

In vitro evaluation of the antioxidant and cytotoxic activities of constituents of the mangrove *Lumnitzera racemosa* Willd.

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Abstract This study performed phytochemical and bioactive assessments of the mangrove *Lumnitzera racemosa* Willd. leaves. Bioassay-guided fractionation of the methanolic extracts led to the identification of thirty-six compounds (**1–36**), their structures were elucidated using detailed NMR spectroscopic and MS analysis. The extracts, fractions, and the isolated compounds were screened for potential antioxidant and cytotoxic activities. Antioxidant assays were performed using peroxyl radical-scavenging and reducing assays, whereas cytotoxicity was measured using MTT

assays in HL-60 and Hel-299 cell lines. The methanolic extract, CH₂Cl₂ and *n*-BuOH fractions (10.0 µg/mL) exhibited potent antioxidant activity, with Trolox equivalent (TE) values of 24.94 ± 0.59, 28.34 ± 0.20, and 27.09 ± 0.37 (µM), respectively. In addition, the isolated compounds exerted cytotoxic effects in a dose-dependent manner; compounds **1** and **14** exhibited the most potent cytotoxicity in HL-60 cells, with IC₅₀ values of 0.15 ± 0.29 and 0.60 ± 0.16 µM, respectively. To clarify the mechanism(s) behind these cytotoxic effects, we measured the time-dependent changes in apoptotic markers including the condensation and fragmentation of nuclear chromatin, and the downregulation of p-ERK1/2, p-AKT, and c-Myc levels.

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Introduction

In recent years, several medicinal plants have been a useful sources of novel biologically active compounds. Many pharmaceutical agents have been discovered by screening natural products from plants, many of which have been developed as new lead agents for pharmaceuticals (Sayed 2000). Examples of successful drugs originally isolated from plants include the Vinca alkaloids from *Catharanthus roseus*, camptothecin from *Camptotheca acuminata*, podophyllotoxin from *Podophyllum peltatum*, and paclitaxel from *Taxus baccata* (Weber 2009).

Cancer, which is defined as the uncontrolled growth of abnormal cells, is a major health problem. Reactive oxygen species (ROS)-induced cellular damage underlies several key steps in the development of the malignant phenotype, including the evasion of apoptosis, angiogenesis,

uncontrolled proliferation, tissue invasion, and metastasis. In addition, many cancers develop resistance to prolonged chemotherapy. Therefore, there is a need to develop more effective and safe therapeutics, such as herbal remedies. Importantly, natural products are a relevant source of novel and potent bioactive compounds with minimal side effects in vivo. Severe oxidative stress has also been implicated in many other chronic and degenerative diseases, including aging, osteoporosis, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Crespo-Ortiz and Wei 2012; Ziech et al. 2012).

Many phytochemical compounds, including carotenoids and flavonoids, exhibit antioxidant properties. The screening of certain phytochemical compounds is simple and rapid, and requires minimal equipment or selective techniques. Natural antioxidants are capable of inhibiting the formation of carcinogens from precursor substances, and thus could exert preventive effects against cancer (Mukhopadhyay 2007). Oxygen radical absorbance capacity (ORAC) is a method of measuring the antioxidant capacity of biological samples in vitro (Kurihara et al. 2004). The ORAC assay involves calculating the mean free radical activity in samples, and combines both the extent and the length of inhibition time of free radical action by antioxidants. It provides important information regarding the antioxidant capacity of various biological samples from pure compounds such as phenolic and flavonoid substances, to complex matrices such as tea, vegetables, fruits, and animal tissues (Cao et al. 1997; Kurihara et al. 2004).

Lumnitzera racemosa Willd. (Combretaceae) is a small tree found in the mangroves or coast of Asia. The wood of *L. racemosa* is used as a fuel for its calorific value, whereas its leaves are eaten on South Pacific Island during periods of scarcity. In addition, the reddish brown bark contains ~15 × 19 % tannins, although the leaves and wood contain smaller quantities. Fluid obtained from incisions made in the stems have been applied externally for the treatment of herpes and itches (Anjaneyulu et al. 2003). The major chemical constituents of *L. racemosa* are polyisoprenoid alcohols, flavonoids, fatty acids, triterpenoids, and tannins (Anjaneyulu et al. 2003). Previous studies reported that extracts from this plant exert various pharmacological activities, including antibacterial (D'Souza et al. 2010), antifungal, antihypertensive (Lin et al. 1993), protein tyrosine phosphatase 1B (PTP1B) inhibitory, hepatoprotective, and antioxidant activities (Ravikumar and Gnanadesigan 2011).

As a part of our ongoing search for bioactive agents in the mangroves, the CH₂Cl₂- and *n*-BuOH-soluble fractions of a *L. racemosa* methanolic extract were selected for phytochemical and biological investigations. We herein wish to report the compounds that may be contributed to the biological activities. The isolation and structural

elucidation of thirty-six compounds **1–36** (Fig. 1) from the leaves of *L. racemosa*, as well as the biological evaluation of these isolates were obtained using peroxy radical-scavenging and reducing assays, and assessing cytotoxicity in HL-60 and Hel-299 cell lines are reported.

Materials and methods

Plant material

The samples of *L. racemosa* Willd. leaves were collected in Xuan Thuy national botanical park, Namdinh, Vietnam, in July 2013 and identified by Dr. Nguyen The Cuong, IEBR. Voucher specimens (No. XT_CB04C) were deposited at the Institute of Marine Biochemistry, VAST, Vietnam.

Compounds

Bioassay-guided fractionation of the methanolic extract of the mangrove *L. racemosa* leaves, led to the isolation and structural elucidation thirty-six compounds (**1–36**). Stock solutions of the tested compounds in DMSO were prepared, kept at –20 °C, and diluted to the final concentration in fresh media before each experiment. For not to affect cell growth, the final DMSO concentration did not exceed 0.5 % in all experiments.

Reducing capacity

Forty microliters of different concentrations of each compound dissolved in ethanol were mixed with 160 µL of a mixture containing 0.5 mM CuCl₂ and 0.75 mM neocuproine, a Cu(I) ion specific chelator, in 10.0 mM phosphate buffer, pH 7.4. Absorbance was measured using a microplate reader at 454 nm for 1 h. Increased absorbance of the reaction mixture indicated greater reducing power.

Western blot analysis

The HL-60 (3 × 10⁵ cells/mL) cell was treated with the IC₅₀ values of compounds **1** and **14** for 24 and 48 h. After treatment, the cell was harvested and washed two times with cold PBS. The cell was lysed with lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2.0 mM EDTA, 1.0 mM EGTA, 1.0 mM NaVO₃, 10.0 mM NaF, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 25.0 µg/mL aprotinin, 25.0 µg/mL leupeptin, 1 % Nonidet P-40] and kept on ice for 30 min at 4 °C. The lysates were centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatants were stored at –20 °C until use. Protein content was determined by the Bradford assay (Bradford 1976). The same amount of lysates were separated on 8–15 % SDS-PAGE gels and then

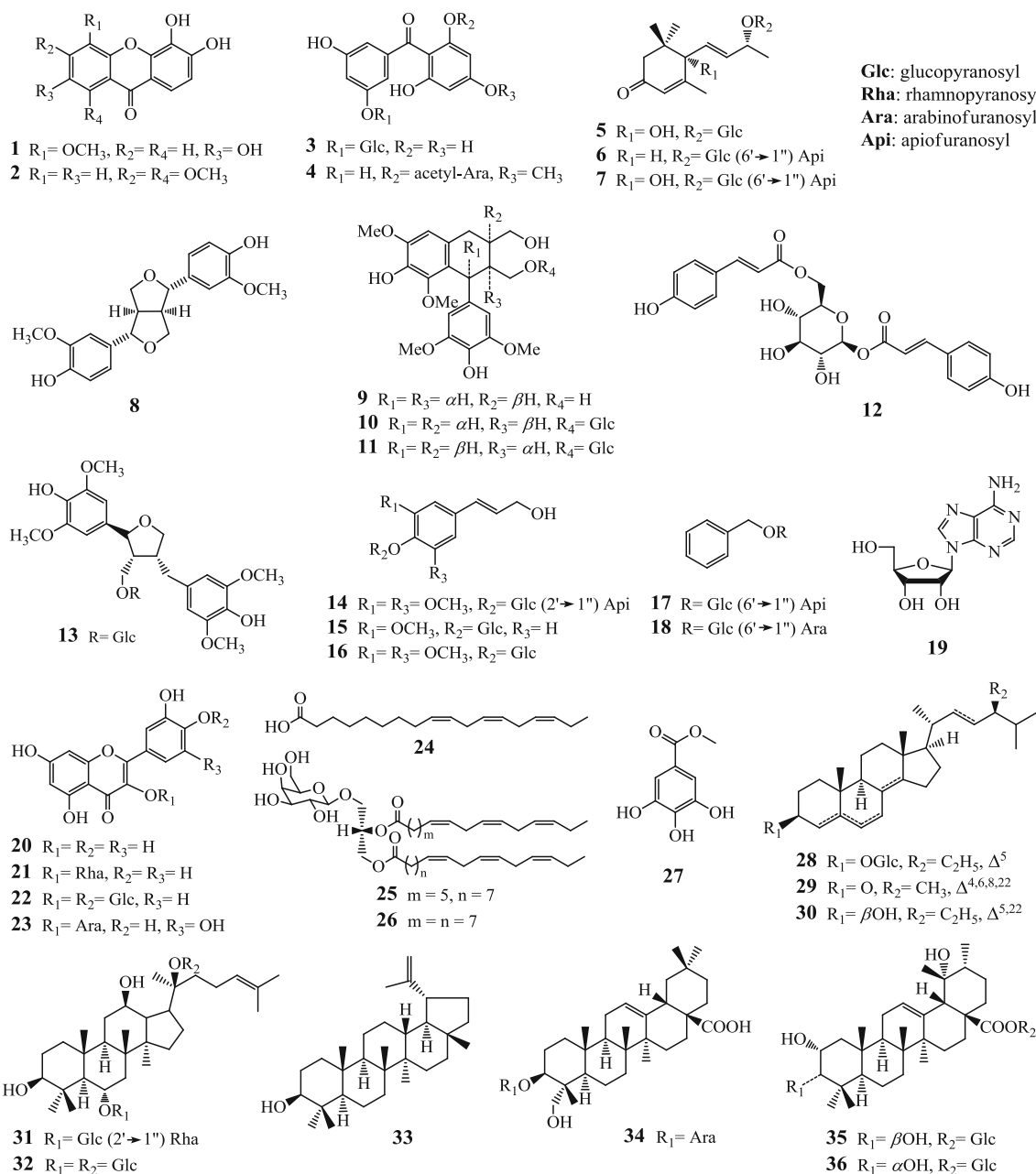


Fig. 1 The chemical structures of compounds 1–36 from the leaves of *L. racemosa*

transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) by glycine transfer buffer [192 mM glycine, 25.0 mM Tris-HCl (pH 8.8), and 20 % MeOH (v/v)] at 200 mA for 2 h. After blocking with 5 % nonfat dried milk, the membrane was incubated with primary antibody against Bcl-2 (1:500), Bax (1:1000), cleaved PARP (1:1000), cleaved caspase-9 (1:1000), cleaved caspase-3 (1:1000), ERK1/2 (1:1000), phospho-ERK1/2 (1:1000), c-Myc (1:1000), and β -actin (1:5000) antibodies and incubated with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, VT, USA) at room temperature. The membrane was exposed on X-ray films (AGFA, Belgium),

and protein bands were detected using a WEST-ZOL[®] plus Western Blot Detection System (iNtRON, Gyeonggi-do, Republic of Korea).

Statistical analysis

Data are presented as the mean \pm SD of at least three independent experiments performed in triplicate. Statistically significant differences were determined by one-way ANOVA followed by Dunnett's multiple test using GraphPad Prism 6 program (GraphPad Software Inc., San Diego, CA, USA).

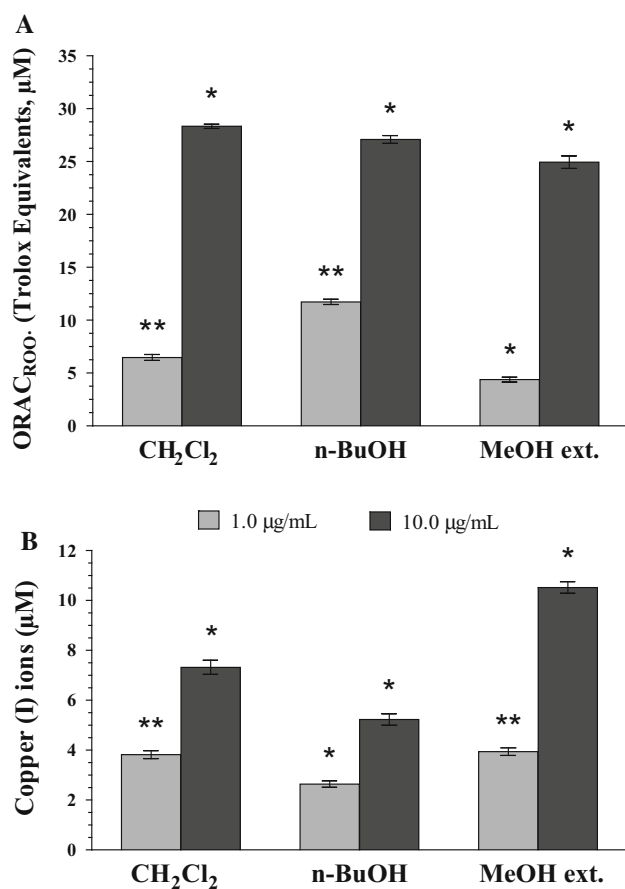


Fig. 2 Peroxyl radical-scavenging capacity (A) and reducing capacity (B) of the extracts. Data are expressed as the mean \pm standard deviation of three individual experiments. Statistical significance is indicated as determined by one-way ANOVA followed by Dunnett's multiple comparison test (* $P < 0.05$, ** $P < 0.01$)

Results and discussion

Phytochemical analysis

A methanolic extract of *L. racemosa* leaves was suspended in water, and then sequentially partitioned using *n*-hexane, CH₂Cl₂, and *n*-BuOH. All of the obtained fractions were evaluated in antioxidant and cytotoxic assays *in vitro*. The methanolic extract, CH₂Cl₂- and *n*-BuOH-soluble fractions exhibited potent cytotoxic activity with IC₅₀ values of 3.48 ± 0.10 , 2.33 ± 0.03 , and 1.27 ± 0.06 μg/mL, respectively; other fractions were considered inactive (IC₅₀s > 50 μg/mL). Similarly, this extract and fractions exhibited antioxidant activity in both peroxyl radical-scavenging and reducing assays, with Trolox equivalent (TE) values of ranging from 24.94 ± 0.59 to 28.34 ± 0.20 μM (Fig. 2).

Therefore, the CH₂Cl₂- and *n*-BuOH-soluble fractions were selected for further separation using various chromatographic methods, which resulted in the isolation of thirty-six secondary metabolites (1–36, Fig. 1). Their structures were identified as

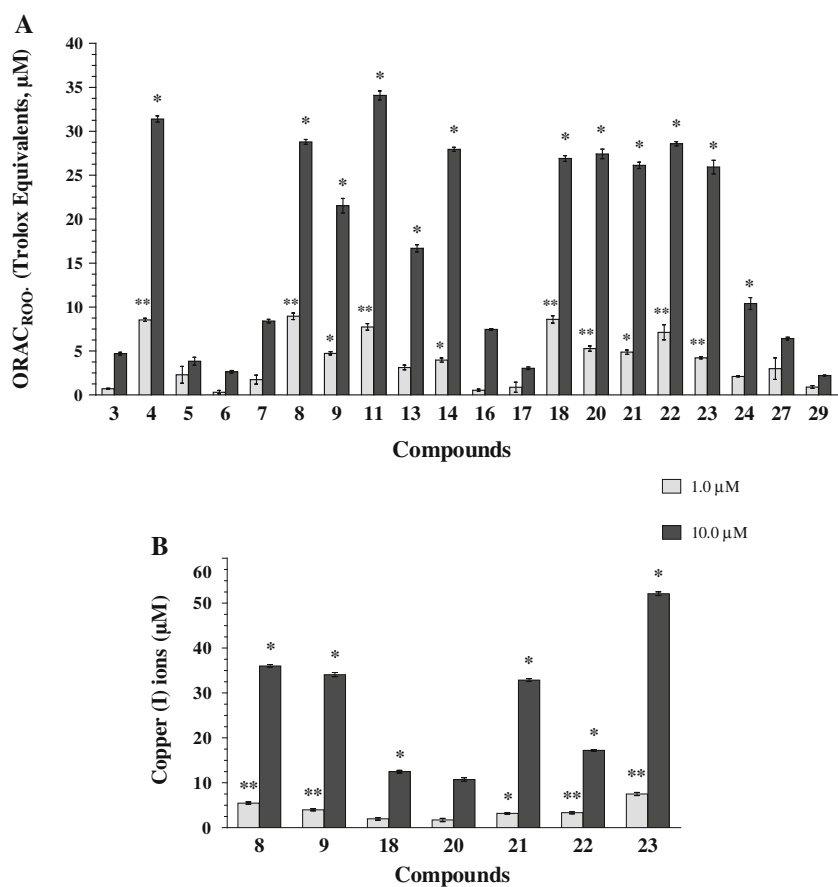
1,5,6-trihydroxy-3-methoxyxanthone (1) (Terreaux et al. 1995), 5,6-dihydroxy-2,4-dimethoxyxanthone (2) (Terreaux et al. 1995), perforaphenonoside A (3) (Li et al. 2013), acetylannulatophenonoside (4) (Nedialkov and Kitanov 2002), (6*S*,9*R*)-6-hydroxy-3-oxo- α -ionol-9-*O*- β -D-glucopyranoside (5) (Otsuka et al. 1995), (6*S*,9*R*)-9-hydroxy-4,7-megastigadien-3-one-9-*O*-(β -D-apiofuranosyl-(1 \rightarrow 6'))- β -D-glucopyranoside (6) (Tommasi et al. 1992), (6*S*,9*R*)-vomifoliol-9-*O*- β -apiofuranosyl-(1'' \rightarrow 6')-*O*- β -glucopyranoside (7) (Ito et al. 2001), (+)-pinoresinol (8) (Yeo et al. 2004), polystachyol (9) (Sadhu et al. 2006), (+)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (10) (Inoshiri et al. 1987), (-)-lyoniresinol-3 α -*O*- β -D-glucopyranoside (11) (Inoshiri et al. 1987), 1,6-di-*O*-*p*-coumaroyl- β -D-glycopyranoside (12), alangilignoside C (13) (Achenbach et al. 1997), polygalatenoside E (14) (Cheng et al. 2006), 3-(4 β -D-glucopyranosyloxy-3-methoxy)-phenyl-2*E*-propenol (15) (Dubeler et al. 1997), lawsoniaside B (16) (Cuong et al. 2010), icaraside F₂ (17) (Miyse et al. 1988), benzyl- α -L-arabinopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (18) (Lee et al. 2012), adenosine (19) (Song et al. 2008), quercetine (20) (Satake et al. 2007), quercitrin (21) (Xi-Ning et al. 1997), quercetin-4'-*O*- β -D-glucopyranosyl-3-*O*- β -D-glucopyranoside (22) (Jaramillo et al. 2011), myricetin-3-arabinoside (23) (Toshimura et al. 2008), linolenic acid (24) (Mats et al. 1999), (2*S*)-1-*O*- α -linolenoyl-2-*O*-[(7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoyl]-3-*O*- β -D-galactopyranosyl-*sn*-glycerol (25) (Nakabayashi et al. 2009), (2*S*)-1,2-di-*O*-[(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoyl]-3-*O*- β -D-galactopyranosyl-*sn*-glycerol (26) (Nakabayashi et al. 2009), methyl ester galic acid (27) (Didem et al. 2012), ipuranol (28) (Voutquenne et al. 1999), ergosta-4,6,8(14),22-tetraen-3-one (29) (Gan et al. 1998), stigmasterol (30) (Jash et al. 2013), ginsenoside Re (31) (Fujioka et al. 1989), ginsenoside Rg1 (32) (Fujioka et al. 1989), 20(29)lupen-3-ol (33) (Sholichin et al. 1980), hederagenin 3-*O*- α -L-arabinopyranoside (34) (Kizu and Tomimori 1982), tormentic acid (35) (Jia et al. 1993), and kaji-ichigoside F₁ (36) (Jia et al. 1993) based on spectral and chemical evidence, consistent with literature data. Of these pure isolates, compounds 1–13 were reported for the first time from the genus *Lumnitzera*, as well as the first report of 14–18 from this plant.

Biological activities

Determination of antioxidant activity

Some biological effects of the mangrove *L. racemosa* are related to its antioxidant properties. To date, there have been no reports of the antioxidative nature of pure compounds from this plant are available in the literature; most of the publications described the antioxidant activities of only extracts (Ravikumar and Gnanadesigan 2011; Vadlapudi and Chandrasekhr 2009). The antioxidant effects of

Fig. 3 Peroxyl radical-scavenging capacity (A) and reducing capacity (B) of the isolated compounds. Data are expressed as the mean \pm standard deviation of three individual experiments. Statistical significance is indicated as determined by one-way ANOVA followed by Dunnett's multiple comparison test (* $P < 0.05$, ** $P < 0.01$)



the isolated compounds were analyzed to evaluate their mode of action. The antioxidant capacity of all isolated compounds (1–36) was determined by measuring their peroxyl radical-scavenging and reducing activities (see Materials and methods and Supporting data).

Our data revealed that 10.0 $\mu\text{g/mL}$ methanolic extract, as well as CH_2Cl_2 and *n*-BuOH fractions exhibited potent reducing (with values of 11.73 ± 0.25 , 7.47 ± 0.28 , and 5.38 ± 0.23 μM TE, respectively), and peroxyl radical-scavenging capacities (with values of 24.94 ± 0.59 , 28.34 ± 0.20 , and 27.09 ± 0.37 μM TE, respectively, Fig. 2). With regard to peroxyl radical-scavenging activity, Fig. 3a shows that the peroxyl radical-scavenging activities of the isolated compounds on peroxyl radicals generated from AAPH were dose-dependent at concentrations of 1.0 and 10.0 μM . Compounds 4, 8–14, 18, and 20–23 exhibited the most potent activities of the isolated compounds, with TE values of 31.4 ± 0.35 , 28.79 ± 0.27 , 21.63 ± 0.82 , 34.08 ± 0.51 , 16.96 ± 0.40 , 27.95 ± 0.23 , 26.90 ± 0.33 , 27.42 ± 0.54 , 26.13 ± 0.36 , 28.58 ± 0.23 , 25.93 ± 0.77 , and 10.40 ± 0.68 (μM), respectively, at a concentration of 10.0 μM . The activities of these compounds were higher than that of Trolox, the positive control. In addition,

compounds 4, 8, 11, 18, 20, and 22 also exerted significant effects against peroxyl radicals at a concentration of 1.0 μM , with values of 8.55 ± 0.17 , 8.96 ± 0.37 , 7.74 ± 0.37 , 8.60 ± 0.41 , 5.28 ± 0.29 , and 7.13 ± 0.85 μM TE, respectively (Fig. 3A). A comparison of the peroxyl radical-scavenging ability of the compounds 1–36 revealed an interesting preliminary structure–activity relationship (SAR), where the scavenging capacity was dependent on the location and number of hydroxy groups, and the location and species of sugar moieties in their structures.

We next assessed the reducing capacity of the compounds by measuring the concentration of Cu (I) ions reduced from Cu (II) ions. The ability of the isolated compounds (1–36) to stimulate the reduction of copper ions was investigated to determine whether their peroxyl radical-scavenging activity, which involves donating a hydrogen atom, was related to their reducing capacity, which entails donating electrons. As the results, compounds 8, 9, 18, and 20–23 (10.0 μM) had meaningful reducing capacity, with 36.02 ± 0.30 , 34.09 ± 0.46 , 12.49 ± 0.30 , 10.73 ± 0.41 , 32.87 ± 0.31 , 17.23 ± 0.17 , and 52.12 ± 0.41 μM of generated copper (I) ions,

Table 1 The cytotoxic effects of the extracts and isolated compounds **1–36** from *L. racemosa*

Compounds	IC ₅₀ values (μM)	
	HL-60	Hel-299
1	0.15 ± 0.29	55.92 ± 3.51
2	6.10 ± 0.50	>100
3	12.36 ± 0.56	>100
4	8.91 ± 2.21	65.05 ± 5.58
5	16.36 ± 1.16	>100
6	19.85 ± 0.65	>100
7	17.17 ± 3.06	>100
8	16.37 ± 2.83	>100
9	2.05 ± 1.20	62.60 ± 6.09
10	9.11 ± 0.53	74.03 ± 6.34
11	6.46 ± 1.07	78.46 ± 2.60
12	8.33 ± 1.11	>100
13	5.77 ± 2.85	81.07 ± 4.22
14	0.60 ± 0.16	54.34 ± 3.17
15	6.66 ± 4.01	>100
16	7.97 ± 2.23	88.36 ± 5.32
17	5.31 ± 0.06	38.31 ± 6.35
18	6.98 ± 3.76	68.52 ± 6.80
19	18.85 ± 0.39	73.61 ± 5.65
20	28.78 ± 0.82	>100
21	15.26 ± 1.09	>100
22	15.02 ± 4.6	>100
23	34.86 ± 1.02	>100
24	30.52 ± 0.92	>100
25	12.56 ± 0.71	>100
26	19.59 ± 1.16	>100
27	8.56 ± 0.39	84.18 ± 2.86
28	57.35 ± 0.41	>100
29	2.69 ± 0.74	25.60 ± 0.29
30	66.60 ± 2.73	>100
31	8.61 ± 1.43	40.64 ± 2.74
32	3.29 ± 1.13	>100
33	43.33 ± 3.04	>100
34	4.02 ± 0.88	42.05 ± 5.15
35	37.85 ± 4.56	>100
36	27.74 ± 1.43	>100
CH₂Cl₂ fra.^a	2.33 ± 0.03	>50
<i>n</i>-BuOH fra.^a	1.27 ± 0.06	3.94 ± 0.55
MeOH ext.^a	3.48 ± 0.10	9.72 ± 0.39
MX^b	6.80 ± 0.09	28.41 ± 2.24

The results are the mean ± SD of three independent experiments in triplicate. The IC₅₀ values equal to or <50 μg/mL (for extracts) and 100 μM (for compounds) were considered active

^a μg/mL; ^b Mitoxantrone (MX) was used as a positive control

respectively. The remaining compounds showed negligible effects, with <2.0 μM of copper (I) ions at different concentration (Fig. 3B).

These results suggest that the ability of compounds **4**, **8–14**, and **18–23** to donate hydrogen atoms or electrons to peroxy radicals and convert them into relatively stable forms may contribute to their peroxy radical-scavenging capacities. Although compounds **4**, **11**, **13**, and **14** exhibited potent peroxy radical-scavenging activities, they demonstrate lower capacities for reducing Cu (II) ions to Cu (I) ions compared with compounds **8**, **9**, **18**, and **20–23**. This suggests that the hydrogen-donating ability of compounds **4**, **11**, **13**, and **14** is strong, but that their single electron-transferring activity is weak. This may be due to the presence of only one hydroxyl substituent on the benzene ring, which results in unfavorable electron transfer to the Cu (II) ions. These results demonstrate that lignans (**8–13**) and flavonoids (**20–23**), which contain the phenolic hydroxyl groups, are the major components responsible for the potent antioxidant activity of *L. racemosa* leaf extracts.

Determination of cytotoxic activity

To date, the cytotoxicities of the extracts and/or compounds isolated from *L. racemosa* have not been reported. Therefore, we assayed the cytotoxic effects of the methanolic extract, as well as CH₂Cl₂-, and *n*-BuOH-soluble fractions against HL-60 and Hel-299 cell lines using MTT assays (Table 1). Treating these cell lines with the CH₂Cl₂ and *n*-BuOH fractions for 72 h yielded IC₅₀ values ranging from 1.27 ± 0.06 to 3.94 ± 0.55 μg/mL. Notably, the *n*-BuOH fraction was potentially cytotoxic on both human cell lines.

Next, the cytotoxic effects of all isolated compounds (**1–36**) from this plant were evaluated against HL-60 and Hel-299 cell lines using MTT assays after treatment for 72 h (Table 1). According to the in vitro results, compounds **1** and **14** exhibited the most potent cytotoxic activity on HL-60 cells, with IC₅₀ values of 0.15 ± 0.29 and 0.60 ± 0.16 μM, respectively. For comparison, the positive control mitoxantrone had IC₅₀ value of 6.80 ± 0.09 μM, respectively. While moderate cytotoxicity was observed with both compounds **1** and **14** against Hel-299 cells (IC₅₀ value of 28.41 ± 2.24 μM), with IC₅₀ values of 55.92 ± 3.51 and 54.34 ± 3.17 μM, respectively. Additionally, compounds **2**, **4**, **9–13**, **15–18**, **27**, **29**, **31**, **32**, and **34** also exhibited significant cytotoxicity in HL-60 cells, with IC₅₀ values ranging from 2.05 ± 1.20 to 9.11 ± 0.53 μM; but moderate or weak effects were observed on Hel-299 cells. Moderate effects were also observed for compounds **3**, **5–8**, **15**, **20–22**, **24–26**, and **36**

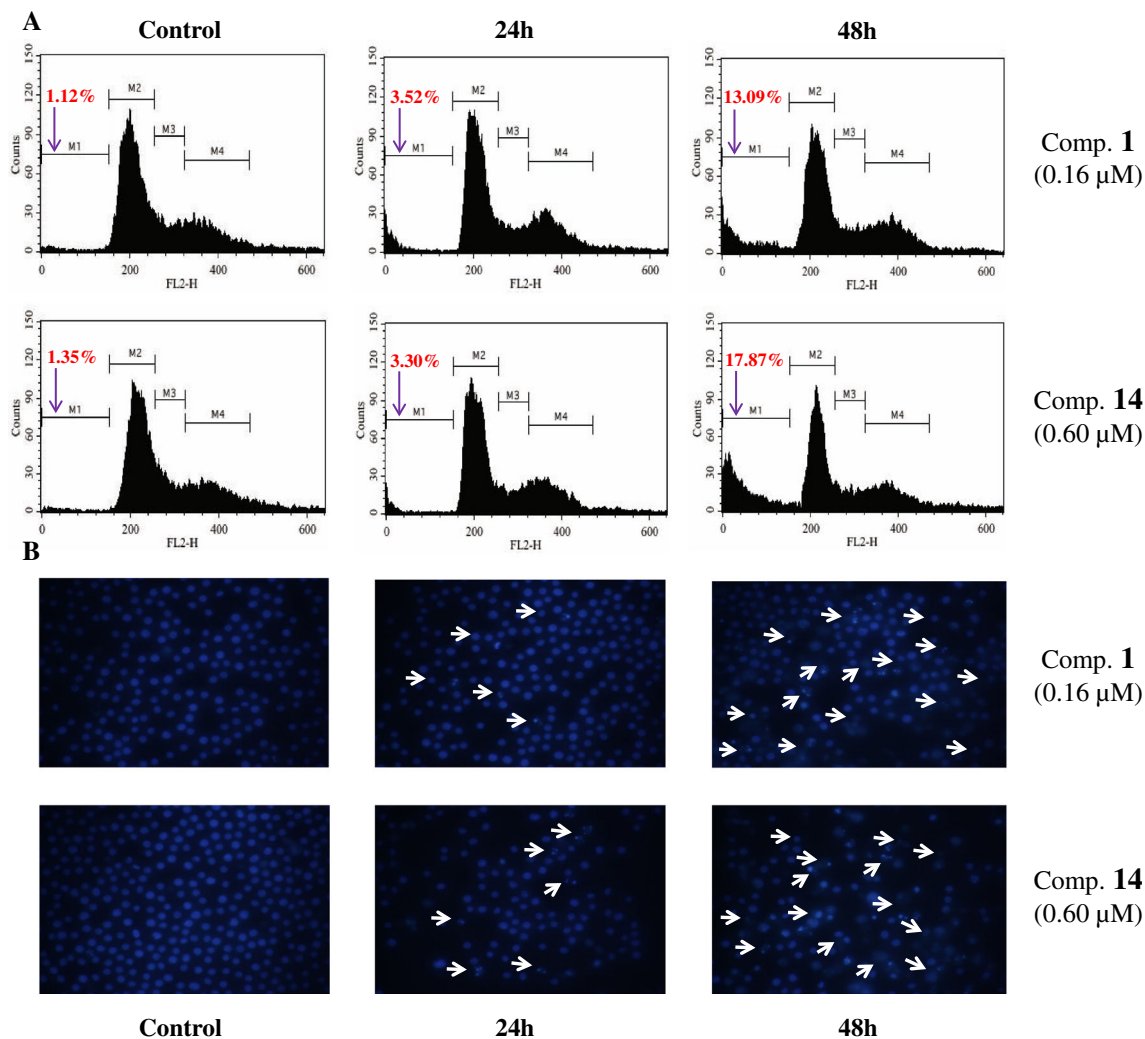


Fig. 4 The degree of apoptosis represented as the DNA content measured by flow cytometric analysis (A) and the fluorescent image of nuclei in HL-60 cell by Hoechst 33342 staining (B). The HL-60 cell was treated with the IC_{50} values of compounds **1** and **14** for 24 and 48 h. DNA-specific fluorescent dye, Hoechst 33342 (culture

medium at a final concentration of 10.0 μ g/mL) was directly added to media and apoptotic bodies were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification \times 200)

(with IC_{50} ranging from 12.36 ± 0.56 to 30.52 ± 0.92 μ M), whereas the effects of compounds **23**, **28**, **30**, **33**, and **35** were weak on both human cell lines compared with the positive control (Table 1). This suggested that active constituents could be isolated from individual compounds, which may become useful for the treatment of leukemia.

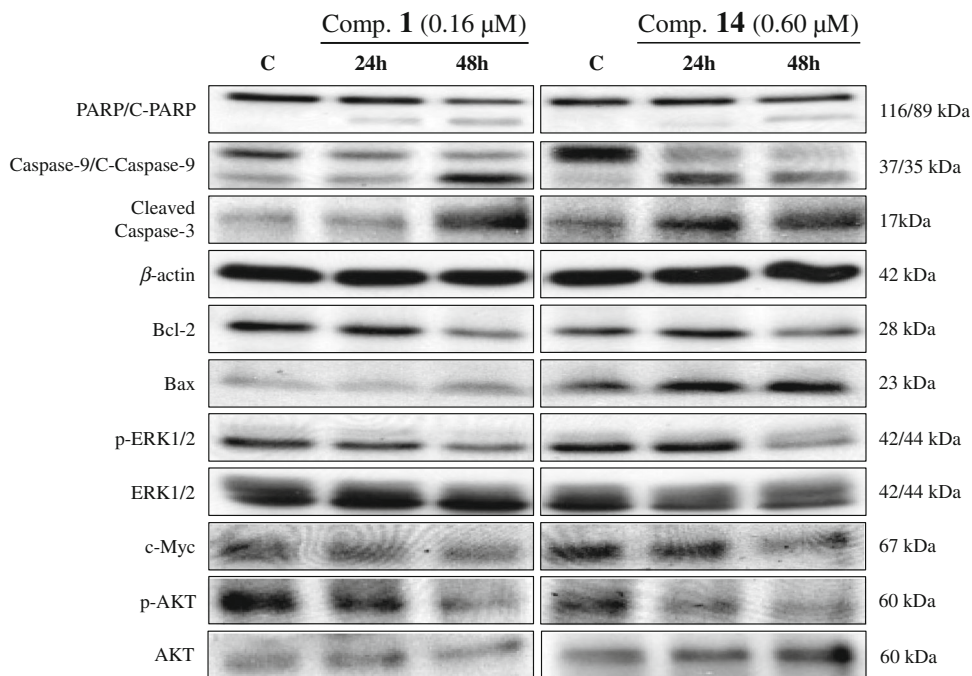
Determination of apoptosis in HL-60 human cancer cell line

Many previous reports revealed that natural products and compounds exert potential antitumor activity by inducing apoptosis (Lee et al. 2006). One of the more attractive strategies being considered in current cancer therapy is dietary and/or pharmaceutical manipulation to induce the

apoptosis death of malignant cells (Tsuda et al. 2004). An accumulating body of evidence suggests that naturally occurring compounds and many chemotherapeutic agents trigger apoptosis in cancer cells (Bold et al. 1997).

To elucidate the cytotoxic mechanism, we investigated whether the inhibitory effects of compounds **1** and **14** on the proliferation of the human leukemia HL-60 cells arose from the induction of apoptosis (see Supporting data). Apoptotic cells exhibit typical characteristics such as membrane blebbing, cell shrinkage, chromatin condensation, and an increased population of sub- G_1 hypodiploid cells (Elmore 2007). Therefore, we examined the apoptotic characteristics of cancer cells following treatment with **1** and **14** at IC_{50} levels for 48 h. Flow cytometry revealed that the percentage of sub- G_1 hypodiploid cells exposed to

Fig. 5 The effects of compounds **1** and **14** on the levels of Bcl-2, Bax, cleaved caspase-9, caspase-3, cleaved PARP, and on the activation of ERK1/2 MAPK and c-Myc in HL-60 cell for 24 and 48 h



1 and **14** was increased significantly after 48 h compared with control (Fig. 4A). These results suggest the induction of apoptosis, which was supported further by an increase in the number of apoptotic bodies in treated cells stained with the cell-permeable DNA dye Hoechst 33342 and visualized using fluorescence microscopy (Fig. 4B).

The Bcl-2 family is divided into two sub-families that either inhibit or promote apoptosis. The pro-apoptotic members of the Bcl-2 family induce apoptosis by stimulating the release of cytochrome *c* from the mitochondria, which results in the cleavage and activation of caspase-9. Upon activation, caspase-9 initiates a protease cascade leading to the rapid activation of caspase-3, an effector caspase in cells undergoing apoptosis (Cheng et al. 2005; Hanahan and Weinberg 2000; Zimmermann and Green 2001). Therefore, to determine the possible mechanism underlying the induction of apoptosis, we monitored the expression of the apoptosis-related proteins Bcl-2, Bax, cleaved-caspase-9, caspase-3, and PARP in HL-60 cells. When treated with the IC₅₀ values of **1** and **14**, the levels of these apoptosis-related proteins were altered. Specifically, Bcl-2 was decreased, and Bax, and cleaved caspase-9, caspase-3, and PARP were increased in a time-dependent manner (Fig. 5). This suggests that compounds **1** and **14** induced apoptosis by modulating the expression of apoptosis-related proteins in human leukemia HL-60 cells.

The MAPK pathway regulates cell survival, cell growth, and apoptosis. Recent studies reported that the oncoprotein c-Myc was stabilized by activation of the ERK1/2 MAPK pathway. In addition, activation of the PI3K/AKT pathway promoted the transcription of c-Myc (Rasola et al. 2010). To determine if the MAPK pathways played a role in the

apoptosis induced by **1** and **14**, we examined the expression of ERK1/2 MAPK in HL-60 cells. Treatment with **1** and **14** significantly decreased phospho-ERK1/2 levels significantly (Fig. 5). Several studies have reported that activation of the ERK1/2 MAPK pathway contributes to the stabilization of c-Myc (Yeh et al. 2004). Interestingly, decreased c-Myc and phospho-ERK1/2 levels were observed in HL-60 cells treated with **1** or **14** (Fig. 5). These results suggest that these compounds induce apoptosis in part by inhibiting ERK1/2 signaling and downregulating c-Myc in HL-60 cells.

Conclusions

In conclusion, we investigated the in vitro antioxidant and cytotoxic activities of various fractions of the methanolic extract, and isolated compounds from *L. racemosa* Willd. leaves. The methanolic extract, CH₂Cl₂- and *n*-BuOH-soluble fractions exhibited potent antioxidant and cytotoxic activities. Importantly, the current study has addressed the cytotoxic effects of chemical constituents isolated from the mangrove *L. racemosa* for the first time. This highlights the need for further investigations of the extracts and isolated compounds from this species for potential use as complementary cancer remedies. An additional mechanism of cancer prevention might be the scavenging of free radical oxygen that are associated with cancer cell development. Therefore, future studies should be performed to investigate the in vitro and/or in vivo mechanisms of action of these compounds.

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Conflict of interest The authors have declared that there is no conflict of interest.

References

- Achenbach, H., M. Benirschke, and R. Torrenegra. 1997. Alkaloids and other compounds from deeds of *Tabernaemontana cymosa*. *Phytochemistry* 45: 325–335.
- Anjaneyulu, A.S.R., Y.L.N. Murthy, V.L. Rao, and K. Sreedhar. 2003. A new aromatic ester from the mangrove plant *Lumnitzera racemosa* Willd. *ARKIVOC (Issue in Honor of Prof. Sukh Dev)* (iii): 25–30.
- Bold, R.J., P.M. Termuhlen, and D.J. McConkey. 1997. Apoptosis, cancer and cancer therapy. *Surgical Oncology* 6: 133–142.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- Cao, G., E. Sofic, and R.L. Prio. 1997. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. *Free Radical Biology and Medicine* 22: 749–760.
- Cheng, A.C., T.C. Huang, C.S. Lai, and M.H. Pan. 2005. Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. *European Journal of Pharmacology* 509: 1–10.
- Cheng, M.C., C.Y. Li, H.C. Ko, F.N. Ko, Y.L. Lin, and T.S. Wu. 2006. Antidepressant principles of the roots of *Polygala tenuifolia*. *Journal of Natural Products* 69: 1305–1309.
- Crespo-Ortiz, M.P., and M.Q. Wei. 2012. Antitumor activity of artemisinin and its derivatives: From a well-known antimalarial agent to a potential anticancer drug. *Journal of Biomedicine and Biotechnology* 247597: 18.
- Cuong, N.X., N.X. Nhiem, N.P. Thao, N.H. Nam, N.T. Dat, H.L.T. Anh, L.M. Huong, P.V. Kiem, C.V. Minh, J.H. Won, W.Y. Chung, and Y.H. Kim. 2010. Inhibitors of osteoclastogenesis from *Lawsonia inermis* leaves. *Bioorganic and Medicinal Chemistry Letters* 20: 4782–4784.
- D'Souza, L., S. Wahidulla, and P. Devi. 2010. Antibacterial phenolics from the mangrove *Lumnitzera racemosa*. *Indian Journal of Marine Sciences* 39: 249–298.
- Didem, S., S.M. Koray, S.S. Atasayar, H. Ozgunes, D. Hayri, and S. Olov. 2012. Antioxidant secondary metabolites from *Geranium lasiopus* Boiss. & Heldr. *Natural Product Research* 26: 1261–1264.
- Dubeler, A., G. Voltmer, V. Gora, J. Lunderstadt, and A. Zeeck. 1997. Phenols from *Fagus sylvatica* and their role in defence against *Cryptococcus fagisuga*. *Phytochemistry* 45: 51–57.
- Elmore, S. 2007. Apoptosis: A review of programmed cell death. *Toxicologic Pathology* 35: 495–516.
- Fujioka, N., H. Kohda, K. Yamasaki, R. Kasai, O. Tanaka, Y. Shoyama, and I. Nishioka. 1989. Dammarane and oleanane saponin from callus tissue of *Panax japonicus*. *Phytochemistry* 28: 1855–1858.
- Gan, K.H., S.H. Kuo, and C.N. Lin. 1998. Steroidal constituents of *Ganoderma applanatum* and *Ganoderma neojaponicum*. *Journal of Natural Products* 61: 1421–1422.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100: 57–70.
- Inoshiri, S., M. Sasaki, H. Kohda, H. Otsuka, and K. Yamasaki. 1987. Aromatic glycosides from *Berchemia racemosa*. *Phytochemistry* 26: 2811–2814.
- Ito, H., E. Kobayashi, S.H. Li, T. Hatano, D. Sugita, N. Kubo, S. Shimura, Y. Itoh, and T. Toshida. 2001. Megastigmane glycosides and an acylated triterpenoid from *Eriobotrya japonica*. *Journal of Natural Products* 64: 737–740.
- Jaramillo, K., C. Dawid, T. Hofmann, Y. Fujimoto, and C. Osorio. 2011. Identification of antioxidative flavonols and anthocyanins in *Sicana odorifera* fruit peel. *Journal of Agricultural and Food Chemistry* 59: 975–983.
- Jash, S.K., A. Gangopadhyay, A. Sarkar, and D. Gorai. 2013. Phytochemical investigation of the hexane extract of stem bark of *Peltophorum pterocarpum*. *Der Pharma Chemica* 5: 49–53.
- Jia, Z.J., X.Q. Liu, and Z.M. Liu. 1993. Triterpenoids from *Sanguisorba alpina*. *Phytochemistry* 32: 155–159.
- Kizu, H., and T. Tomimori. 1982. Studies on the constituents of *Clematis* species. V. On the saponin of the root of *Clematis chinensis* Osbeck. *Chemical and Pharmaceutical Bulletin* 30: 3340–3346.
- Kurihara, H., H. Fukami, S. Asami, Y. Toyoda, M. Nakai, H. Shibata, and X.S. Yao. 2004. Effects of oolong tea on plasma antioxidative capacity in mice loaded with restraint stress assessed using the oxygen radical absorbance capacity (ORAC) assay. *Biological and Pharmaceutical Bulletin* 27: 1093–1098.
- Lee, E.O., J.R. Lee, K.H. Kim, N.I. Baek, S.J. Lee, B.H. Lee, K.D. Cho, K.S. Ahn, and S.H. Kim. 2006. The methylene chloride fraction of *Trichosanthis fructus* induces apoptosis in U937 cells through the mitochondrial pathway. *Biological and Pharmaceutical Bulletin* 29: 21–25.
- Lee, S.Y., K.H. Kim, K. Lee, K.H. Lee, S.U. Choi, and K.R. Lee. 2012. A new flavonol glycoside from *Hylomecon vernalis*. *Archives of Pharmacal Research* 35: 415–421.
- Li, W., Y. Ding, T.H. Quang, N.T.T. Ngan, Y.N. Sun, X.T. Yan, S.Y. Yang, C.W. Choi, E.J. Lee, K.Y. Paek, and Y.H. Kim. 2013. NF- κ B inhibition and PPAR activation by phenolic compounds from *Hypericum perforatum* L. adventitious root. *The Bulletin of the Korean Chemistry Society* 34: 1407–1413.
- Lin, T.C., F.L. Hsu, and J.T. Cheng. 1993. Antihypertensive activity of corilagin and chebulinic acid, tannins from *Lumnitzera racemosa*. *Journal of Natural Products* 56: 629–632.
- Mats, H., S. Ana, and C. Carmen. 1999. α -Oxidation of fatty acids in higher plants. Identification of a pathogen-inducible oxygenase (PIOX) as an α -dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. *The Journal of biological chemistry* 274: 24503–24513.
- Miyase, T., A. Ueno, N. Takizawa, H. Kobayashi, and H. Oguchi. 1988. Studies on the glycosides of *Epimedium grandiflorum* Morr. var. *Chemical and Pharmaceutical Bulletin* 36: 2475–2484.
- Mukhopadhyay, A.K. 2007. *Antioxidants: Natural and synthetic*. Berlin: Amani International.
- Nakabayashi, R., M. Kusano, M. Kobayashi, T. Tohge, K. Yonekura-Sakakibara, N. Kogure, M. Yamazaki, M. Kitajima, K. Saito, and H. Takayama. 2009. Metabolomics-oriented isolation and structure elucidation of 37 compounds including two anthocyanins from *Arabidopsis thaliana*. *Phytochemistry* 70: 1017–1029.
- Nedialkov, P.T., and G.M. Kitanov. 2002. Two benzophenone O-arabinosides and a chromone from *Hypericum annulatum*. *Phytochemistry* 59: 867–871.
- Otsuka, H., M. Yao, K. Kamada, and Y. Takeda. 1995. Alangionosides G-M: Glycosides of megastigmane derivatives from the

- leaves of *Alangium premnifolium*. *Chemical and Pharmaceutical Bulletin* 43: 754–759.
- Rasola, A., M. Sciacovelli, F. Chiara, B. Pantic, W.S. Brusilow, and P. Bernardi. 2010. Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. *Proceedings of the National Academy of Sciences* 107: 726–731.
- Ravikumar, S., and M. Gnanadesigan. 2011. Hepatoprotective and antioxidant activity of a mangrove plant *Lumnitzera racemosa*. *Asian Pacific Journal Tropical Biomedicine* 1: 348–352.
- Sadhu, S.K., P. Phattanawasin, M.S.K. Choudhuri, T. Ohtsuki, and M. Ishibashi. 2006. A new lignan from *Aphanamixis polystachya*. *Journal of Natural Medicines* 60: 258–260.
- Satake, T., K. Kamiya, Y. An, T. Oishi, and J. Yamamoto. 2007. The anti-thrombotic active constituents from *Centella asiatica*. *Chemical and Pharmaceutical Bulletin* 30: 935–940.
- Sayed, K.A.E. 2000. Natural products as antiviral agents. *Studies in Natural Products Chemistry* 24: 473–572.
- Sholichin, M., K. Tamasaki, R. Kasai, and O. Tanaka. 1980. ¹³C nuclear magnetic resonance of lupane-type triterpenes, lupeol, betulin and betulinic acid. *Chemical and Pharmaceutical Bulletin* 28: 1006–1008.
- Song, M.C., H.J. Yang, T.S. Jeong, K.T. Kim, and N.I. Baek. 2008. Heterocyclic compounds from *Chrysanthemum coronarium* L. and their inhibitory activity on hACAT-1, hACAT-2, and LDL-oxidation. *Archives of Pharmacal Research* 31: 573–578.
- Terreaux, C., M. Maillard, M.P. Gupta, and K. Hostettmann. 1995. Xanthones from *Schultesia lisianthoides*. *Phytochemistry* 40: 1791–1795.
- Tommasi, N.D., R. Aquino, F.D. Simone, and C. Pizza. 1992. Plant metanolites. New sesquiterpene and ionone glycosides from *Eriobotrya japonica*. *Journal of Natural Products* 55: 1025–1032.
- Toshimura, M., Y. Amakura, M. Tokuhara, and T. Yoshida. 2008. Polyphenolic compounds isolated from the leaves of *Myrtus communis*. *Journal of Natural Medicines* 62: 366.
- Tsuda, H., Y. Ohshima, H. Nomoto, K. Fujita, E. Matsuda, M. Iigo, N. Takasuka, and M.A. Moore. 2004. Cancer prevention by natural compounds. *Drug Metabolism and Pharmacokinetics* 19: 245–263.
- Vadlapudi, V., and N.K. Chandrasekhr. 2009. Evaluation of antioxidant potential of selected mangrove plants. *Journal of Pharmacy Research* 2: 1742–1745.
- Voutquenne, L., C. Lavaud, G. Massiot, T. Sevent, and H.A. Hadi. 1999. Cytotoxic polyisoprenes and glycosides of long-chain fatty alcohols from *Dimocarpus fumatus*. *Phytochemistry* 50: 63–69.
- Weber, M.L. 2009. New therapeutic aspects of flavones: The anticancer properties of *Scutellaria* and its main active constituents Wogonin, Baicalein and Baicalin. *Cancer Treatment Reviews* 35: 57–68.
- Xi-Ning, Z., O. Hideaki, T. Ide, E. Hirata, and A. Takushi. 1997. Three flavonol glycosides from leaves of *Myrsine seguinii*. *Phytochemistry* 46: 943–946.
- Yeh, E., M. Cunningham, H. Arnold, D. Chasse, T. Monteith, G. Ivaldi, W.C. Hahn, P.T. Stukenberg, S. Shenolikar, T. Uchida, C.M. Counter, J.R. Nevins, A.R. Means, and R. Sears. 2004. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nature Cell Biology* 6: 308–318.
- Yeo, H., Y.W. Chin, S.Y. Park, and J. Kim. 2004. Lignans of *Rosa multiflora* roots. *Archives of Pharmacal Research* 27: 287–290.
- Ziech, D., I. Anastopoulos, R. Hanafi, G.P. Voulgaridou, R. Franco, A.G. Georgakilas, A. Pappa, and M.I. Panayiotidis. 2012. Pleiotropic effects of natural products in ROS-induced carcinogenesis: The role of plant-derived natural products in oral cancer chemoprevention. *Cancer Letters* 327: 16–25.
- Zimmermann, K.C., and D.R. Green. 2001. How cells die: Apoptosis pathways. *The Journal of Allergy and Clinical Immunology* 108: S99–S103.