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Antioxidant Activity of *Mayodendron igneum* Kurz and Cytotoxicity of Isolated Terpenoids

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ABSTRACT

A petroleum ether extract of *Mayodendron igneum* Kurz, (Family Bignoniaceae) yielded five compounds identified as sitosterol, erythrodiol, oleanolic acid, ursolic acid and 5', 6' norigeumone. Antioxidant activity of total ethanol extracts of the plant was equal to 77.02% of the antioxidant activity of ascorbic acid (100%) as measured by electron spin resonance (ESR) and using DPPH as the stable free radical. The antioxidant activity of successive fractions of *M. igneum* leaves were tested with the strongest inhibition of DPPH stable free radicals exhibited by the successive ethanol fraction (equal to 100% of free radical scavenging activity relative to ascorbic acid). The ethyl acetate fraction had 64.96% of the antioxidant activity of ascorbic acid, and the petroleum ether and chloroform fractions had 12.46% and 0%, respectively, of the antioxidant activity of ascorbic acid. An examination of isolated oleanolic acid demonstrated moderate cytotoxic activity against four tested cancer cell lines (liver, cervix, breast, and colon). The highest activity level was against the cervix cancer cell line (HELA) (IC_{50}= 32 µg/mL), followed by the liver cancer cell line (HEPG2) (IC_{50}= 33 µg/mL), the colon cancer cell line (HCT-116) (IC_{50}= 35 µg/mL), and the breast cancer cell line (MCF7) (IC_{50}= 37 µg/mL).

INTRODUCTION

The Trumpet Creeper family (Bignoniaceae), which includes approximately 750 species of plants in 120 genera within 8 tribes, is distributed throughout tropical and subtropical regions of South America, Africa, and India, growing as both a wild plant and a cultivated ornamental (Von Poser et al., 2000). Plants in the Bignoniaceae family are notably rich in terpenoids and flavonoids and are used medicinally in traditional medicine and pharmaceutical preparations.

Species within the Bignoniaceae, have yielded a number of phytochemicals. Sitosterol and other compounds have been isolated from the bark of *Tecomella undulata*, and the heartwood of *Heterophragma adenophylum* and *Millingtonia hortensis* (Singh, et al., 1972) and β-sitosterol has been isolated from the trunkwood of *Tabebuia ochracea* (Zani et al., 1991) and the roots of *Incarvillea arguta* (Luo et al., 2004). In addition to β-sitosterol, an aqueous extract of *Jacaranda caucana* has yielded the triterpene jacarandic acid, ursolic acid, 2 *α*-hydroxyursolic acid and 2 *α*,3 *α*-dihydroxyurs-12-en-28-oic acid (Ogura et al., 1977). The diterpene, 2-*α*-hydroxy-12 *β*-hydroxy-isopimara-8(14), 15-diene, along with six known triterpenes, sterol. and fatty acids, has been isolated from the

stem bark of *Tanaecium jaroba*, a Bolivian plant used in traditional medicine (Mitaine-Offer et al., 2002). Jin et al. (2004) has isolated five pentacyclic triterpenoids from leaves of *Campsis grandiflora* that have characterized as oleanolic acid, hederagenin, ursolic acid, tormentic acid, and myrianthic acid using spectroscopic methods. Work by Ogura et al. (1977) and Hashem and Sleem (2006) led, respectively, to the isolation of the triterpenoid ursolic acid from the petroleum ether extract of *Jacranda caucana* and *Tecoma radicans*.

The biological activities of phytochemicals extracted from plants in the Bignoniaceae family have been demonstrated in a number of studies. For example, ursolic acid is known to have cytotoxic activity *in vitro* (El-Hossary et al., 2000). Biological investigations on *Tecoma stans* fruit extract indicates a strong scavenging activity to DPPH, peroxyl, and hydroxyl radicals plus cytotoxic effects on human hepatocarcinoma cells and growth inhibition of human breast carcinoma cells (Marzouk et al., 2006). In a study on the coumarin fraction of *Macfadyena unguis–cati* by Hashem et al. (2007), demonstrated 93.9% inhibition of the stable DPPH free radical, level similar to that of vitamin C at 95.5% inhibition. Additional studies by Hashem (2007a) demonstrated the coumarin fraction of *Tecoma radicans* and peucenin had active antioxidant activity. A later study by Hashem (2007b) showed that the most potent free radical scavenger of the five identified flavonoids in *Teocoma radicans* was quercetin 3-methyl ether.

Of special interest in the current study are the phytochemical constituents of *Mayodendron igneum* Kurz. (also known as *Radermachera ignea*), a member of the Bignoniaceae family used in traditional medicine. *M. igneum* grows wild in Northern Myanmar, Northern Thailand, Laos, Vietnam, and South China (Chun-Lin, 1990). The plant, which is frequently grown in gardens as an ornamental and vegetable, is known to contain a number of minerals, vitamins, and amino acids (Youkai et al., 2005). Earlier studies have shown the plant bark of *M. igneum* to contain a number of phytochemicals, including the meroditerpenoid igeumone and 18 other chemical compounds: quercetin, naringenin, naringin, vanillic acid**,** ethyl caffeate**,** eugenin, syringaldehyde**,** phlogacantholide B**,** lup-20(29)-ene-3α, 27β-diol**,** paederoside, nemoroside, picroside, 2-hydroxy-3-hydroxymethyl-9,10 anthraquinone, 1-methoxy-3-hydroxy-2-carbon-methoxy-9,10-anthraquinone, lucidin primeveroside, β-daucosterol, β-sitosterol, and auranamide (Guo et al., 2007).

In a study of a total alcohol and successive extracts of *M. igneum* leaves against human cancer cell lines Hashem, et al. (2011), the total ethanol extract demonstrated strong cytotoxic activity against the cervix cancer cell line (IC_{50}= 0.60 µg/mL), breast cancer cell line (IC_{50}=0.54 µg/mL) and liver cancer cell line (IC_{50}=0.87 µg/mL) when compared with the anticancer drug doxorubicin. A petroleum ether extract showed cytotoxic activity against a liver cancer cell line (IC_{50}= 0.37 µg/mL) and breast cancer cell line (IC_{50}= 2.15 µg/mL). Therefore, in this study, the petroleum ether extract was subjected to a phytochemical investigation to determine the active constituents that contribute to the observed cytotoxic activity.

MATERIALS AND METHODS

Plant material. Fresh leaves from plants of *Mayodendron igneum* Kurz. (Bignoniaceae) growing in the Giza Zoo, Giza, Egypt, were used in this study. The plants were identified by Dr. M. El-Gibaly, a plant taxonomist, and Mrs. Trease Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of Orman Botanical Garden, Giza, Egypt. A voucher specimen of the plant material (Voucher number M.20) has been placed in the herbarium at the Orman Botanical Garden, Giza Egypt.

Plant extracts. A total ethanol extract was prepared from 400 g of air-dried, powdered leaves of *M. igneum* by repeatedly extracting in a Soxhlet apparatus with 80% ethanol until exhaustion. The extract was recovered and filtered to remove debris before concentrating under reduced pressure to dryness at 55°C, yielding 95 g of crude extract. Successive fractions of the 80% ethanol extract were made using 90 g of the crude extract suspended in water and partitioned successively with petroleum ether (60-80^{o}C), chloroform, and ethyl acetate. The solvents were evaporated to dryness under reduced pressure at 55°C yielding fractions of 25 g of

petroleum ether extract, 19 g of chloroform extract, 10 g of ethyl acetate extract, and 30 g of remaining residue that was considered the ethanol extract.

Phytoconstituent isolation and identification. The petroleum ether fraction from the leaves of *M. igneum* (15 g) was fractionated using an open glass column (100 cm L x 4.5 cm ID) packed with 300 g of activated silica gel as a stationary phase. The elution process was in a gradient mode, beginning with benzene as an eluent and followed by a benzene/ethyl acetate mixture of increasing polarity until reaching 100% ethyl acetate.

The collected fractions were subjected to thin layer chromatography (TLC) using two separate solvent systems S_1 (benzene:ethyl acetate, 9:1, v:v) and S_2.(chloroform:methanol, 9:1, v:v). After development, the TLC plates were examined under UV light and visualized by spraying with 20% H_2SO_4 and heating at 110°C for 5 min. Similar fractions were pooled and further purified using preparative layer chromatography (PLC) with S_1 as the solvent system yielding five pure compounds (Table 1).

Table 1. Chromatographic properties of isolated compounds.

Constituent	Rf	Spot color	
		UV-Visible	20% H_2SO_4 spray
I	0.40	Colorless	Red
II	0.45	Colorless	Pink
III	0.48	Colorless	Pink
IV	0.63	Colorless	Pink
V	0.82	Colorless	Green

Constituent characterization. The five extracted compounds were characterized by IR, UV-visable mass, NMR, and ESR spectra (Table 2).

Table 2. Analytical equipment used in compound identification.

Analytical equipment	Model	Source
Infrared spectrophotometer	Jasco FT/IR-6100	Jasco International, Tokyo, Japan
UV-Vis double beam spectrophotometer	Labomed UVD–3500	Labomed, Inc., Culver City, CA, USA
NMR spectrometer	JEOL ECA-500 MHz & 125 MHZ	JEOL, LTD, Tokyo, Japan
ESR spectrometer	Bruker ELEXSYS E500	Bruker, GmbH. Breman, Germany
Mass spectrometer	Finnigan Model 3200 at 70 eV	Finigan, Sunnyvale, CA, USA
ELISA reader	Sunrise with Magellan 4 software	TECAN, Männedorf, Switzerland

Compound I:

IR Spectrum: The IR spectrum shows the following signals, IR υ_{max} (KBr) cm^{-1}: 3423.9 (OH), 2925.2, 2854.3 both for aliphatic C-H, 1612.3 (C=C), 1421.8 $[CH_2]_n$.

Mass Spectrum: EI-MS shows the following peaks: m/z (rel. int. %): 414 (100) $[M]^+$, 399 (69), 396 (84), 381(25), 329 (32), 303 (20), 279 (56), 273 (26), 255 (30), 213 (26), 189 (41).

Compound II:

IR spectrum**:** The IR spectrum shows the following signals, IR υ_{max} (KBr) cm^{-1} : 3446 (OH), 2924.4 , 2854.4 both for aliphatic C-H, 1624.4 (C=C) and 1132.4.

Mass spectrum**:** EI-MS shows the following peaks: m/z (rel. int. %): 442 (52) $[M]^+$, 411 (8), 386 (17), 256 (25), 234 (21), 207 (19), 204 (17), 203 (100), 189 (15), 175 (41), 133 (25), 95 (20), 69 (25), 55 (29).

Compound III:

IR spectrum**:** The IR spectrum shows the following signals, IR υ_{max} (KBr) cm^{-1} : 3446.17 (OH), 2924 , 2853 both for aliphatic C-H, 1678.73 (C=O),1606.41 (C=C),1308.46 and 1054.87.

Mass spectrum**:** EI-MS shows the following peaks: m/z (rel. int. %): 456 (17) $[M]^+$, 411 (2.2), 248 (100), 233 (2.09), 207 (26.2), 203 (36.3),189 (6.6), 175 (7.7), 133 (4.4), 105 (7.8), 81 (10.4), 69 (17.6), 55 (20.3).

Proton NMR: The chemical shifts in hydrogen were determined ($CDCL_3$, 500 MHz) (Table 3).

Compound IV:

IR spectrum**:** The IR spectrum shows the following signals, IR_{max} (KBr) cm^{-1} : 3432.67 (OH), 2923.56, 2854.16 both for aliphatic C-H, 1631.48 (C=O), 1216.86 and 768.494.

Mass spectrum**:** EI-MS shows the following peaks: m/z (rel. int. %): 456 (4) $[M]^+$, 411 (5), 248 (100), 233 (4), 207 (25), 203 (63),189 (38), 175 (6), 133 (15), 105 (16), 81 (17), 69 (31), 55 (37).

Proton NMR: The chemical resonances in hydrogen were determined ($CDCL_3$, 500 MHz) (Table 3).

Compound V:

UV spectrum**:** UV λ_{max} (Chloroform)**:** 210, 285

IR spectrum**:** The IR spectrum shows the following signals, IR υ_{max} (KBr) cm^{-1}: 3444.24 (OH), 2922.59,

2853.17 both for aliphatic C-H, 1741.41, 1646.91, 1541.81, 1517.70, 1218.79 and 771.39.

Mass spectrum**:** EI-MS shows the following peaks: m/z (rel. int. %): 400 (4) $[M]^+$, 382 (5), 324 (4), 255 (12), 129 (11), 97 (17), 83 (60), 69 (100).

NMR: The chemical resonances in carbon were determined (^{1}H, 500 MHz; ^{13}C, 125 MHz) (Table 4).

Table 3. ^{1}H-NMR spectrums of compounds III and IV.

Proton No.	δ ^{1}H (ppm) (*multiplicity*, *J* in Hz)	
	Compound III	Compound IV
H-3	3.19 (1H, *dd*, *J*= 11.5, 4.5 Hz)	3.54 (1H, *dd*, *J*= 10, 5 Hz)
H-12	5.24 (1H, *t*, *J*= 3.5 Hz)	5.21 (1H, *br*. *s*)
H-18	2.83 (1H, *dd*, *J*= 14,4 Hz)	2.32 (1H, *d*, *J*= 11 Hz)
H-23	1.07 (3H, *s*)	1.25 (3H, *s*)
H-24	0.76 (3H, *s*)	0.91 (3H, *s*)
H-25	0.91 (3H, *s*)	1.05 (3H, *s*)
H-26	0.97 (3H, *s*)	1.03 (3H, *s*)
H-27	1.12 (3H, *s*)	1.27 (3H, *s*)
H-29	0.78 (3H, *s*)	1.03 (3H, *d*, *J*= 6.5 Hz)
H-30	0.86 (3H, *s*)	0.94 (3H, *d*, *J*= 6.5 Hz)

Antioxidant activity. The antioxidant activity of the total ethanol extract and successive fractions of the *M. igneum* ethanol extract were accessed by measuring the ability of each fraction to scavenge the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), a compound known to abstract labile hydrogen (Constantin et al., 1990; Matsubara et al., 1991), using electron spin resonance (ESR) spectroscopy (Calliste, et al., 2001).

A 5 x 10^{-4} M solution of DPPH (Sigma-Aldrich, Inc., St. Louis, MO, USA) was prepared along with a solution of each extract in methanol (0.5 g/mL). Ascorbic acid (El-Nasr Pham. Chem. Co., Adwic, Egypt) was used as a reference compound and a mixture of 1 mL DPPH and 1 mL of methanol was used as a control. Aliquots (1 mL) of DPPH and the chemical compound or ascorbic acid were mixed for 2 min, and the loss of absorbance measured at 523 nm.

Due to paramagnetic properties, DPPH exhibits an ESR signal. The decrease of this signal after mixing with the solution of each fraction was taken as an indication of the antioxidant activity, measured as a double integration area (DIA).

$$\% \text{ Inhibition} = \frac{[\text{DIA (DPPH)} - \text{DIA (DPPH + fraction)}] \times 100}{\text{DIA (DPPH)}}$$

Table 4. ^{1}H- and C-NMR spectrums of for compound V.

Carbon No.	δ ^{1}H (ppm) (*multiplicity*, *J* in Hz)	δ ^{13}C (ppm)
C-1		30.34
C-2		29.69
C-3	3.54 (1H, *t*, *J*= 1.5 Hz)	74.75
C-4		38.72
C-5		51.63
C-6		14.10
C-7		39.12
C-8		39.90
C-9	1.30 -2.33 (hump for protons of CH_2× 6 and -CH× 2)	57.25
C-10		37.50
C-11		18.20
C-12		34.35
C-13		130.88
C-14		132.42
C-15	1.02 (3H, *s*)	28.92
C-16	0.88 (3H, *s*)	22.98
C-17	0.89 (3H, *s*)	14.12
C-18	1.21 (3H, *s*)	22.68
C-19	1.25 (3H, *s*)	20.30
C-20	4.20 (1H, *d*, *J*= 16.5 Hz, H-20β), 4.30 (1H, *d*, *J*= 16.5 Hz, H-20α)	23.72
C-2′		167.76
C-3′		100.16
C-4′		163.20
C-5′	7.52 (1H, *d*, *J*= 11 Hz)	109.89
C-6′	7.69 (1H, *d*, *J*= 11 Hz)	128.79

Cytotoxic activity. The potential cytotoxicity of compound III, isolated from petroleum ether fraction of *M. igneum*, was tested for activity against four human tumor cell lines (HEPG2-liver, HCT116-colon, HELA-cervix, and MCF7-breast). The tumor cell lines were plated in individual 96 - multi-well plate (10^4 tumor cells /well) for 24 h before to allow attachment of the tumor cells to the well walls before treatment with the chemical compound to be tested.

The extracted chemical compounds were solubilized in DMSO, and concentrations (0, 62.5, 125 and 250 μg/mL) of the compound in DMSO were added to the cell monolayer. Triplicate wells were prepared for each individual dose. The monolayer cells were incubated with the compound under test for 48 h at 37^{o}C in a 5% CO_2 atmosphere.

At the end of the 48 h incubation, the cells were fixed with trichloroacetic acid and washed and stained with Sulfo-Rhodamine B (SRB) (Sigma-Aldrich, Inc., St. Louis, MO). Only surviving cells are colored with SRB stain (Skehan et al., 1990), and excess stain was

washed away with acetic acid. Attached stain was recovered with Tris-EDTA buffer (Sigma-Aldrich, Inc., St. Louis). The color intensity (absorbance) of the attached stain was measured in an ELISA reader at 570 nm and was directly related to a measure of the surviving tumor fraction. The relationship between the surviving fraction tumor cells and the concentration of the compound under test was plotted to get the survival curve of each tumor cell line after treatment. The inhibitory concentration, the drug dose (μg/mL) that reduces the tumor cell line to 50% survival (IC_{50}), was deduced from the survival curve. The potency was compared with reference anticancer agent Doxorubicin (Merck KGaA, Damstadt, Germany).

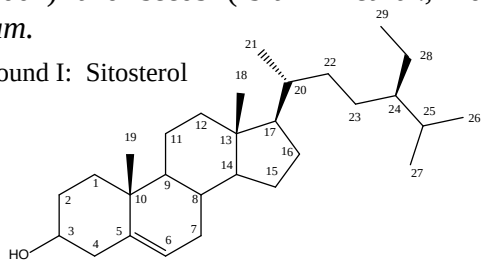
Results and Discussion

Identification. Identification of Compound I was based on several pieces of evidence. The IR spectrum showed an absorption band at 3423.9 cm^{-1} (characteristic for stretching vibration of a hydroxyl group appearing as a broad band due to loss of hydrogen bonding). Absorption bands at 2925.2 and 2854.3 cm^{-1} indicated aliphatic C-H stretching vibration. Other absorption frequencies included 1612.3 cm^{-1} indicative of an olefinic group (C=C) stretching vibration, and 1421.8 cm^{-1}, indicating a bending vibration for cyclic $[CH_2]_n$. These absorption frequencies resembled those observed for sitosterol, (Baskar et al., 2010; Pateh et al., 2009; Abd El-Razek, 2006).

The electron impact mass (EI-MS) mass spectrum for compound I showed $[M]^+$ peak at m/z 414, corresponding to the molecular formula ($C_{29}H_{50}O$) for sitosterol. The fragmentation pattern of compound was typical for sterols where the most characteristic cleavage is that of the side chain and C-15, 16, 17. Thus, characteristic fragment ions peaks were observed at m/z 399 ($[M]^+$- CH_3), 396 ($[M]^+$- H_2O), 381 ($[M]^+$- CH_3- H_2O), 329 and 303 are diagnostic for sterols having Δ^5-unsaturation, 273 ($[M]^+$- side chain), 255 ($[M]^+$-side chain-H_2O),231 ($[M]^+$-side chain-ring D), 213 (231-H_2O).

Compound I was identified as sitosterol with molecular formula of $C_{29}H_{50}O$. The spectrum of compound I agreed with that for sitosterol (Baskar et al.,2010; Pateh et al.,2009; Abd El-Razek, 2006). Sitosterol was previously isolated from the bark (Guo et al., 2007) and seeds (Ibrahim et al., 2002) of *M. igneum.*

Compound I: Sitosterol

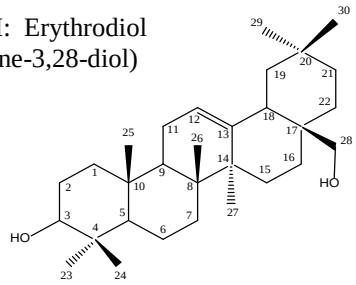


Identification of Compound II was based on the analytical evidence. The IR spectrum showed an absorption band at 3446 cm^{-1}, a characteristic of stretching vibration of a hydroxyl group appearing as a broad band due to hydrogen bonding. Absorption bands at 2924.4 and 2854.4 cm^{-1} were due to aliphatic C-H stretching vibration. An absorption frequency at 1624.4 cm^{-1} results from an olefinic group (C=C) stretching vibration. The EI-MS mass spectrum showed an $[M]^+$ peak at m/z 442, corresponding to the molecular formula ($C_{30}H_{50}O_2$) for erythrodiol.

The fragmentation pattern of compound II was typical for triterpenes with a Δ^{12}-amyrin skeleton that undergoes Retro-Diels-Alder (RDA) reaction to form fragments containing ABC*- and C*DE-rings **(**C* indicates a portion of ring C). In the RDA reaction, compound II yielded a fragment at m/z 234, characteristic for erythrodiol (Burnouf-Radosevich et al., 1985). Other fragments at m/z 203, 189, 175, 133 were typical for Δ^{12}-amyrin triterpenes with hydroxyl groups in ring A and/or B (Kwon et al., 1997; Hui and Sung, 1968). Another fragment ion appeared at m/z 411 ($[M]^+$- CH_2.OH).

Compound II was identified as erythrodiol molecular formula of $C_{30}H_{50}O_2$, a compound not previously isolated from Bignoniaceae. The spectrum agreed with that published for erythrodiol (Budzikiewicz et al., 1963; Burnouf-Radosevich *et al.*, 1985).

Compound II: Erythrodiol
(Olean-12-ene-3,28-diol)



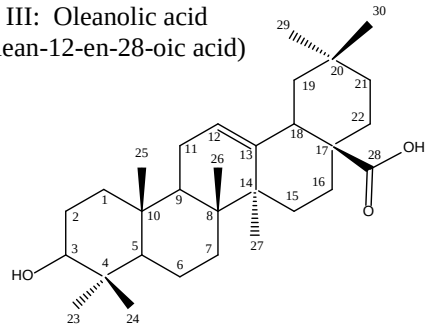
Identification of Compound III was based on analytical evidence. The IR spectrum had absorption bands 3446.17 cm^{-1}, characteristic for stretching vibration of hydroxyl group that appears as a broad band due to hydrogen bonding. Absorption at 2924 and 2853 cm^{-1} could be due to aliphatic C-H stretching vibration, while the absorption at 1678.73 cm^{-1} could be attributed to carboxylic group. The absorption band at 1606.41 cm^{-1} would be due to an olefinic group (C=C) stretching vibration. The EI-MS mass spectrum showed $[M]^+$ peak at m/z 456, corresponding to the molecular formula ($C_{30}H_{48}O_3$) for oleanolic acid.

The fragmentation pattern of compound III is typical for triterpenes with Δ^{12}-amyrin skeleton which undergo RDA reaction as did compound II**.** Oleanolic acid undergoes RDA reaction giving fragments at m/z 248 (base peak) and at m/z 207 (Burnouf-Radosevich et al., 1985)**.** Other fragments appear at m/z 233, 203, 189,175,133 characteristic for Δ^{12}-amyrin triterpenes with hydroxyl groups in ring A and/or B (Kwon et al., 1997) & (Hui and Sung, 1968). Another fragment ion appears at m/z 411 ($[M]^+$- COOH). The ^{1}H-NMR spectrum indicated an olefinic proton (H-12) at δ: 5.24 ppm as triplet signal with *J*=3.5 Hz. While the proton geminal to the hydroxyl group (H-3) is observed as doublet of doublet signal at δ: 3.19 ppm (*J*=11.5, 4.5 Hz). The methine proton (H-18) appears as doublet of doublet at δ: 2.83 ppm (*J*=14, 4 Hz). In addition, seven methyl functionalities attached to saturated carbons appear as singlets at δ: 0.76-1.12 ppm.

The data are consistent with compound III being oleanolic acid with the molecular formula $C_{30}H_{48}O_3$. Comparison of the current data with published data (Abd El-Razek, 2006; Kwon et al., 1997) confirms the identification. While oleanolic acid has not previously been reported as a constituent of *M. igneum*, the compound has been isolated from other species in the Bignoniaceae family, including from leaves of *Campsis grandiflora* (Jin et al., 2004), stem and root bark of *Tabebuia pentaphylla* (Prakash and Singh, 1980), and from various tissues of other species *Newbouldia laevis*, *Markhamia acuminata*, *Spathodea campanulata* (Gormann et al., 2004).

Ursolic acid and oleanolic acid both belong to pentacyclic triterpenoid acids and have similar molecular structure, differing only at the sites of the methyl group on the E ring (methyl group at C_{19} is ursolic acid, at C_{20} is oleanolic acid). Ursolic acid is widely distributed in Bignoniaceae. In Korean traditional medicine, ursolic acid has been used in anti-tumor therapy. Recent tests in and outside China indicate ursolic acid has definitive antitumor activity by various metabolic routes (Hollosy et al., 2000). In the present study, cytotoxic activity of oleanolic acid was noted on four cancer cell lines mentioned earlier.

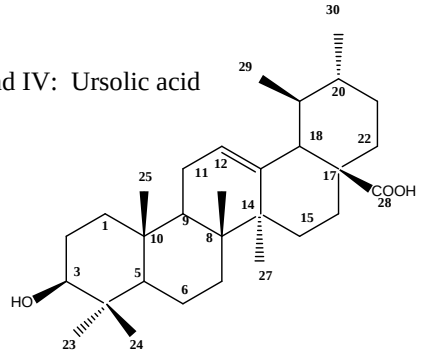
Compound III: Oleanolic acid
(3*β*-Hydroxyolean-12-en-28-oic acid)



Identification of Compound IV was based on the following analytical evidence. The IR spectrum showed the absorption band at 3432.67 cm^{-1} that is characteristic for stretching vibration of hydroxyl group, appearing as broad band due to hydrogen bonding. Absorption at 2923.56 and 2854.16 cm^{-1} would be due to aliphatic C-H stretching vibration. The absorption frequency at 1631.48 could be attributed to a carboxylic group. The EI-MS mass spectrum showed $[M]^+$ peak at m/z 456, corresponding to the molecular formula ($C_{30}H_{48}O_3$) for ursolic acid. The fragmentation pattern of compound IV was typical for triterpenes with Δ^{12}-amyrin skeleton which undergo RDA reaction**.** Ursolic acid undergoes RDA reaction giving fragments at m/z 248 (base peak) and at m/z 207 (Burnouf-Radosevich et al., 1985). Other fragments appeared at m/z 233, 203, 189, 175, and 133, characteristic for Δ^{12}-amyrin triterpenes with hydroxyl groups in ring A and/or B, (Kwon et al., 1997; Hui and Sung, 1968). Another fragment ion appears at m/z 411 ($[M]^+$- COOH). The ^{1}H-NMR

spectrum shows an olefinic proton (H-12) at δ: 5.21 ppm as broad singlet signal. While the proton geminal to the hydroxyl group (H-3) is observed as doublet of doublet signal at δ: 3.54 ppm (*J*=10, 5 Hz). The methine proton (H-18) appears as doublet at δ: 2.32 ppm (*J*=11 Hz). Also, five tertiary methyl functionalities appear as singlets at δ: 0.91, 1.03, 1.05, 1.25, 1.27 ppm. While the signals at δ: 0.94 (3H, *d*, *J*= 6.5 Hz) and 1.03 (3H, *d*, *J*= 6.5 Hz) were indicative of a ursane skeleton.

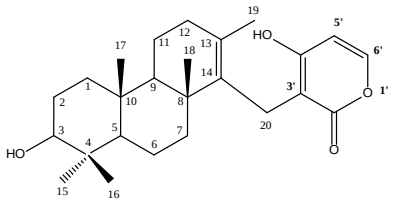
Compound IV: Ursolic acid



Compound V, isolated as colourless amorphous powder, was determined to have the molecular formula ($C_{25}H_{36}O_4$) from $[M]^+$ peak at m/z 400 in the EI-MS mass spectrum. The fragmentation pattern of compound V indicated the presence of a lactone ring due to the presence of base peak at m/z 69 corresponding to (C_3HO_2). IR spectrum showed absorption bands for hydroxyl group (3444.24 cm^{-1}) and an unsaturated lactone ring (1741.41, 1646.91 cm^{-1}) which was confirmed by UV absorption at λ_{max} 210. UV absorption at λ_{max} 285 indicated the presence of pyran-2-one moiety

The ^{1}H-NMR spectrum showed five methyl singlets at δ: 0.88, 0.89, 1.02, 1.21, 1.25 (each 3H, *s*). Two doublets were observed at δ: 7.52 and 7.69 ppm and assigned for lactone ring protons (H-5′ and H-6′, respectively). A hump that appeared at δ: 1.30-2.33 ppm is characteristic for CH and CH_2 protons in the phenanthrene cycloaliphatic ring of terpenoids. ^{13}C-NMR signals at δ: 167.76, 163.20, 132.42, 130.88, 128.79, 109.89 and 100.16 ppm suggested the presence of an unsaturated lactone carbonyl and three double bonds. Thus, compound V was identified as 5', 6' norigeumone. Earlier, Guo et al., (2007) had isolated the meroditerpenoid, igeumone from an ethanolic extract of the bark of *M. igneum*.

Compound V: 5',6' norigeumone



Antioxidant activity. In a preliminary screening, the total ethanol extract of *M. igneum* demonstrated significant antioxidant activity, giving 77.02% inhibition in comparison with ascorbic acid (100%) (Table 5). The strongest inhibition of DPPH stable free radicals was exhibited by the successive ethanol fraction that gave 100% free radical scavenging activity relative to ascorbic acid followed by ethyl acetate fraction giving 64.96% and the petroleum ether and chloroform fractions showing 12.46% and 0% inhibition, respectively.

Table 5. Antioxidant activity of *M. igneum* extract fractions.

Extract fraction[1]	Concentration (mg/mL MeOH)	Double integration area (DIA)	Inhibition (%)
DPPH	0.197	502809	---
Total ethanol	0.5	115503	77.02
Petroleum ether	0.5	440123	12.46
Chloroform	0.5	502809	0
Ethyl acetate	0.5	176156	64.96
Ethanol	0.5	0	100
Ascorbic acid	0.5	0	100

[1]DPPH and ascorbic acid are controls.

Cytotoxic activity of oleanolic acid. The cytotoxicity of oleanolic acid isolated from the petroleum ether extract of *M. igneum* leaves against human cancer cell lines was based on criteria of the National Cancer Institute (NCI) in which a pure compound has strong activity if the IC_{50} is <30μg/mL. In the current study, oleanolic acid showed moderate cytotoxic activity against the cancer cell lines (liver, cervix, breast and colon) tested (Table 6). The highest activity was against cervix cancer cell line (HELA) (IC_{50} = 32 μg/mL), followed by liver cancer

cell line (HEPG2) (IC_{50} = 33 µg/mL), colon cancer cell line (HCT-116) (IC_{50} = 35 µg/mL) and then breast cancer cell line (MCF7) (IC_{50} = 37 µg/mL). The cytotoxic activity of the petroleum ether fraction was higher than that of oleanolic acid, which implies that petroleum ether fraction contains other effective ingredients that work synergistically with oleanolic acid.

Table 6. Cytotoxicity of oleanolic acid.

Cancer cell line	Concentration (µg/mL)	Surviving fraction	IC_{50}[1] oleanolic acid (µg/mL)
HEPG2 (Liver)	0.00	1.000	33
	62.50	0.075	
	125.00	0.078	
	250.00	0.118	
HELA (Cervix)	0.00	1.000	32
	62.50	0.047	
	125.00	0.093	
	250.00	0.113	
MCF7 (Breast)	0.00	1.000	37
	62.50	0.128	
	125.00	0.153	
	250.00	0.154	
HCT-116 (Colon)	0.00	1.000	35
	62.50	0.121	
	125.00	0.186	
	250.00	0.065	

[1]IC_{50} is the drug dose that reduces survival to 50%.

The mechanism of action reported for cytotoxic activity of oleanolic acid apparently includes inhibition of tumorigenesis, inhibition of tumor promotion, and inhibition of tumor cell differentiation. Oleanolic acid effectively inhibits angiogenesis, invasion of tumor cells, and metastasis (Ovesna et al., 2004). Stimulation of NO secretion and TNF-α release are able to upregulate iNOS and TNF-α expression, which may be the mechanism for the anti-tumor effect (Choi et al., 2001).

Triterpenes that possess free carboxylic group at C-28, exhibit moderate to considerable cytotoxic activity against tumor lines, whereas their sugar-bonded esters are inactive. In addition, differences in cytotoxicity between oleanane and lupane triterpenes have been observed with the cytotoxicity of the lupane-type generally much weaker than those of the oleanane-type (Bang et al., 2005).

Conclusion

The phytochemical investigations of petroleum ether extract of *M.* igneum in this study focused on the phytoconstituents that may have contributed to cytotoxic activity observed in a previous study (Hashem et al., 2011). The four terpenes and one sterol isolated from a petroleum ether extract of *M. igneum* proved to contain a number of terpenoids, similar to other plants in the Bignoniaceae family.

Compound V, identified as 5',6' norigeumone, had a chemical structure similar to igeumone, a meroterpenoid isolated from an ethanolic extract of *M. igneum* bark (Guo et al., 2007). The biological activity of terpenoids is interesting, especially as related to cytotoxic activity. For this reason, cytotoxic activity of oleanolic acid isolated from the petroleum ether extract of *M. igneum* leaves was studied against four human cancer cell lines.

As a natural occurring compound, oleanolic acid is present in leaves, roots, and barks of several plants and has been associated with a number of pharmaceutical activities (Fai and Tau, 2009). In our study, the oleanolic acid demonstrated moderate cytotoxic activity against the four cancer cell lines (liver, cervix, breast and colon). The first indications of oleanolic acid and ursolic acid having antitumor activity have been reviewed by Liu (1995). El-Hossary et al. (2000) demonstrated ursolic acid had cytotoxic activity in vitro. Unfortunately, cytotoxic activity could not be done for the other isolated compounds in the current study as the small amounts available were used for compound identification, leaving no material for any other studies.

Biological screening of *M. igneum* leaf extracts revealed strong antioxidant activity in an ethanol extract (100% free radical scavenging activity equivalent to ascorbic acid). Successive extracts of the original ethanol extract. The ethyl acetate extract also exhibited antioxidant activity. These observations demonstrated that the family Bignoniaceae has significant antioxidant activity. Phytochemical investigations on these phytoactive, antioxidant extracts are being continued by us in exploration of the active phytoconstituents of *M. igneum*.

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