), magnesium (ranged from 42 to 102 mg/100 g), and iron (ranged from 20.5 to 65 mg/100 g). However, zinc, manganese, and copper metals were found at lower levels. These results differed mainly in the amount of potassium, magnesium,

Table 3: Fatty acid (%) extracted from cress, mustard, black cumin, fenugreek, black pepper, and clove seeds*.

Fatty acid	Cress	Mustard	Black cumin	Fenugreek	Black pepper	Clove
$C_{14:0}$	1.55	—	1.0	1.38	—	1.29
$C_{16:0}$	5.86	3.2	10.5	3.85	3.15	6.21
$C_{16:1}$	2.02	—	—	8.29	11.91	20.96
$C_{18:0}$	6.56	1.18	2.04	1.78	—	—
$C_{18:1}$ (Cis)	15.35	18.32	16.23	8.29	16.17	13.0
$C_{18:1}$ (Trans)	4.05	—	—	10.76	9.89	6.20
$C_{18:2}$	11.79	23.57	68.07	34.85	33.03	44.73
$C_{18:3}$	48.43	23.92	2.16	30.8	10.34	2.93
$C_{20:0}$	2.79	—	—	—	15.51	4.68
$C_{20:2}$	1.60	—	—	—	—	—
$C_{22:1}$	—	29.81	—	—	—	—
TSF	16.76	4.38	13.54	7.01	18.66	12.18
TUSF	83.24	95.62	86.46	92.99	81.34	87.82

calcium and iron, as compared to previous results obtained by Nergiz and Otles [28], for black cumin and fenugreek seeds (El-Mahdy, and El-Sebaiy [29]). However, Gokavi et al. [9] found that cress seeds contain 1193 mg/100 g potassium. While, clove seeds contain high levels of potassium (1102 mg/100 g) and magnesium (268 mg/100 g) as reported by Milind and Deepa [24]. The differences in the results obtained and that reported in previous studies may be due to environmental factors that prevail in production areas, cultivars used to produce seeds and also due to the different methods used to prepare these local spices.

Fatty acids composition of cress, mustard, black cumin, fenugreek, black pepper, and clove seed oils are presented in Table 3. The major fatty acids in cress and mustard were lenolenic acid (48.43%) and erucic acid (29.81%), respectively. While, linoleic acid was the major fatty acid in black cumin, fenugreek, black pepper, and clove oils being 68.07%, 34.85%, 33.03%, and 44.73%, respectively. Total unsaturated fatty acids were 83.24, 95.62, 86.46, 92.99, 81.34, and 87.82% for cress, mustard, black cumin, fenugreek, black pepper, and clove, respectively. These results are in good agreement with most of the previous studies. Moser et al., [10] reported that oleic (30.60%) and linolenic (29.3%) acids were the major fatty acids in cress seed oil. However, erucic acid (30–40%) was the major fatty acid in mustard oil according to Ali and McKay [11] and Ildikó et al. [12]. Nergiz and Otles [28], Tulukcu [16], and Sultan et al. [30] demonstrated that the predominant fatty acid in black cumin, fenugreek, black pepper and clove seed oils was linoleic acid.

From the results of this study, it could be concluded that the spices and herbs under investigation contain appreciable amounts of nutrients which may serve as beneficial health sources if consumed regularly specially cress and fenugreek and can be used as food supplements for edible oils, besides its uses as a condiments in home. Spices and herbs are used at relatively low levels in foods, these data indicate that spices may provide a meaningful level of protein, fat, and minerals when consumed in a variety of foods. Also, these results were obtained with relatively different results of others and this requires further studies on the impact of soil and weather conditions on the composition of these crops.

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Review Article

Interaction of Herbal Compounds with Biological Targets: A Case Study with Berberine

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Berberine is one of the main alkaloids found in the Chinese herb Huang lian (*Rhizoma Coptidis*), which has been reported to have multiple pharmacological activities. This study aimed to analyze the molecular targets of berberine based on literature data followed by a pathway analysis using the PANTHER program. PANTHER analysis of berberine targets showed that the most classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor-linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved. In addition, berberine was found to interact with a mixture of biological pathways, such as Alzheimer's disease-presenilin and -secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Hungtington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways. We also explored the possible mechanism of action for the anti-diabetic effect of berberine. Further studies are warranted to elucidate the mechanisms of action of berberine using systems biology approach.

1. Introduction

The majority of clinical drugs achieve their effect by binding to a cavity and regulating the cavity, of its protein targets [1]. In general, drugs act on four main types of regulatory proteins that mediate the actions of hormones, neurotransmitters, and autacoids. These four types of regulatory proteins are carriers, proteins, ion channels, and receptors [2]. Certain characteristics are expected for therapeutic targets [3]. A potential target needs only not to be druggable but also linked to disease, most preferably playing critical and inimitable roles in disease state. Binding sites are to have certain structural and physiochemical properties to accommodate high-affinity site-specific binding and subsequent regulation of protein activity by drugs. They are not significantly involved in other important biological processes to avoid potential side effects. Useful information about these targets may be investigated by analysing their sequence properties, protein families, structural folds, biochemical classes, similarity proteins, gene location in the human genome, and associated pathways [4]. This information can be potentially useful in derivation of rule and developing predictive tools in the search for druggable and potential targets [4].

The number of molecular targets acted on by current drug therapy is still in dispute. In 1996, Drews and Ryser identified a total of 483 drug targets addressed by drug therapy [5, 6]. Approximately 45% are cell membrane receptors, 28% are enzymes, and the remaining classes comprise

hormones (11%), ion channels (5%), nuclear receptors (2%), and DNA (2%). About 7% of the targets are not known biochemically. Later, Hopkins and Groom challenged this figure and suggested that "rule-of-five" compliant drugs acted primarily through only 120 underlying molecular targets [3, 7]. However, the statistical analysis of disease genes and related proteins suggested that the total number of the estimated potential targets in the human genome ranges from 600 to 1,500 [3]. In the meantime, another report showed the estimated total number of distinct targets is in the range of 1,700–3,000 [8]. Chen et al. reported targets collected in the Therapeutic Target Database [9] is 997 distinct proteins, 1,494 distinct protein subtypes, and 41 nucleic acids, which are only targeted by at least one marketed drug and 1,267 research targets, which are only targeted by investigational agents that are not approved for clinical use at present [4]. Targets for neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. An increase in target numbers is made possible by advances in genomics, proteomics, better molecular understanding of diseases, and increased effort in the exploration of new therapeutic targets as well as increased knowledge of unknown or unreported targets of previous existing drugs. An improvement in technology for target identification and validation also contributes greatly.

Chinese herbal medicine (CHM) has always been an integral part of traditional Chinese medicine (TCM), which has been practiced in the east for thousands of years. Chinese herbs are usually in the forms of dried whole plants or parts of the plants (roots, leaves, body, etc.); sometimes shells and even minerals are used. Chinese herbs are often used in a compound formula, consisting of several different herbs hosting different roles according to the principle of Jun-Chen-Zuo-Shi described by the ancient Chinese. Each of Jun, Chen, Zuo, and Shi function together to harmonise the body, with Shi (courier) herbs are included in many formulae to ensure that all components in the prescription are well absorbed and to help to deliver or guide them to the target organs [10]. On some level, the guiding function of Shi herbs relates to modern drug delivery techniques, guiding the drug compound to target tissues. In the modern world, complementary medicine has gained vast popularity in the West. There has been increased use of herbal medicine to manage chronic diseases and promote wellbeing, in countries such as Australia, New Zealand, USA, and Europe [11]. Reports show that 18.9% of the American population used natural products in the precedent year [12]. This increase in popularity is closely related to its proven effectiveness in clinical practice over the past centuries. To date, more than 11,000 species of plants are used medicinally and about 300 are commonly used [13].

Despite its widespread use, CHM is associated with high levels of uncertainty. This is mainly due to lack of evidence, base of efficacy, targets, and safety data. During the process of therapeutic drug development, owing to the preselection of targets, researchers have a basic if not full understanding of which molecular structures the drug will react with or which biological pathway in the body it might alter. Knowledge on molecular interactions and modulations of the drug is anticipated and researched on. However, this is not the case for CHM. There is no preselection of molecular targets in the body but CHM has been used for thousands of years and is proven to be effective. The exact mechanism of the herbs actions is yet to be elucidated.

The proven clinical efficacy of some herbal medicines is considered to be due to the interaction of pharmacologically active components from the herbs with molecular targets in the body. Similar to synthetic drugs, active compounds of herbal medicine may bind to and undergo interactions with molecular structures or herbal targets to produce therapeutic or adverse effects. However, there is a lack of understanding of how CHMs exert their biological and clinical effects at a molecular level, which impedes development of CHMs and the incorporation of CHMs into mainstream medicine in the West.

Berberine (Figure 1, molecular formula $C_{20}H_{19}NO_5$ and a molecular weight of 353.36) is an isoquinoline alkaloid found in many medicinal plants [14]. It is a major constituent of many medicinal plants of families Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, and Annonaceae [15]. It is present in *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). The berberine alkaloid can be found in the roots, rhizomes, and stem bark of the plants. Berberine is one of the main alkaloids found in the Chinese herb Huang Lian (*Rhizoma coptidis*) [16]. Huang Lian has traditionally been used to treat diarrhoea and diabetes. In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhoea [14, 17]. In recent years, there has been a growing interest in the pharmacological activities of berberine and many studies have been carried out to elucidate the mechanisms of action of berberine. This study aims to review molecular targets of berberine based on *in vitro* studies. Berberine has shown to have good hypoglycaemic effects, so we also reviewed the effects of berberine in animal and human studies, with a focus on diabetes mellitus.

2. Methods

2.1. Data Retrieval from the Literature. *In vitro* studies related to berberine and its targets were searched using Pubmed (from inception to April 2012). Search terms used were a combination of "berberine," "*in vitro*," "human cell," and "mechanism." Only studies using human cell lines were used to extract current berberine targets. Studies using animal cell lines or berberine derivatives or in a language other than English were excluded. Information extracted from these studies includes molecular targets of berberine (name and gene symbols), cell type, effects of berberine, and possible clinical applications.

2.2. PANTHER Analysis. Using the PANTHER Classification System, *in vitro* berberine targets were analysed using three

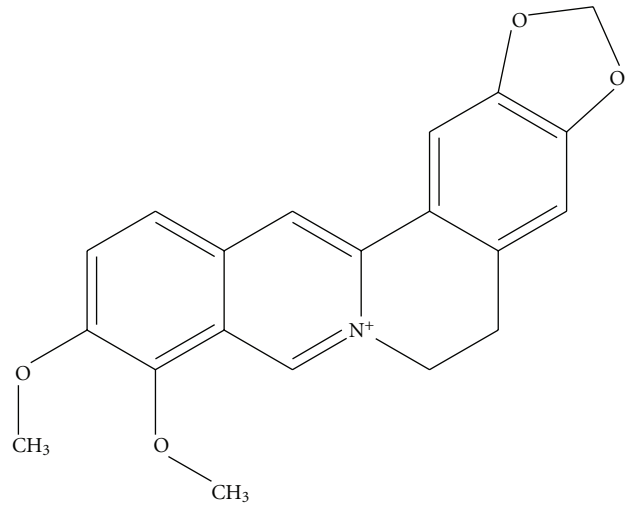

Figure 1: Chemical structure of berberine.

approaches: molecular function, biological process, and pathway involvement Table 2. PANTHER is a publicly available database that relates protein sequence evolution to evolution of protein functions and biological roles (http://www.pantherdb.org/).

3. Results

3.1. Targets of Berberine. A total of 90 berberine targets were identified in our literature search, as shown in Table 1.

Extensive research has been carried out to study the effects of berberine on cancer cells *in vitro*. This may be related to recent discovery of anti-cancer drugs with natural compound origin, for example, paclitaxel and topotecan.

Various human cancer cell lines were used to demonstrate the anti-cancer effects of berberine *in vitro*. These include cancer cell lines of the tongue, stomach, lung, colon, liver, breast, prostate, nasopharyngeal, neurones, epidermal, and blood [18–28]. Berberine has shown to induce cancer cell death via several mechanisms such as regulation of apoptosis proteins and cell cycle arrest.

Berberine treatment increased the expression of apoptotic cell death proteins, promotes cell cycle arrest, and induces cell death in human cancer cell lines. For instance, in human prostate epithelial cells (PWR-1E), berberine-increased expression of BCL2-associated X protein (Bax) was observed after berberine treatment, inducing cell death and demonstrating pro-apoptotic properties [29]. Similar effects of berberine were observed in prostate carcinoma cells (DU145, PC-3, and LNCaP) [21, 30]. Berberine also increased levels of Bax in promyelocytic leukemia cells [31], gastric carcinoma cells [24], and lung cancer cells [20].

Berberine can also promote cell death by the regulation of antiapoptotic proteins. Decreased expression of antiapoptotic Bcl-2 protein was observed in human oral squamous cell carcinoma after berberine treatment [23]. Studies done in other cancer cell lines such as lung cancer, gastric cancer, and prostate cancer also showed reduced levels of Bcl-2 after berberine treatment [20, 21, 24, 30]. Cell cycle arrest at different phases has also been observed in human cancer cell lines after treatment with berberine. In giant cell carcinoma and prostate carcinoma cells, berberine also decreased G_0/G_1 phase-associated cyclins (D_1, D_2, E, Cdk2, Cdk4, and Cdk6), inducing G_0/G_1 arrest and suppressing cell proliferation [21, 25, 30, 32]. Further, in HepG2 cells, berberine acted on B-cell CLL/lymphoma 2 (BCL2), procaspase-3 and -9, and poly (ADP-ribose) polymerase (PARP), induced cell cycle arrest at G_2/M phase and inhibited cell proliferation [22].

Further, berberine can promote cell death via the regulation of pro- and antiapoptotic proteins. In addition to this, berberine can also promote apoptosis via mitochondrial/caspase pathway. In cancer cell lines (tongue cancer, oral squamous cell carcinoma and prostate epithelial) [18, 23, 29, 33], activation of caspases-3 & -9 promotes G_1 cell cycle arrest in different human cancer cell lines (lung, stomach, and prostate) [20, 21, 24, 30, 33].

Berberine also showed anti-metastatic properties in several cancer cell lines, acting on 72 kDa type IV collagenase (MMP2), Cdc42 effector protein 1 (CDC42EP1), and ras-related C3 botulinum toxin substrate 1 (RAC1), transforming protein RhoA (RHOA) and urokinase-plasminogen activator A (PLAU) [34, 35]. Further, berberine showed antitopoisomerase I properties [36]; this observation can be useful as topoisomerase I is responsible for DNA replication and antitopoisomerase I compounds can be effective in cancer treatments.

In addition to its effects on cancer cells, berberine also acts on molecular targets related to insulin resistance. In free-fatty-acid-induced insulin resistance muscle cells, berberine improves insulin resistance and improves glucose uptake by decreasing PPARγ and FAT/CD36 protein expression [37]. Another study reported increased insulin receptor (InsR) mRNA and protein expression increases insulin sensitivity in liver cells after berberine treatment [38]. In Caco-2 cells, berberine inhibited alpha-glucosidase and disaccharidases activities, leading to reduced glucose levels [39]. In Hep G_2

Table 1: Potential targets of berberine.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
72 kDa type IV collagenase	*MMP2*	HUVECs, tongue cancer SCC-4 cells, gastric carcinoma SNU-5 cells, lung cancer A549 cells, and U-87 glioma cells	Downregulation of MMP2 mRNA and protein expression, reduced MMP-2 levels	Antimetastatic	[18, 69–72]
Acetyl-Coenzyme A carboxylase-α	*ACACA*	HepG2 hepatoma cells	Phosphorylation	Antihyperlipidemic	[73]
α-Fetoprotein	*AFP*	HepG2 hepatoma cells	Reduced secretion of alpha fetoprotein	Apoptosis	[74]
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Neuroglioma H4 cells	Reduces amyloid-β peptide (Aβ) levels via modulation of APP	Alzheimer disease	[75]
Bcl-X	*BCL2L1*	Colonic carcinoma cells, HepG2 cells/A549 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells	JNK/p38 pathway and induction of ROS production. Decreased expression	Cell apoptosis, anticancer, and anti-inflammatory	[19–21, 30, 76, 77]
Arylamine *N*-acetyltransferase 1	*NAT*	Leukemia HL-60 cells, colon tumour cells, brain tumour cells (G95/VGH and GBM 8401)	Decrease in *N*-acetyltransferase (NAT) protein and expression of mRNA	Anticancer (leukemia, colon cancer, brain tumour, etc.)	[78–80]
ATP-binding cassette subfamily G member 2	*ABCG2*	MCF-7 breast cancer cells	Decrease in ABCG2 expression	Breast cancer	[81]
Baculoviral IAP repeat-containing protein 2 (antiapoptosis factor c-IPA-1)	*BIRC2*	Jurkat cells, colonic carcinoma cells (SW620)	Suppresses expression of antiapoptosis factor IAP1	Anticancer	[19, 77]
Baculoviral IAP repeat-containing protein 3	*BIRC3*	Jurkat cells	Suppresses expression of antiapoptosis factor IAP2	Anticancer	[77]
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	Jurkat cells	Suppresses expression of survivin	Anticancer and anti-inflammatory agent	[77]
B-cell CLL/lymphoma 2	*BCL2*	HepG2 cells, oral squamous cell carcinoma, tongue cancer SCC-4 cells, colonic carcinoma cells, lung cancer cells, breast cancer MCF-7 (estrogen receptor+) cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Bcl-2 Downregulation	Cell apoptosis, cancer, and ER antagonist adjuvant therapy	[19–23, 30, 34, 82, 83]
B-cell lymphoma 3-encoded protein	*Bcl-3*	Gastric carcinoma SNU-5 cells	Downregulation of Bcl-3	Gastric cancer	[24]
Bcl2 antagonist of cell death	*BAD*	Human oral squamous cell carcinoma	Increased expression of proapoptotic BAD protein	Antitumour	[23]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
BCL2-associated X protein	*BAX*	Gastric carcinoma SNU-5 cells, prostate carcinoma cells (DU145, PC-3, LNCaP and PWR-1E), leukemia HL-60, tongue cancer SCC-4 cells, lung cancer cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Upregulation of Bax, increased expression. G2/M phase arrest	Cell apoptosis, gastric cancer	[18, 20, 21, 24, 29, 30, 83, 84]
BH3-interacting domain death agonist p11	*BID*	Colonic carcinoma cells/HepG2 cells	JNK/p38 pathway and induction of ROS production	Induction of apoptosis	[19, 76]
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	Cervical cancer Ca Ski cells	Induced expression of GADD153	Cervical cancer	[85]
CASP8 and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Jurkat cells	Suppresses expression of cFLIP	Anticancer and anti-inflammatory	[77]
Caspase 3	*CASP3*	Tongue cancer SCC-4 cells, neuroblastoma (SK-N-SH), glioblastoma T98G cells, gastric carcinoma SNU-5 cells, HL-60 cells, prostate carcinoma cells (DU145, PWR-1E, PC-3 and LNCaP), colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma, promonocytic U937 cells, lung cancer A549, H1301 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase-3, G2/M phase arrest	Anticancer	[18–21, 23–25, 30, 33, 76, 83, 86–89]
Caspase 8	*CASP8*	Tongue cancer SCC-4 cells, colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma	Activated caspase 8	Anticancer	[18, 19, 23, 76]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 9	*CASP9*	Tongue cancer SCC-4 cells, glioblastoma T98G, oral squamous carcinoma, promonocytic U937 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase 9	Cell apoptosis, anticancer	[18, 21, 23, 30, 33, 83, 86, 87, 89, 90]
Cdc42 effector protein 1	*CDC42EP1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Cdc42)	Cancer metastasis inhibition	[91]
Cell division protein kinase 6	*CDK6*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk6	Cell apoptosis, cancer	[21, 30, 83]
Cellular tumor antigen p53	*TP53*	Gastric carcinoma SNU-5 cells, osteosarcoma	Increased expression of p53 protein, cell cycle arrest at G1G2/M phase arrest	Anticancer (gastric cancer, osteosarcoma)	[24, 92]
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Retinal pigment epithelial cell line	Inhibits CCL2 (MCP-1) expression	Anti-inflammatory	[93]
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	Colorectal cancer cells	Induces ATF3 expression	Colorectal cancer	[94]
Cyclin-dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	HL-60 cell, gastric carcinoma SNU-5 cells	Inactivation of Cdc2 (CDK1) or decreased protein expression	Antiproliferative and proapoptotic	[24, 95]
Cyclin E1	*CCNE1*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Anticancer	[25, 83, 90]
Cyclin-dependent kinase 2	*CDK2*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk2	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclin-dependent kinase 4	*CDK4*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk4	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Brest cancer MCF-7 (estrogen receptor+) cells, epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of p21	Breast cancer, ER antagonist adjuvant therapy	[21, 30, 82, 83]
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of Cdki proteins	Cell apoptosis, cancer	[21, 30, 83]
Cytochrome c-1	*CYC1*	Tongue cancer SCC-4 cells, colonic carcinoma cells, promyelocytic leukemia HL-60 cells	Release of cytochrome c-1	Anticancer	[18, 19, 84, 86]
CYP2C9	*CYP2C9*	Recombinant CYP	Inhibition of CYP2C9	Drug interactions	[96]
CYP2D6	*CYP2D6*	Recombinant CYP	Inhibition of CYP2D6	Drug interactions	[96]
CYP3A4	*CYP3A4*	Caco-2 cells, patients	CYP3A4 Downregulation and inhibition	Drug interactions	[97, 98]
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Recombinant DPP4	Inhibition of DPP4	—	[99]
Early activation antigen CD69	*CD69*	Human peripheral lymphocytes	Reduced expression of CD69	Immunosuppressive agent	[100]
Epidermal growth factor receptor	*EGFR*	Brest cancer MCF-7 (estrogen receptor+) cells	EGFRdownregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Ezrin	*EZR*	Nasopharyngeal carcinoma 5–8F cells	Ezrin inhibition	Anticancer	[26]
G1/S-specific cyclin-D1	*CCND1*	Giant cell carcinoma cell line, HL-60 cell, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells, neuroblastoma (SK-N-SK), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Inhibits expression of cyclin D1	Antiproliferative and proapoptotic, anticancer, anti-inflammatory	[21, 25, 30, 32, 77, 83, 95]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
G1/S-specific cyclin-D2	*CCND2*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin D2	Cell apoptosis, cancer	[21, 30, 83]
G1/S-specific cyclin-E1	*CCNE1*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Cell apoptosis, cancer	[21, 30, 83]
G2/mitotic-specific cyclin-B1	*CCNB1*	Gastric carcinoma SNU-5 cells	Decreased cyclin B, G2/M phase arrest	Cell apoptosis, anticancer	[24]
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	NCI-H716 cells	Enhanced glucagon-like peptide (GLP)-1	Antidiabetic agent	[42]
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Colorectal cancer cells	Induces NAG-1 expression	Colorectal cancer	[94]
Hypoxia-inducible factor 1α	*HIF1A*	HUVECs, HepG2 cells	Prevention and reduction of HIF-1 alpha expression	Tumour angiogenesis	[101, 102]
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Oral cancer cells	Inhibition of Mcl-1 expression	Induced apoptosis	[103]
Inhibitor of NF-κB kinase subunit alpha (IκB kinase)	*CHUK(IKK)*	Jurkat cells	Inhibition of IκB kinase (IKK)	Anticancer and anti-inflammatory agent	[77]
Interferon-γ	*IFNB1*	Brest cancer MCF-7 (estrogen receptor+) cells	IFN-beta upregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Interleukin 8	*IL8*	Retinal pigment epithelial cell line	Inhibits IL-8 expression	Anti-inflammatory	[93]
Interleukin-1β	*IL1B*	Fibroblasts (HFL1)	Induces IL-1B productions	Pulmonary inflammation	[104]
Interleukin-2 receptor α-chain	*IL2RA/CD25*	Human peripheral lymphocytes	Reduced expression of CD25	Immunosuppressive agent	[100]
Interleukin-6	*IL6*	Keratinocytes	Reduces and IL-6 expression	Antiskin ageing agent	[105]
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	HepG2 cells	Increased mRNA and protein expression	Hyperlipidemia	[106–108]
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Dermal fibroblasts, U-87 glioma cells	MMP-1 expression decreased	Antiskin ageing agent, anticancer	[70, 109]
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Tongue cancer SCC-4 cells, keratinocytes, gastric carcinoma SNU-5	Inhibition	Anticancer	[34, 70, 105]
Matrix metalloproteinase-16	*MMP16*	Jurkat cells	Suppresses expression of MMP-16	Anticancer and anti-inflammatory agent	[77]
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Peripheral blood monocytes (PBMC)	ERK1 protein expression inhibition	Antiatherosclerotic effects	[110]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Peripheral blood monocytes (PBMC)	ERK2 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Peripheral blood monocytes (PBMC)	Jun N-terminal kinase (JNK) protein expression inhibited at high levels of BBR	Antiatherosclerotic effects	[19, 110]
M-phase inducer phosphatase 1	*CDC25A*	HL-60 cell	Phosphorylation and degradation of Cdc25A	Antiproliferative and proapoptotic	[95]
Multidrug resistance protein 1 (P-gp, P-gp-170)	*ABCB1*	Tumour cell lines	Significant inhibited P-gp multidrug resistance (MDR) activity	MDR activity reversal	[111]
		Hepatoma HepG2 cells	Upregulated multidrug resistance transporter (P-gp-170) expression	Reduced retention of chemotherapeutic agents	[112]
Myc proto-oncogene protein	*MYC*	U-87 glioma cells	Myc level decreased	Malignant glioma and cancer development	[71]
NF-κB inhibitor-α	*NFKBIA*	Lung epithelial cells (A-549)	Inhibition of κB-α phosphorylation and degradation	Pulmonary inflammation	[104]
Nuclear factor NF-κB p50 subunit (NF-κB)	*NFKB1*	Jurkat cells, osteoblastic cells, HepG2 cells	Inhibit NF-κB production and suppress NF-κB	Anticancer and anti-inflammatory agent, alcohol liver disease, osteoclast formation	[77, 113–115]
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	HepG2 cells	Reduced GR levels	Cell growth arrest	[74]
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	Leukemia HL-60 cells	Downregulation of nucleophosmin/B23 and telomerase activity	Cancer	[116]
Peroxisome proliferator-activated receptor-γ	*PPARG*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes, 3T3-L1 preadipocytes	Decreased expression	Antidiabetic	[37, 117]
Platelet glycoprotein 4	*CD36/FAT*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes	Decreased expression	Antidiabetic	[37]
Poly (ADP-ribose) polymerase family, member 1	*PARP*	HepG2 cells/hepatoma cells, colonic carcinoma cells, prostate cancer cells (PC-3), prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Cleavage of poly (ADP-ribose) polymerase. Activation of PARP	Cell apoptosis, Anticancer	[19, 21, 22, 30, 76, 83, 87]
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Leukemic stem cells (LSCs)	Inhibits HERG1 K (+) channels of leukemic cells	Inhibits AML cell migration	[35]

Table 1: Continued.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Processed sterol regulatory element-binding protein 2	*SREBP2*	HepG2 cells	Reduction of SREBP2	Hyperlipidemia	[101]
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	HepG2 cells	Suppression of PCSK9 mRNA and protein levels	Hyperlipidemia	[101, 107]
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Peripheral blood monocytes (PBMC), oral cancer cell lines OC2 and KB cells, breast cancer MCF-7 (estrogen receptor+) cells, Jurkat cells, colon cancer cells	Decrease of Cox-2 mRNA and protein expression	Antiatherosclerotic effects, anti-inflammatory, anticancer, breast cancer ER antagonist adjuvant therapy, Anticancer	[27, 77, 82, 103, 110, 118]
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	HUVECs	Inhibition of ROS generation	Protects LDL oxidation and prevents ox-LDL-induced cellular dysfunction	[19, 119]
Ras-related C3 botulinum toxin substrate 1	*RAC1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Rac1)	Cancer metastasis inhibition	[91]
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Brest cancer MCF-7 (estrogen receptor+) cells	HER2 downregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Rho-associated protein kinase 1	*ROCK1/RHO*	Nasopharyngeal carcinoma 5–8F cells	Suppression of Rho kinase activity	Anticancer	[91]
Runt-related transcription factor 2	*RUNX2*	Osteoblast cells	Promotes transcriptional activity of Runx2	Osteoblast differentiation and bone formation in osteoporosis	[120]
SDF-1-α (3–67) (SDF-1)	*CXCL12*	Acute myeloid leukemia (AML)	Reduces SDF-1 chemokine	Inhibits AML cell migration	[35]
Sucrase-isomaltase (α-glucosidase)	*SI*	Caco-2 cells	Inhibit alpha-glucosidase	Antihyperglycaemic	[39]
Topoisomerase (DNA) I	*Top1*	Recombinant human topoisomerase I	Top1 inhibition	Anticancer	[121]
Transcription factor AP-1	*AP-1*	Hepatoma cells, MDA-MB-231 breast cancer cells, giant cell carcinoma cell line, colon cancer cells, U-87 glioma cells, HeLa cells	Inhibition of AP-1 activity, AP-1 DNA suppression	Antitumor activity, Anticancer	[27, 32, 71, 115, 118, 122–124]
Transforming protein RhoA	*RHOA*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (RhoA)	Cancer metastasis inhibition	[91]
Tumor necrosis factor-α	*TNFA*	Macrophages, fibroblasts (HFL1)	Inhibition of TNF-α	Anti-inflammatory	[104, 125]
Urokinase-plasminogen activator	*PLAU*	Lung cancer A549 cells, tongue cancer SCC-4 cells	Reduced urokinase-plasminogen activator (u-PA)	Antimetastatic, Anticancer	[34, 72]
Vascular endothelial growth factor A	*VEGFA*	HUVECs	Prevention of VEGF expression	Tumour angiogenesis	[102]
Wee1-like protein kinase	*Wee1*	Gastric carcinoma SNU-5 cells	Increased expression of Wee1protein, G2/M phase arrest	Gastric cancer	[24]

Table 2: Berberine's target classification based on PANTHER.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Multidrug resistance protein 1 (Pgp, Pgp-170)	*ABCB1*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity	Immune system process, extracellular transport, carbohydrate metabolic process, response to toxin	ATP-binding cassette (ABC) transporter
ATP-binding cassette sub-family G member 2	*ABCG2*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity, anion channel activity	Immune system process, anion transport, lipid transport, oxygen and reactive oxygen species, metabolic process, lipid metabolic process, response to stress	N/A
Acetyl-coenzyme A carboxylase-α	*ACACA*	Other ligase	Gluconeogenesis, monosaccharide metabolism, fatty acid biosynthesis, coenzyme metabolism	N/A
α-Fetoprotein	*AFP*	Other transfer/carrier protein	Transport, mesoderm development, oncogenesis	N/A
Transcription factor AP-1	*AP-1*	DNA binding, transcription factor activity	Cell cycle, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle, signal transduction	Toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway, oxidative stress response, angiogenesis, TGF-beta signaling pathway, T-cell activation, B-cell activation, Ras Pathway, FAS signaling pathway, PDGF signaling pathway
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Other signaling molecules	Other signal transduction, cell communication, other intracellular protein traffic	Alzheimer disease-amyloid secretase pathway, Alzheimer disease-presenilin pathway, blood coagulation, Alzheimer disease-presenilin pathway, Alzheimer disease-amyloid secretase pathway
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	DNA binding, transcription factor activity	Transcription factor activity, immune system process, neurological system process, induction of apoptosis, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Apoptosis signaling pathway
Bcl2 antagonist of cell death	*BAD*	N/A	N/A	PDGF signaling pathway, apoptosis signaling pathway, angiogenesis, PI3 kinase pathway, VEGF signaling pathway, interleukin signaling pathway
BCL2-associated X protein	*BAX*	Other signaling molecule	Induction of apoptosis, gametogenesis, hematopoiesis, cell cycle control, cell proliferation and differentiation, tumor suppressor	p53 pathway, apoptosis signaling pathway, Huntington disease

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
B-cell CLL/lymphoma 2	*BCL2*	Other signaling molecule	Inhibition of apoptosis, oncogenesis	Oxidative stress response, apoptosis signaling pathway
Apoptosis regulator Bcl-X	*BCL2L1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
B-cell lymphoma 3-encoded protein	*Bcl-3*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Inflammation mediated by chemokine and cytokine signaling pathway
BH3-interacting domain death agonist p11	*BID*	N/A	N/A	Apoptosis signaling pathway, FAS signaling pathway
Baculoviral IAP repeat-containing protein 2 (anti-apoptosis factor c-IPA-1)	*BIRC2*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 3	*BIRC3*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	N/A	N/A	Angiogenesis
Caspase 3, apoptosis-related cysteine peptidase	*CASP3*	Cysteine protease	Proteolysis, apoptosis	Huntington disease, FAS signaling pathway, apoptosis signaling pathway
Caspase 8, apoptosis-related cysteine peptidase	*CASP8*	Cysteine protease	Proteolysis, apoptosis	Apoptosis signaling pathway, FAS signaling pathway, Huntington disease
Caspase 9, apoptosis-related cysteine peptidase	*CASP9*	Cysteine protease	Proteolysis, apoptosis	Angiogenesis, apoptosis signaling pathway, FAS signaling pathway, VEGF signaling pathway, PI3 kinase pathway
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Nonreceptor serine/threonine, protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
G2/mitotic-specific cyclin-B1	*CCNB1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	Cell cycle, p53 pathway
G1/S-specific cyclin-D1	*CCND1*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle, Wnt signaling pathway
G1/S-specific cyclin-D2	*CCND2*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle
Cyclin E1	*CCNE1*	Kinase activator	Cell cycle control, mitosis, cell proliferation and differentiation	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
G1/S-specific cyclin-E1	*CCNE1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2
Interleukin-2 receptor alpha chain	*IL2RA/CD25*	Cytokine receptor activity	Immune system process, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Interleukin signaling pathway
Platelet glycoprotein 4	*CD36/FAT*	Receptor activity	Macrophage activation, lipid transport, apoptosis, signal transduction, cell adhesion, lipid metabolic process, signal transduction, cell adhesion, cellular component, morphogenesis	N/A
Early activation antigen CD69	*CD69*	Receptor activity, receptor binding	B-cell-mediated immunity, natural killer cell activation, cellular defense response	Membrane-bound signaling molecule
M-phase inducer phosphatase 1	*CDC25A*	Hydrolase activity, acting on ester bonds, phosphatase activity	Phosphatase activity cell cycle, phosphate metabolic process, protein metabolic process, cell cycle	p53 pathway
Cdc42 effector protein 1	*CDC42EP1*	N/A	N/A	N/A
Cyclin dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	p53 pathway
Cyclin-dependent kinase 2	*CDK2*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	p53 pathway, p53 pathway feedback loops 2
Cyclin-dependent kinase 4	*CDK4*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
Cell division protein kinase 6	*CDK6*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	N/A
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway, p53 pathway feedback loops 2, p53 pathway
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway
CASP8-and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Peptidase activity, protein binding, peptidase inhibitor activity	Apoptosis, protein metabolic process	Apoptosis signaling pathway, FAS signaling pathway
Inhibitor of NF-κB kinase subunit alpha (IκB kinase)	*CHUK(IKK)*	Kinase activity	Immune response, intracellular signaling cascade, protein metabolic process, signal transduction, response to stimulus	Interleukin signaling pathway, apoptosis signaling pathway, T-cell activation, toll receptor signaling pathway, B-cell activation

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
SDF-1-α (3–67) (SDF-1)	*CXCL12*	N/A	N/A	Axon guidance-mediated by Slit/Robo
Cytochrome c-1	*CYC1*	Reductase	Oxidative phosphorylation	FAS signaling pathway, ATP synthesis, Huntington disease
Cytochrome P450, family 2, subfamily C, polypeptide 9	*CYP2C9*	Oxygenase	Fatty acid metabolism, steroid metabolism, electron transport	N/A
Cytochrome P450, family 2, subfamily D, polypeptide 6	*CYP2D6*	Oxygenase	Other lipid, fatty acid and steroid metabolism, steroid metabolism, electron transport	Vitamin D metabolism and pathway
Cytochrome P450, family 3, subfamily A, polypeptide 4	*CYP3A4*	Oxygenase	Steroid hormone metabolism, electron transport	N/A
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Serine protease	Proteolysis, cell surface receptor mediated signal transduction, T-cell-mediated immunity	N/A
Epidermal growth factor receptor	*EGFR*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Apoptosis signaling pathway, Alzheimer disease-amyloid secretase pathway, B-cell activation, Ras pathway, interleukin signaling pathway, angiogenesis, T-cell activation, toll receptor signaling pathway, insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, FGF signaling pathway, Parkinson disease, PDGF signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, VEGF signaling pathway, interferon-gamma signaling pathway, endothelin signaling pathway, angiogenesis, TGF-beta signaling pathway, integrin signalling pathway, EGF receptor signaling pathway
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, interleukin signaling pathway, angiogenesis, VEGF signaling pathway, integrin signalling pathway
Ezrin	*EZR*	Structural constituent of cytoskeleton	Cellular component, morphogenesis	N/A
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	N/A	N/A	Oxidative stress response
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	Receptor binding	Signal transduction, cell-cell signalling, carbohydrate metabolic process, lipid metabolic process, signal transduction, cell-cell signalling, cellular glucose homeostasis	Peptide hormone

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Receptor binding	Female gamete generation, cell surface receptor linked signal transduction, signal transduction, ectoderm development, mesoderm development, skeletal system development, heart development, muscle organ development	TGF-beta signaling pathway
Hypoxia-inducible factor 1α	*HIF1A*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, ectoderm development, nervous system development	Hypoxia response via HIF activation, VEGF signaling pathway, angiogenesis
Interferon-β	*IFNB1*	Receptor binding	Response to interferon-gamma, induction of apoptosis, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Toll receptor signaling pathway
Interleukin-1β	*IL1B*	Receptor binding	Immune response, macrophage activation, cell surface receptor linked signal transduction, cell-cell signalling, signal transduction, cell-cell signalling, response to stimulus	Inflammation mediated by chemokine and cytokine signaling pathway
Interleukin-6	*IL6*	Receptor binding	Immune system process, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling signal transduction, cell-cell signaling	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Interleukin 8	*IL8*	Chemokine	Cytokine- and chemokine-mediated signaling pathways, calcium-mediated signalling, NF-kappaB cascade, ligand-mediated signalling, T-cell-mediated immunity, macrophage-mediated immunity, granulocyte-mediated immunity, angiogenesis, cell proliferation and differentiation, cell motility	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Receptor activity, cation transmembrane transporter activity, voltage-gated potassium channel activity, cation channel activity, cyclic nucleotide-gated ion channel activity	Cation transport, signal transduction	Ligand-gated ion channel
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	Other receptor	Oogenesis	Alzheimer disease-presenilin pathway

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, Ras pathway, EGF receptor signaling pathway, Parkinson disease, angiogenesis, FGF signaling pathway, FAS signaling pathway, toll receptor signaling pathway, TGF-beta signaling pathway, PDGF signaling pathway, Huntington disease, integrin signalling pathway, T-cell activation, B-cell activation, interferon-gamma signaling pathway, oxidative stress response, apoptosis signaling pathway, integrin signalling pathway
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Peptidase activity	Protein metabolic process	Plasminogen activating cascade, Alzheimer disease-presenilin pathway, plasminogen activating cascade
Matrix metalloproteinase-16	*MMP16*	Peptidase activity	Protein metabolic process	Alzheimer disease-presenilin pathway
72 kDa type IV collagenase	*MMP2*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway, plasminogen activating cascade
Myc proto-oncogene protein	*MYC*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle	Oxidative stress response, p53 pathway feedback loops 2, Wnt signaling pathway, interleukin signaling pathway, PDGF signaling pathway
Arylamine *N*-acetyltransferase 1	*NAT*	Acyltransferase activity	Metabolic process	Acetyltransferase

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Nuclear factor NF-κB p50 subunit (NF-κB)	*NFKB1*	DNA binding, transcription factor activity	B-cell-mediated immunity, negative regulation of apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, cellular defense response	T-cell activation, B-cell activation, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway
NF-κB inhibitor-α	*NFKBIA*	Protein binding	Immune system process, intracellular protein transport apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, response to stress	Apoptosis signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, T-cell activation, B-cell activation
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	N/A
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	Nuclear hormone receptor, transcription factor, nucleic acid binding	N/A	N/A
Poly(ADP-ribose) polymerase family, member 1	*PARP*	Glycosyltransferase	DNA repair, protein ADP-ribosylation, stress response	FAS signaling pathway
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	Serine protease	Proteolysis	N/A
Urokinase-plasminogen activator	*PLAU*	Peptidase activity	Immune system process, signal transduction, protein metabolic process, cell motion, signal transduction, blood coagulation	Blood coagulation, plasminogen activating cascade
Peroxisome proliferator-activated receptor-γ	*PPARG*	Nuclear hormone receptor, transcription factor, nucleic acid binding	Monosaccharide metabolism, regulation of lipid, fatty acid, and steroid metabolism, mRNA transcription regulation, ligand-mediated signalling, stress response, developmental processes, cell proliferation and differentiation	N/A
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Oxidoreductase activity	Immune system process	Endothelin signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Ras-related C3 botulinum toxin substrate 1	*RAC1*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, integrin signalling pathway, inflammation mediated by chemokine and cytokine signaling pathway, Huntington disease, axon guidance mediated by Slit/Robo, FGF signaling pathway, T-cell activation, axon guidance mediated by netrin, EGF receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase, aAxon guidance mediated by semaphorins, cytoskeletal regulation by Rho GTPase, B-cell activation, Ras pathway
Rho-associated protein kinase 1	*ROCK1/RHO*	Kinase activity	Mitosis, intracellular signaling cascade, cell adhesion, protein metabolic process, cell motion, mitosis, signal transduction, cell adhesion, embryonic development	Inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase
Transforming protein RhoA	*RHOA*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, angiogenesis, heterotrimeric G-protein signaling pathway-Gq alpha; and Go alpha mediated pathway, axon guidance mediated by semaphorins, inflammation mediated by chemokine and cytokine signaling pathway, integrin signalling pathway, Ras pathway, cytoskeletal regulation by Rho GTPase, PDGF signaling pathway
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, visual perception, sensory perception, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cycle, signal transduction, ectoderm development, mesoderm development, embryonic development, nervous system development	N/A

Table 2: Continued.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Runt-related transcription factor 2	*RUNX2*	DNA binding, transcription factor activity	Mesoderm development, skeletal system development, hemopoiesis	N/A
Sucrase-isomaltase (Alpha-glucosidase)	*SI*	Hydrolase activity, hydrolyzing O-glycosyl compounds	Carbohydrate metabolic process, protein metabolic process	N/A
Processed sterol regulatory element-binding protein 2	*SREBP2*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, lipid metabolic process	Basic helix-loop-helix transcription factor
Tumor necrosis factor/tumor necrosis factor-α	*TNFA*	Tumor necrosis factor family member	Cytokine- and chemokine-mediated signaling pathways, ligand-mediated signalling, immunity and defense, induction of apoptosis	Wnt signaling pathway, apoptosis signaling pathway
Topoisomerase (DNA) I	*Top1*	DNA topoisomerase	DNA replication, general mRNA transcription activities	DNA replication
Cellular tumor antigen p53	*TP53*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, cell cycle	Apoptosis signaling pathway, Huntington disease, P53 pathway feedback loops 1, p53 pathway, p53 pathway by glucose deprivation, p53 pathway feedback loops 2, Wnt signaling pathway
Vascular endothelial growth factor A	*VEGFA*	Receptor binding	Immune system process, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell cyclesignal transduction, mesoderm development, angiogenesis, response to stress	Angiogenesis, VEGF signaling pathway
Wee1-like protein kinase	*Wee1*	Kinase activity	Mitosis, protein metabolic process	Protein kinase

cells, berberine also improved insulin signal transduction through various mechanisms such as decreased phosphorylation of PERK and eLF2-α, increased phosphorylation of IRS-1 tyrosine and AKT serine [40]. In intestinal NCI-H716 cells, berberine enhanced glucagon-like peptide 1 (GLP-1) release and promotes proglucagon mRNA expression [41]. These results demonstrate that berberine has great potential for insulin resistance treatment and should be explored further in animal and human studies.

3.2. PANTHER Analysis of Berberine Targets. Distribution of berberine therapeutic targets *in vitro* varied in each of these functional classifications. Tables 3, 4, and 5 show various distributions of the most frequent occurring berberine targets *in vitro* based on molecular functions, biological processes, and pathways, respectively.

As shown in Table 3, berberine acts on a diverse range of molecular targets *in vitro*. The most common classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Known berberine targets *in vitro* from the receptor binding class include epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGFA), interleukin-1β (IL1B) and interleukin-6 (IL6), growth/differentiation factor 15 (NAG-1), and glucagon-like peptide (GLP1).

Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved (Table 4). *In vitro* berberine targets involved signal transduction include cyclin-dependant kinases (CDK1 and CDK6), inhibitor of nuclear factor kappa-B kinase subunit alpha (CHUK), epidermal growth factor receptor (EFGR), receptor tyrosine-protein kinase (ERBB2), glucagon-like peptide (GCG), growth/differentiation factor 15 (GDF15), interferon beta (IFNB1), interleukins (IL1B, IL2RA, and IL6), potassium voltage-gated channel subfamily H member 2 (KCNH1), mitogen-activated protein kinases (ERK1, ERK2, and MAPK8), nuclear factor-kappa-B p50 subunit (NFKB1), NF-kappa-B inhibitor alpha (NFKB1A), urokinase-plasminogen activator (PLAU), Ras-related C3 botulinum toxin substrate 1 (RAC1), Rho-associated protein kinase 4 (RHO), transforming protein RhoA (RHOA), proto-oncogene tyrosine-protein kinase ROS (ROS1), vascular endothelial growth factor A (VEGFA).

According to the PANTHER Classification System, *in vitro* berberine targets correlate with a mixture of biological pathways, such as Alzheimer disease-presenilin and secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Huntington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways (Table 5).

The targets of berberine distributed across a large number of PANTHER classifications of molecular functions, biological processes, and pathways. This can be an advantage in terms of drug discovery using berberine. Seen that berberine targets are involved in a wide range of molecular activities, in turn, can alter many pathological states of the body. Thus, berberine can be explored for the treatment

Table 3: Distribution of berberine's targets *in vitro* according to molecular functions.

PANTHER molecular function	Number of targets
Acyltransferase activity	1
Anion channel activity	1
ATPase activity, coupled to transmembrane movement of substances	2
Cation channel activity	1
Cation transmembrane transporter activity	1
Chemokine	1
Cyclic nucleotide-gated ion channel activity	1
Cysteine protease	3
Cytokine receptor activity	1
DNA binding	9
DNA topoisomerase	1
Glycosyltransferase	1
GTPase activity	2
Hydrolase activity, acting on ester bonds	1
Hydrolase activity, hydrolyzing O-glycosyl compounds	1
Kinase activator	1
Kinase activator activity	4
Kinase activity	11
Kinase inhibitor activity	2
Kinase regulator activity	6
Metalloprotease	2
Not classified	10
Non-receptor serine/threonine protein kinase	3
Nuclear hormone receptor	2
Nucleic acid binding	2
Other extracellular matrix	2
Other ligase	1
Other receptor	1
Other signaling molecule	3
Other transfer/carrier protein	1
Oxidoreductase activity	1
Oxygenase	3
Peptidase activity	4
Peptidase inhibitor activity	1
Phosphatase activity	1
Protein binding	10
Receptor activity	3
Receptor binding	12
Reductase	1
Serine protease	2
Structural constituent of cytoskeleton	1
Transmembrane transporter activity	2
Transcription factor	2
Transcription factor activity	9
Transmembrane receptor protein kinase activity	3
Transmembrane receptor protein tyrosine kinase activity	3
Tumor necrosis factor family member	1
Voltage-gated potassium channel activity	1

Table 4: Distribution of berberine's targets *in vitro* according to biological functions.

PANTHER biological functions	Number of targets
Angiogenesis	2
Anion transport	1
Apoptosis	6
B-cell-mediated immunity	2
Blood coagulation	1
Calcium-mediated signaling	1
Carbohydrate metabolic process	7
Cation transport	1
Cell adhesion	3
Cell communication	1
Cell cycle	11
Cell cycle control	5
Cell cycle intracellular signaling cascade	1
Cell cycle signal transduction	1
Cell motility	1
Cell motion	10
Cell proliferation and differentiation	3
Cell proliferation and differentiation	1
Cell surface receptor linked signal transduction	14
Cell surface receptor-mediated signal transduction	1
Cell-cell adhesion	3
Cell-cell signaling	9
Cellular component morphogenesis	2
Cellular defense response	4
Cellular glucose homeostasis	1
Coenzyme metabolism	1
Cytokine- and chemokine-mediated signaling pathways	2
Developmental processes	1
DNA repair	1
DNA replication	2
Dorsal/ventral axis specification	1
Ectoderm development	1
Ectoderm development	8
Electron transport	3
Embryonic development	7
Endocytosis	2
Extracellular transport	2
Fatty acid biosynthesis	1
Fatty acid metabolism	1
Female gamete generation	4
Gamete generation	2
Gametogenesis	1
General mRNA transcription activities	1
Gluconeogenesis	1
Granulocyte-mediated immunity	1
Heart development	1
Hematopoiesis	1
Hemopoiesis	3
Immune response	2
Immune system process	16
Immune system processMitosis	1
Immunity and defense	1
Induction of apoptosis	9
Intracellular protein transport	3
Intracellular signaling cascade	18
Ligand-mediated signaling	3
Lipid metabolic process	4
Lipid transport	2
Macrophage activation	2
Macrophage-mediated immunity	1
Mesoderm development	12
Metabolic process	1
Mitosis	4
Monosaccharide metabolism	2
mRNA transcription regulation	1
Muscle organ development	1
Not classified	9
Natural killer cell activation	1
Negative regulation of apoptosis	8
Nervous system development	7
Neurological system process	1
NF-κB cascade	1
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	10
Oncogenesis	3
Other intracellular protein traffic	1
Other lipid, fatty acid and steroid metabolism	1
Other signal transduction	1
Oxidative phosphorylation	1
Oxygen and reactive oxygen species metabolic process	1
Phosphatase activity cell cycle	1
Phosphate metabolic process	1
Protein ADP-ribosylation	1
Protein metabolic process	17
Protein phosphorylation	3
Proteolysis	7
Regulation of lipid, fatty acid and steroid metabolism	1
Response to interferon-γ	1
Response to stimulus	2
Response to stress	8
Response to toxin	2
Segment specification	3
Sensory perception	1
Signal transduction	25

Table 4: Continued.

PANTHER biological functions	Number of targets
Skeletal system development	2
Spermatogenesis	2
Steroid hormone metabolism	1
Steroid metabolism	2
Stress response	2
T-cell-mediated immunity	2
Transcription factor activity immune system process	1
Transport	1
Tumor suppressor	1
Visual perception	1

of different diseases. On the other hand, the nature of multitargeting of berberine lacks in target specificity which can become difficult for drug design. Further, because berberine can have interactions with so many molecular structures and involve in different pathways, much attention must be paid to avoid interactions with other therapeutic drugs.

3.3. Data from In Vivo Studies with a Focus on Diabetes Mellitus. In China, Huang Lian (*Rhizoma coptidis*) has been used to treat diabetes for more than 1,400 years [16]. Berberine is one of the main active alkaloids present in *Rhizoma Coptidis* and has shown to have good hypoglycaemic effects *in vitro* [37–39, 42]. Further, the chemical structure of berberine is different from the commonly used other hypoglycaemic agents such as sulphonylureas, biguanides, thiazolidinediones, or acarbose [14]. Thus, it is meaningful to investigate the efficacy and safety of berberine treatments for diabetes mellitus to confirm the possibility of berberine serving as a new class of antidiabetic medications. Extensive research has been done to investigate the hypoglycaemic effects of berberine in animal models. This section will highlight the effects of berberine in diabetic animal studies, focusing on different mechanisms of actions of berberine.

Hyperglycemia is a hallmark metabolic abnormality associated with metabolic diseases such as type 2 diabetes. Berberine has shown to significantly decrease fasting blood glucose levels in diabetic rats (diet or drug induced), this has been observed in a number of studies [43–46]. Berberine can reduce fasting blood glucose level via different mechanisms. For example, Liu et al. [43] reported that berberine reduced fasting blood glucose (FBG) levels by inhibiting intestinal disaccharidases in a concentration-dependent manner. Xia et al. [46] reported berberine reduced fasting glucose level via the inhibition of gluconeogenesis, via decreased *PEPCK* and *G6Pase* genes in the liver, reduced hepatic steatosis, and inhibition of FAS expression.

Current diabetes therapies do not address the key driver of this condition, β-cell dysfunction [47, 48], and do not alter the progressive nature of insulin secretory deficit [49]. Berberine increased pancreatic β-cell numbers and β-cell mass in streptozotocin-induced diabetic rats [41, 50]. It also reversed pathological changes of pancreatic β-cells in diabetic rats induced by streptozotocin and diet [51]. Further,

Table 5: Distribution of berberine's targets *in vitro* according to pathway categories.

PANTHER pathway categories	Number of targets
Acetyltransferase	1
Alzheimer disease-amyloid secretase pathway	11
Alzheimer disease-presenilin pathway	14
Angiogenesis	11
Apoptosis signaling pathway	21
ATP synthesis	1
ATP-binding cassette (ABC) transporter	2
Axon guidance mediated by netrin	1
Axon guidance mediated by semaphorins	1
Axon guidance mediated by Slit/Robo	4
B-cell activation	7
Basic helix-loop-helix transcription factor	1
Blood coagulation	3
Cadherin signaling pathway	2
Cell cycle	4
Cytoskeletal regulation by Rho GTPase	3
DNA replication	2
EGF receptor signaling pathway	4
Endothelin signaling pathway	2
FAS signaling pathway	13
FGF signaling pathway	4
Heterotrimeric G-protein signaling pathway—Gq alpha- and Go alpha-mediated pathway	1
Huntington disease	9
Hypoxia response via HIF activation	1
Inflammation mediated by chemokine and cytokine signaling pathways	13
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	1
Integrin signalling pathway	6
Interferon-gamma signaling pathway	2
Interleukin signaling pathway	10
Ligand-gated ion channel	1
Membrane-bound signaling molecule	1
Pathway unclassified	19
Oxidative stress response	5
p53 pathway	12
p53 pathway by glucose deprivation	1
p53 pathway feedback loops	1
P53 pathway feedback loops 1	1
p53 pathway feedback loops 2	4
Parkinson disease	3
PDGF signaling pathway	6
Peptide hormone	1
PI3 kinase pathway	4
Plasminogen activating cascade	8

Table 5: Continued.

PANTHER pathway categories	Number of targets
Protein kinase	1
Ras Pathway	5
T-cell activation	7
TGF-β signaling pathway	4
Toll receptor signaling pathway	9
VEGF signaling pathway	7
Vitamin D metabolism and pathway	1
Wnt signaling pathway	4

in berberine treated diabetic rats, the pancreatic and plasma insulin levels increased after glucose load, reducing blood glucose levels [41, 50]. These observations are significant as berberine may be explored further as an additional therapy to existing antidiabetic drugs to effectively preserve β-cell functions, reverse β-cell damage, and promote insulin secretion in diabetes patients.

Further to β-cell dysfunction and insulin secretory deficit in diabetes, defects in insulin receptor (InsR) expression or function can cause insulin resistance and diabetes mellitus [52]. Thus, regulation of InsR expression may improve insulin resistance in diabetes mellitus. Berberine increases InsR mRNA and protein expression in human liver cells and in animal model in a dose- and time-dependent manner [38]. Berberine upregulates InsR and leads to enhanced insulin signalling pathway, confirming berberine as an insulin sensitiser.

Glucagon-like peptide 1 (GLP-1) is an intestinal peptide hormone released in response to food ingestion [53]. GLP-1 enhances meal-related insulin secretion and promotes glucose tolerance. In streptozotocin-induced rats, berberine enhanced GLP-1 release and promotes proglucagon mRNA expression, increased beta cell mass and pancreas insulin levels after glucose load [41]. This observation was in line with the groups, previous experiments *in vitro*. Lu et al. [50] also reported that berberine increased proglucagon mRNA expression and plasma insulin levels in streptozotocin-induced diabetic rats. The glucagon gene encodes GLP-1 and the increased expression of proglucagon mRNA assists in controlling the blood glucose homeostasis.

Berberine also reduced body weight and caused a significant improvement in glucose tolerance without altering food intake in *db/db* mice [54]. Oral glucose tolerance improvement in diabetic rats after berberine treatment has also been observed in other studies [55, 56].

Long-term hyperglycaemia can lead to increased risk of cardiovascular complications. In hyperglycemia and hypercholesterolemia rats with injured cardiac functions, berberine (15, 30 mg/kg/day, i.g for 6 weeks) increased cardiac output, left ventricular systolic pressure, and $+dp/dt_{\max}$ by 64, 16, and 79%, but decreased left ventricular end diastolic pressure and $-dp/dt_{\max}$ by 121 and 61% in the rats receiving HSFD/streptozotocin, respectively, when compared with the untreated rats of hyperglycemia and hypercholesterolemia [57]. Berberine caused significant increase in cardiac fatty acid transport protein-1 (159%), fatty acid transport proteins (56%), fatty acid beta-oxidase (52%), and glucose transporter-4. These results demonstrate the cardioprotective functions of berberine in hyperglycemia/hypercholesterolemia through alleviating cardiac lipid accumulation and promoting glucose transport 4 [57]. Another study also showed improved vasorelaxation in impaired aorta in diabetic rats after berberine treatment (100 mg/kg/day, 8 weeks) [45]. Thus, in addition to its hypoglycaemic effects, berberine can also be investigated for cardiomyopathy in diabetes.

Berberine also regulates lipid metabolism which is closely related to diabetes. In rats with induced diabetic hyperlipidemia, berberine (75, 150, 300 mg/kg/day for 16 weeks) effectively reduced liver weight and liver/body weight ratio, levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol [58]. In rats with a high fat diet, berberine significantly reduced body weight, alleviated liver steatosis, and improved insulin resistance [59]. This observation indicates that berberine can be an effective treatment for diabetes with obesity.

Clinically, preeminent factors for monitoring glycaemia and evaluating the risks of complications of diabetes include FBG, haemoglobin A_{1c} (HbA_{1c}) [60]. Triglyceride synthesis is closely associated with glucose metabolism so serum triglyceride levels are determined. Clinical studies often measure FBG, HbA_{1c}, and triglyceride levels, along with other factors to study the hypoglycaemic effects of berberine. The efficacies of berberine in type 2 diabetes patients have been reported. Through literature search, key clinical studies on berberine effects on type 2 diabetes patients are summarised.

Zhang et al. [61] conducted a randomized, double-blind, placebo-controlled multicenter trial ($n = 116$). The authors found that when berberine (1.0 g daily) was administered for 3 months in type 2 diabetes patients with dyslipidemia, the fasting and postload plasma glucose levels decreased from 7.0 ± 0.8 to 5.6 ± 0.9 and from 12.0 ± 2.7 to 8.9 ± 2.8 mM/L, HbA_{1c} from $7.5 \pm 1.0\%$ to $6.6 \pm 0.7\%$. Further, in the treatment group, triglyceride levels were reduced from 2.51 ± 2.04 to 1.61 ± 1.0 mM/L, total cholesterol from 5.31 ± 0.98 to 4.35 ± 0.96 mM/L, and LDL-cholesterol from 3.23 ± 0.81 to 2.55 ± 0.77 mM/L. Results from the treatment group was significant compared to the control group. In the treatment group, patient's body weight was also significantly reduced. Mild-to-moderate constipation was reported in 5 patients from the treatment group and 1 patient from the control group; however, this finding was not statistically significant. No other adverse events were reported. At 3 months, berberine was found to be effective in lowering blood glucose, lipids, body weight, and blood pressure with a good safety profile.

Yin et al. reported a 3-month study comparing berberine to antidiabetic drug metformin (0.5 g t.i.d) [14]. In this study, berberine exhibited identical effect as metformin in the regulation of glucose metabolism, significant decreases in HbA_{1c} (by 2%, $P < 0.01$), FBG (by 3.8 mmol/L; $P < 0.01$), and postprandial blood glucose (PBG) (by 8.8 mmol/L; $P < 0.01$). Further, the regulation of lipid metabolism was

better in the berberine group than the metformin group. Triglycerides and total cholesterol levels were significantly lower than in the metformin group ($P < 0.05$). At the same time, the same group of researchers used berberine as a combination therapy to evaluate its additive or synergistic effects on the commonly used hypoglycemic agents, such as sulphonylureas, biguanides, thiazolidinediones, and acarbose. Patients were given 500 mg berberine three times daily for 3 months in addition to their previous treatment. At week 5, berberine significantly ($P < 0.01$) reduced HbA_{1c} (from 8.1% to 7.3%), FBG, PBG, and fasting insulin levels. Blood lipids including triglyceride, total cholesterol, and LDL-C decreased significantly lowered compared to baseline. In both studies, incidences of gastrointestinal adverse events were observed, including diarrhea, constipation, flatulence, and abdominal pain. Interestingly, patients did not suffer from severe gastrointestinal adverse events when berberine was used alone and in combination therapy; adverse effects disappeared after berberine dosage was reduced. No pronounced elevation in liver enzymes or creatinine was observed, suggesting that berberine did not cause damage to the liver or kidneys.

Another clinical study [62] randomly divided 97 type 2 diabetes mellitus patients into berberine treatment (1 g/day) for 2 months, using metformin therapy (1.5 g/day) and rosiglitazone group (4 mg/b.i.d) as reference groups. Blood samples were taking before and after treatments to measure FBG, HbA_{1c}, triglyceride, and serum insulin levels. Compared to values prior to treatment, berberine significantly lowered FBG by 25.9% ($P < 0.001$), HbA_{1c} by 18.1% ($P < 0.00$), and triglycerides by 17.6% ($P < 0.01$). The hypoglycaemic effects of berberine were comparable to metformin and rosiglitazone. Serum insulin level was declined significantly ($P < 0.01$) by 28.2%; this indicates increased insulin sensitivity in peripheral tissues by berberine treatment. Peripheral blood lymphocytes from berberine treated patients were isolated to examine the InsR expression. The surface expression of InsR significantly elevated by 3.6-fold after berberine treatment.

Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. So the effect of berberine was tested in hyperglycaemic patients with hepatitis. Hepatitis B and C patients with hyperglycaemia received berberine at 1 g/day for 2 months. In both diabetic hepatitis B and C patients, berberine significantly reduced FBG and triglyceride levels. Berberine treatment also reduced the elevated alanine transaminase and aspartate aminotransferase levels in these patients. Overall, berberine is safe and effective in hyperglycaemic patients with liver function damage.

Table 6 compares clinical studies of berberine in diabetes patients. Across the studies, berberine has shown to significantly reduce FBG, PBG, and HbA_{1c} levels. Berberine also demonstrated ability to reduce triglyceride and cholesterol levels. Minimal gastrointestinal side effects were shown but no liver or kidney damage was observed. These observations in diabetes patients demonstrate that berberine is a safe and effective antidiabetic agent.

4. Discussion

The "rule-of-five" analysis by Lipinski et al. [7] shows that poor absorption or permeation of a compound is more likely when there are more than five hydrogen-bond donors; the molecular mass is more than 500 Da; the lipophilicity is high (expressed as $c\mathrm{Log}\,P > 5$); the sum of nitrogen and oxygen is more than 10. Specific structural and physiochemical properties, such as "rule-of-five," are required for clinical drugs to have sufficient levels of efficacy, bioavailability, and safety, which define target sites to which drug-like molecules can bind [4].

Plant compounds exhibit enormous structural diversity and only a small portion of the diversity has been explored for its pharmacological potential [64]. In recent years, herbal compounds have been source of new drugs [64]. Approximately 28% of new molecular entities (NMEs) between 1981 and 2002 were natural products or natural product derived; further to this, 20% of these NMEs were natural product mimics [65]. There are a number of successful plant-derived drugs, especially in anti-cancer treatment. Medicinal herbal compounds have become an important source for the discovery of new drugs. Further, drugs derived from medicinal plants can also be used as drug leads suitable for optimization by medicinal and synthetic chemists [65].

As Chinese herbal medicine becomes increasingly popular in the west, researchers are spending more time looking into mechanisms of actions of crude extracts and herbal compounds such as berberine. In recent years, extensive research has been done to explore the effects of berberine on various cell lines *in vitro*. In cell-based studies, berberine has shown effects on multiple molecular targets and alters various biological pathways. Berberine associates with a range of conditions, particularly diabetes, hyperlipidemia, and cancer. Many *in vitro* studies showed potent anticancer properties of berberine against various cancer cells. This observation is valuable in the search for new anti-cancer therapeutics with potent anti-cancer effects but reduced side effect. So berberine may potentially be developed into an anticancer agent, like other natural compounds (taxol, camptothecin) that have been developed and used as anticancer agents.

Diabetes mellitus is a major health problem around the world and its prevalence is on the rise. Diabetes mellitus drug therapy is limited by availability of effective medications, as existing oral hypoglycaemic agents often have side effects and fails in long-term administration [14]. Berberine has shown significant results in fasting blood glucose levels reduction, increase in insulin sensitivity, and improvement in insulin resistance *in vitro*, in diabetic animal models and in diabetic patients. Further, berberine shows mechanism that current antidiabetic drugs do not have. For instance, berberine has shown effects on pancreatic β-cell number and mass improvement [41, 50, 51]. In addition, berberine has a good safety profile and does not show side effects such as hypoglycaemia, weight gain, or liver and kidney damage. Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. Berberine has shown to be effective in the reduction of blood glucose level and is safe in diabetic patients with

Table 6: Comparison of clinical studies of berberine in diabetes patients.

Study type	Study subjects	Berberine dosage	Control treatment	Major findings	Side effects	Reference
Randomised, double-blind, placebo-controlled, multiple-center	Type 2 diabetes and dyslipidemia ($n = 116$)	0.5 g, b.i.d for 3 months	Placebo	Significantly reduced fasting and postload plasma glucose, HbA_{1c} Significantly reduced triglyceride, total cholesterol, and LDL-cholesterol	Mild to moderate constipation in 5 patients	[61]
Randomised, blinded, placebo-controlled	Type 2 diabetes ($n = 36$)	0.5 g, t.i.d for 3 months	Metformin (0.5 g t.i.d)	Significantly reduced FBG, PBG, and HbA_{1c} Significantly reduced plasma triglycerides	Transient gastrointestinal adverse effects. No liver or kidney damage	[14]
	Type 2 diabetes poorly controlled ($n = 48$)	0.5 g, t.i.d for 3 months	Existing anti-diabetic treatment	Lowered FBG and PBG Significantly decreased HbA_{1c} Significantly reduced fasting plasma insulin and HOMA-IR		
Randomised	Type 2 diabetes ($n = 97$)	1 g/day for 2 months	Metformin (1.5 g/day); rosiglitazone (4 mg/day)	Significantly reduced FBG, HbA_{1c}, and triglycerides Serum insulin level was declined significantly ($P < 0.01$), increased insulin sensitivity in peripheral tissues. Significantly elevated surface expression of InsR by 3.6-fold	No adverse events	[62]
	Type 2 diabetes with chronic hepatitis C virus infection ($n = 35$)	1 g/day for 2 months	N/A	Significantly reduced FBG and triglyceride levels Reduced the elevated ALT and aspartate aminotransferase levels		

b.i.d: twice daily; t.i.d: three times daily; FBG: fasting blood glucose; HOMA-IR: homeostasis model of assessment—insulin resistance; PBG: postprandial blood glucose.

viral hepatitis [62]. Berberine can therefore be investigated as an effective diabetes therapy with patients with liver function damage. In addition to its hypoglycemic effects in diabetic patients, berberine also reduced triglyceride and cholesterol levels. Abnormalities in lipid metabolism often deteriorate diabetes and cause complications. The regulation of lipid metabolism in diabetes patients by berberine may have clinical significance in managing diabetic patients with hyperlipidemia. Although there are only a small number of clinical studies and evidence is limited, current reports still show a promising future for berberine being developed into a new antidiabetic agent.

In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhea [14, 17]. However, *in vitro* and *in vivo* studies have shown that berberine has potent anti-cancer, antidiabetic, antilipidemic, and anti-inflammatory effects. Therefore, further clinical studies are warranted to investigate the potential of berberine in the application of cancer and diabetes treatments in the future.

Pharmacological activity of CHMs begins with the binding of the active components to their molecular targets. CHMs are considered as typical multitherapeutics that can interact simultaneously with multiple targets. The origins and the progression of diseases are multifactorial. Complex disorders such as cancer, cardiovascular disease, and depression tend to result from multiple molecular abnormalities, not from a single defect [66]. Biochemical and genetic studies revealed the molecular mechanism that underlie common illnesses [66]. Reports show that targets for neoplasm diseases, circulatory system diseases, infectious diseases, and nervous

system and sense organs disorders constitute the largest number of targets [1]. Because drug targets are presented at the molecular level, increased knowledge of herbal targets can facilitate deeper understanding of complex diseases at its fundamental level. In turn, it is likely to determine the optimal molecular targets for therapeutic intervention [6].

Further to assisting the molecular dissection of the mechanism of action of CHMs, knowledge on herbal targets makes it possible to use disease specific targets and design more desirable herbal drugs/formulas with increased specificity and efficacy. Target-oriented synthesis in drug discovery involves in preselected protein targets [67]. Binding of drugs to preselected protein target/s is dependent on which biological pathway the drug is aimed to modulate the target or the diseased pathway(s) [67]. Target and disease specific drug design results in improved efficacy and reduced side effects, especially in high impact diseases that require more effective and more treatment options. However, due to the fact that diseases often involve in multiple molecular abnormalities, diversity-oriented syntheses are used in efforts to identify simultaneously therapeutic protein targets and their small-molecule regulators [67]. Target-oriented drug design allows more focused drug design, which in turn costs less time and money for pharmaceutical companies.

Protein structure of well-validated old and new targets should be able to guide the chemical effort directed at new drugs [68]. Study of various aspects of known targets including molecular mechanism of their binding agents and related adverse effects is useful for finding clues to new target identification [9]. Based on the knowledge of molecular targets and molecular understanding of disease state and using this knowledge will allow some direction in identifying potential targets. Potential herbal targets may come from the same class as confirmed therapeutic targets and have similar physiological functions, or maybe a structure along a biological pathway. Additionally, with increased number of potential targets from ~500 to >5,000, the nature of pharmaceutical research has changed. This increase in numbers has given researchers more opportunities to discover and design new and improved drugs.

Target selection may be one of the most important determinants of attrition and the overall R&D productivity. There are few ways to overcome this challenge and improve the target selection process, in turn, improving R&D productivity. First of all, researchers can discover new target classes. Targets of herbal medicine are becoming a popular resource to find new target classes. In addition, increased understanding of genetic variations/polymorphisms of drug targets or drug metabolising enzymes can assist in target selection and drug metabolism. Further, the use of new technology can help to speed up the early exploratory discovery phase of drug discovery.

In summary, updated knowledge of herbal targets is valuable contribution to complex disease understanding and clinical responses. Further, drug discovery and development from herbal medicines can be supported by new target discovery and target-focused drug design. This will speed up the exploratory phase of drug R&D and benefit the pharmaceutical industry in terms of cost and time.

Abbreviations

Bax: BCL2-associated X protein
FBG: Fasting blood glucose
GLP-1: Glucagon like peptide 1
HbA_{1c}: Hemoglobin A_{1c}
InsR: Insulin receptor
PBG: Postprandial blood glucose.

Authors' Contribution

Xiao-Wu Chen and Yuan Ming Di contributed equally to this work.

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Research Article

Activated *PTHLH* Coupling Feedback Phosphoinositide to G-Protein Receptor Signal-Induced Cell Adhesion Network in Human Hepatocellular Carcinoma by Systems-Theoretic Analysis

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Studies were done on analysis of biological processes in the same high expression (fold change ≥2) activated *PTHLH* feedback-mediated cell adhesion gene ontology (GO) network of human hepatocellular carcinoma (HCC) compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection). Activated *PTHLH* feedback-mediated cell adhesion network consisted of anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism, cell adhesion, cell differentiation, cell-cell signaling, G-protein-coupled receptor protein signaling pathway, intracellular transport, metabolism, phosphoinositide-mediated signaling, positive regulation of transcription, regulation of cyclin-dependent protein kinase activity, regulation of transcription, signal transduction, transcription, and transport in HCC. We proposed activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network. Our hypothesis was verified by the different activated *PTHLH* feedback-mediated cell adhesion GO network of HCC compared with the corresponding inhibited GO network of no-tumor hepatitis/cirrhotic tissues, or the same compared with the corresponding inhibited GO network of HCC. Activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network included *BUB1B, GNG10, PTHR2, GNAZ, RFC4, UBE2C, NRXN3, BAP1, PVRL2, TROAP,* and *VCAN* in HCC from GEO dataset using gene regulatory network inference method and our programming.

1. Introduction

PTHLH is one of our identified significant high expression (fold change ≥2) genes in human hepatocellular carcinoma (HCC) compared with low expression no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection) from GEO data set GSE10140-10141 [1].

Study of *PTHLH* is presented in some papers, such as Mouse pthlh gene-specific expression profiles distinguish among functional allelic variants in transfected human cancer cells [2]; parathyroid hormone-like protein alternative messenger RNA splicing pathways in human cancer cell lines [3]; parathyroid hormone-like peptide in pancreatic endocrine carcinoma and adenocarcinoma associated with hypercalcemia [4]; parathyroid hormone and parathyroid hormone-like peptide bioactivity in situ biochemistry [5]; parathyroid hormone-like protein polypeptides immunological identification and distribution in normal and malignant tissues [6]; dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression [7]; all major lung cancer cell types produce parathyroid hormone-like protein [8]; parathyroid hormone-like peptide in normal and neoplastic mesothelial cells [9]. Yet the high expression activated *PTHLH* feedback-mediated cell adhesion mechanism in HCC is not clear and remains to be elucidated.

In this study, biological processes and occurrence numbers of the same activated high expression (fold change

≥2) *PTHLH* feedback-mediated cell adhesion GO network in HCC were identified and computed compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection), the different compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues, and the same compared with the corresponding inhibited GO network of HCC, respectively. Simultaneous occurrence of biological processes was identified between the same activated *PTHLH* feedback-mediated cell adhesion GO network of HCC (compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues) and the different (compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues), or the same (compared with the corresponding inhibited GO network of HCC) for putting forward hypothesis of activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network. Activated *PTHLH* feedback-mediated cell adhesion molecular network and numbers in HCC were extracted and computed from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues. *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion molecular relationship in HCC was identified including different molecules but same GO term and same molecule but different GO terms from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues.

2. Materials and Methods

Microarray 6,144 genes were used for analyzing activated *PTHLH* feedback-mediated cell adhesion mechanism of HCC based on GEO data set GSE10140-10141 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10140, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10141). The raw microarray data was preprocessed by log base 2.

225 significant high expression (fold change ≥2) molecules in HCC compared with no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection) were identified using significant analysis of microarrays (SAM) (http://www-stat.stanford.edu/~tibs/SAM/) [10]. We selected two classes paired and minimum fold change ≥2 under the false-discovery rate was 0%.

Activated *PTHLH* feedback-mediated cell adhesion mechanism of HCC was analyzed by using Molecule Annotation System, MAS (CapitalBio Corporation, Beijing, China; http://bioinfo.capitalbio.com/mas3/). The primary databases of MAS integrated various well-known biological resources, such as Gene Ontology (http://www.geneontology.org/), KEGG (http://www.genome.jp/kegg/), BioCarta (http://www.biocarta.com/), GenMapp (http://www.genmapp.org/), HPRD (http://www.hprd.org/), MINT (http://mint.bio.uniroma2.it/mint/Welcome.do), BIND (http://www.blueprint.org/), Intact (http://www.ebi.ac.uk/intact/), UniGene (http://www.ncbi.nlm.nih.gov/unigene), OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), and disease (http://bioinfo.capitalbio.com/mas3/).

Biological processes and occurrence numbers of the same activated high expression (fold change ≥2) *PTHLH* feedback-mediated cell adhesion GO network in HCC were identified and computed compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection), the different compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues, and the same compared with the corresponding inhibited GO network of HCC by our programming, respectively.

Simultaneous occurrence of biological processes was identified between the same activated *PTHLH* feedback-mediated cell adhesion GO network of HCC (compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues) and the different (compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues), or the same (compared with the corresponding inhibited GO network of HCC) for putting forward hypothesis of activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network by our programming, respectively.

Activated *PTHLH* feedback-mediated cell adhesion molecular network and numbers in HCC were extracted and computed from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues by our programming, respectively.

At last, *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion molecular relationship in HCC was identified including different molecules but same GO term and same molecule but different GO terms from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues, and constructed network by GRNInfer [11] and our articles [12–25] and illustrated by GVedit tool.

3. Results

Biological processes and occurrence numbers of the same activated high expression (fold change ≥2) *PTHLH* feedback-mediated cell adhesion GO network in HCC were identified and computed compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection), the different compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues, and the same compared with the corresponding inhibited GO network of HCC, respectively.

The same biological processes of activated *PTHLH* feedback-mediated cell adhesion GO network in HCC included anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism, cell adhesion, cell differentiation, cell-cell signaling, endothelial

cell migration, G-protein-coupled receptor protein signaling pathway, G-protein signaling, intracellular transport, metabolism, phosphoinositide-mediated signaling, positive regulation of transcription, protein amino acid phosphorylation, regulation of cyclin-dependent protein kinase activity, regulation of transcription, signal transduction, transcription, and transport compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues.

The different biological processes of activated *PTHLH* feedback-mediated cell adhesion GO network in HCC contained integrin-mediated signaling pathway, intracellular transport, microtubule cytoskeleton organization and biogenesis, regulation of cell growth, regulation of cyclin-dependent protein kinase activity compared with the corresponding inhibited GO network of no-tumor hepatitis/cirrhotic tissues.

The same biological processes of activated *PTHLH* feedback-mediated cell adhesion GO network in HCC included anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism, cell adhesion, cell differentiation, cell-cell signaling, DNA repair, G-protein-coupled receptor protein signaling pathway, integrin-mediated signaling pathway, metabolism, nucleotide and nucleic acid metabolism, oxidation reduction, phosphoinositide-mediated signaling, positive regulation of transcription, protein modification, proteolysis, regulation of cyclin-dependent protein kinase activity, regulation of transcription, signal transduction, and transcription, transport compared with the corresponding inhibited GO network of HCC, as shown in Table 1.

Activated *PTHLH* feedback-mediated cell adhesion molecular network and numbers in HCC were extracted and computed from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues. Our result showed that *PTHLH* feedback-mediated cell adhesion molecular network consisted of *BUB1B, GNG10, PTHR2, GNAZ, RFC4, UBE2C, NRXN3, BAP1, PVRL2, TROAP, VCAN, CCNA2, CDC6, CDKN2C,* and *ENAH* in HCC, as shown in Table 2.

4. Discussion

Our aim is to study novel high expression-activated *PTHLH* feedback-mediated cell adhesion mechanism in HCC. In this study, biological processes and occurrence numbers of the same activated high expression (fold change ≥ 2) *PTHLH* feedback-mediated cell adhesion GO network in HCC were identified and computed compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection), the different compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues, and the same compared with the corresponding inhibited GO network of HCC, respectively (Table 1).

Simultaneous occurrence of biological processes was identified between the same activated *PTHLH* feedback-mediated cell adhesion GO network of HCC (compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues) and the different (compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues), or the same (compared with the corresponding inhibited GO network of HCC) for putting forward hypothesis of activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network, respectively.

Simultaneous occurrence of biological processes consisted of intracellular transport, regulation of cyclin-dependent protein kinase activity between the same activated *PTHLH* feedback-mediated cell adhesion GO network of HCC (compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues) and the different (compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues).

Simultaneous occurrence of biological processes included anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism, cell adhesion, cell differentiation, cell-cell signaling, G-protein-coupled receptor protein signaling pathway, metabolism, phosphoinositide-mediated signaling, positive regulation of transcription, regulation of cyclin-dependent protein kinase activity, regulation of transcription, signal transduction, transcription, transport between the same activated *PTHLH* feedback-mediated cell adhesion GO network of HCC (compared with the corresponding activated GO network of no-tumor hepatitis/cirrhotic tissues), and the same (compared with the corresponding inhibited GO network of HCC).

The studies of phosphoinositide with adhesion are presented as follows. Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion [26], TAPP2 links phosphoinositide 3-kinase signaling to B-cell adhesion through interaction with the cytoskeletal protein utrophin: expression of a novel cell adhesion-promoting complex in B-cell leukemia [27], neuregulin-1 regulates cell adhesion via an ErbB2/phosphoinositide-3 kinase/Akt-dependent pathway: potential implications for schizophrenia and cancer [28], stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C [29], and functional association of platelet endothelial cell adhesion molecule-1 and phosphoinositide 3-kinase in human neutrophils [30]. Therefore, we proposed activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network in HCC.

Activated *PTHLH* feedback-mediated cell adhesion molecular network and numbers in HCC were extracted and computed from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues (Table 2). *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion molecular relationship in HCC was identified including different molecules but same GO term and same molecule

Table 1: (a) Biological processes and occurrence numbers of the same activated high expression (fold change ≥2) *PTHLH* feedback-mediated cell adhesion GO network in HCC compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection), (b) the different compared with the corresponding inhibited *PTHLH* feedback-mediated cell adhesion GO network of no-tumor hepatitis/cirrhotic tissues, and (c) the same compared with the corresponding inhibited GO network of HCC by our programming.

(a) Biological process and occurrence number of GO term	
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism	5
Cell adhesion	8
Cell differentiation	2
Cell-cell signaling	5
Endothelial cell migration	2
G-protein-coupled receptor protein signaling pathway	4
G-protein signaling	2
Intracellular transport	2
metabolism	4
Phosphoinositide-mediated signaling	4
Positive regulation of transcription	3
Protein amino acid phosphorylation	8
Regulation of cyclin-dependent protein kinase activity	8
Regulation of transcription	8
Signal transduction	10
Transcription	8
Transport	2
(b) Biological process and occurrence number of GO term	
Integrin-mediated signaling pathway	2
Intracellular transport	2
Microtubule cytoskeleton organization and biogenesis	2
Regulation of cell growth	2
Regulation of cyclin-dependent protein kinase activity	8
(c) Biological process and occurrence number of GO term	
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism	5
Cell adhesion	8
Cell differentiation	2
Cell-cell signaling	5
DNA repair	2
G-protein-coupled receptor protein signaling pathway	4
Integrin-mediated signaling pathway	2
Metabolism	4
Nucleotide and nucleic acid metabolism	2
Oxidation reduction	5
Phosphoinositide-mediated signaling	4
Positive regulation of transcription	3
Protein modification	2
Proteolysis	5
Regulation of cyclin-dependent protein kinase activity	8
Regulation of transcription	8
Signal transduction	10
Transcription	8
Transport	2

Table 2: Activated *PTHLH* feedback-mediated cell adhesion molecular network and numbers in HCC from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues by our programming.

Molecular name and number	
BUB1B, GNG10, PTHR2, GNAZ, RFC4, UBE2C, NRXN3, BAP1, PVRL2, TROAP, VCAN, CCNA2, CDC6, CDKN2C, ENAH	15

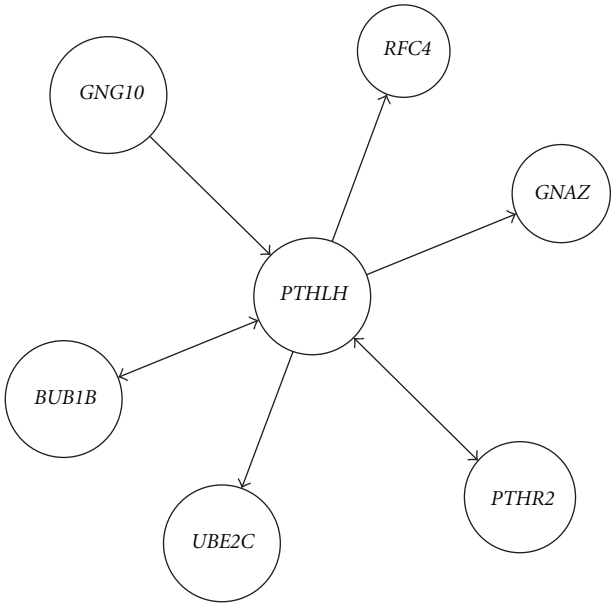

Figure 1: Activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal network construction including different molecules but same GO term and same molecule but different GO terms in HCC from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues by GRNInfer and our programming.

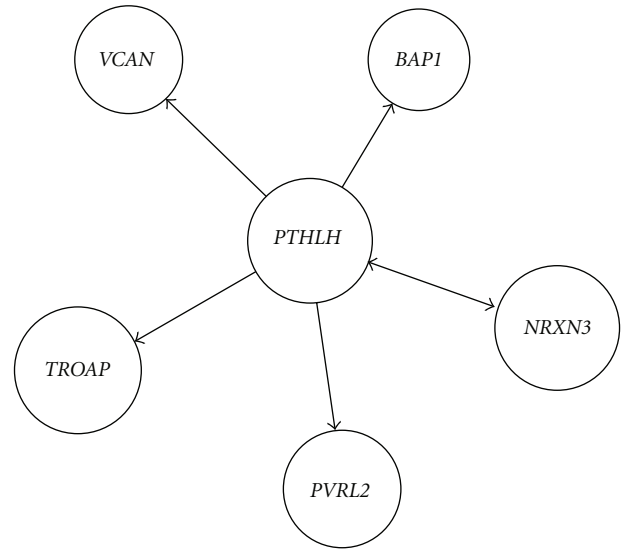

Figure 2: Activated *PTHLH* feedback cell adhesion network construction including different molecules but same GO term and same molecule but different GO terms in HCC from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues by GRNInfer and our programming.

but different GO terms from the same activated *PTHLH* GO-molecular network of HCC compared with the corresponding activated GO-molecular network of no-tumor hepatitis/cirrhotic tissues. Activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal network included *BUB1B, GNG10, PTHR2, GNAZ, PTHR2, BUB1B, RFC4,* and *UBE2C* and activated *PTHLH* feedback cell adhesion network *NRXN3, BAP1, NRXN3, PVRL2, TROAP,* and *VCAN* in HCC, as shown in Figures 1 and 2.

In summary, studies were done on analysis of biological processes in the same high expression (fold change ≥ 2) activated *PTHLH* feedback-mediated cell adhesion GO network of HCC compared with the corresponding low expression activated GO network of no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection). Activated *PTHLH* feedback-mediated cell adhesion network consisted of anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolism, cell adhesion, cell differentiation, cell-cell signaling, G-protein-coupled receptor protein signaling pathway, intracellular transport, metabolism, phosphoinositide-mediated signaling, positive regulation of transcription, regulation of cyclin-dependent protein kinase activity, regulation of transcription, signal transduction, transcription, and transport in HCC. We proposed activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network. Our hypothesis was verified by the different activated *PTHLH* feedback-mediated cell adhesion GO network of HCC compared with the corresponding inhibited GO network of no-tumor hepatitis/cirrhotic tissues, or the same compared with the corresponding inhibited GO network of HCC. Activated *PTHLH* coupling feedback phosphoinositide to G-protein receptor signal-induced cell adhesion network included *BUB1B, GNG10, PTHR2, GNAZ, RFC4, UBE2C, NRXN3, BAP1, PVRL2, TROAP,* and *VCAN* in HCC from GEO data set using gene regulatory network inference method and our programming.

Authors' Contribution

Equal contribution.

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