Growth inhibition and induction of apoptosis in human cancerous HeLa cells by *Maytenus procumbens*

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Abstract

The possible biochemical activities of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens* (L.M.P), and its isolated compounds were investigated in the present study. In cytotoxicity assay, L.M.P showed IC_{50} of 68.79, 51.22, 78.49, 76.59, and 76.64 µg/ml on Caco-2, HeLa, HT29, NIH3T3, and T47D cells, respectively. Bioassay guided fractionation led to the isolation and identification of a new triterpene: '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) in addition to a known terpenoid: 'asiatic acid' (AA). HMO exhibited the most cytotoxicity against HeLa cells and was further investigated for its ability to induce apoptosis in HeLa cells. HMO induced apoptosis up to 20.41% in HeLa cells versus control group (0.40%). Antioxidant/oxidative properties of L.M.P and HMO were investigated using extracellular (DPPH), and intracellular (ROS) assays. Experimental samples represented a time and

concentration-dependent formation of ROS in Hela cells. Generation of ROS seems one of the mechanisms by which HMO induces apoptosis in Hela cells. Conclusion is that the active components in L.M.P might serve as a mediator of the ROS scavenging system and have the potential to act as prooxidant or antioxidant depending on the biological environment of the cells.

Keywords: *Maytenus procumbens*; Cytotoxicity; Apoptosis; Oxidative stress

Highlights

- *Maytenus procumbens* (L.M.P) showed the highest cytotoxicity on HeLa cells.
- A new triterpene: '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) was isolated.
- HMO caused DNA damage and appeared genotoxic to HeLa cells.
- HMO induced apoptosis in HeLa cells through ROS generation.

***Abbreviations**: (alphabetically)*

Annexin V-FITC, Annexin V-fluorescein isothiocyanate; AA, asiatic acid; ^{13}CNMR, carbon nuclear magnetic resonance; DCFH-DA, 2,7-dichlorofluorescin diacetate; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulphoxide; DPPH,1,2-diphenyl-2-picrylhydrazyl; FBS, fetal bovine serum; FRAP, ferric-reducing antioxidant power; HMO, 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one; ^{1}HNMR, proton nuclear magnetic resonance; L.M.P, leaves of *Maytenus procumbens*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NMR, nuclear magnetic resonance; PI, propidium iodide; RSC, radical scavenging capacity; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substance; TLC, thin layer chromatography.

***Abbreviations**:* leaves of *Maytenus procumbens;* L.M.P, 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one; HMO, asiatic acid; AA, thin layer chromatography; TLC, nuclear magnetic resonance; NMR, proton nuclear magnetic resonance; ^{1}HNMR, carbon nuclear magnetic resonance; ^{13}CNMR, fetal bovine serum; FBS, Dulbecco's Modified Eagle's Medium; DMEM, dimethyl sulphoxide; DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide;

MTT, Annexin V-fluorescein isothiocyanate; Annexin V-FITC, propidium iodide; PI, radical scavenging capacity; RSC, 1,2-diphenyl-2-picrylhydrazyl; DPPH, ferric-reducing antioxidant power; FRAP, thiobarbituric acid reactive substance; TBARS, reactive oxygen species; ROS, 2,7-dichlorofluorescin diacetate; DCFH-DA.

Introduction

The genus *Maytenus* belongs to Celastraceae family which is indigenous to tropical and subtropical regions of the world, including North Africa, South America and East Asia. The family constitutes approximately about 90-100 genera and 1300 species. The great majority of the genera are tropical while only *Celastrus* (the staff vines), *Euonymus* (the spindles) and *Maytenus* are widespread in temperate climates. The member of genus *Maytenus* is distributed throughout Central and South America, Southeast Asia, Micronesia and Australasia, the Indian Ocean and Africa. They grow in a very wide variety of climates, from tropical to subpolar. Celastraceae generally grows as small trees, bushes, or lianas and has resinous stems and leaves (Spivey et al., 2002).

The variety of bioactivities reported in literature of the Celastraceae family in traditional medicine and agriculture is astonishing, which includes stimulant, appetite suppressive, sedative, emetic, purgative, memory restorative, fertility-regulating agent, male contraceptive, antitumour, anti-leukemic, antibacterial, insecticidal, and insect repellent activities (Costa et al., 2008; Ghazanfar, 1994; Spivey et al., 2002; Souza-Formigonia et al., 1991). Cytotoxicity and antitumor activities of *Maytenus ilicifolia, M. ovatus, M. cuzcoina, M. serrate*, *M. diversifolia, M*. *Molina*, *M. rigidi*and, *M. emarginata* have been reported in literature earlier (Cragg and Newman, 2005; Gonzalez et al., 2000; Hong, 2000; Hui et al., 2009; Lee et al., 1982; Martucciello et al., 2010; Spivey et al., 2002). *M. heterophylla* and *M. senegalensis* are used to treat respiratory ailments and inflammation (da Silva et al., 2011). The *in vivo* antiplasmodial (Muregi et al., 2007) and *in vitro* antileishmanial activities (Matu and van Staden, 2003) of different species of the genus *Maytenus* have been reported previously. Antimicrobial activities of various *Maytenus ss*p. have

been previously found (Avilla et al., 2000; da Silva et al., 2011; Kloucek et al., 2005; Lindsey et al., 2006).

Different plant species belonging to the genus *Maytenus* are extensively investigated for bioactive compounds as they are widely used in folk medicine as antiseptic, antiasthmatic, fertility-regulating agent, antitumor, as well as for stomach problems (Ghazanfar, 1994). Diverse types of secondary metabolites, including triterpenes (Shirota et al., 1996), oligo-nicotinate sesquiterpenes and sesquiterpene pyridine alkaloids (Corsino et al., 1998), phenolic glucosides (Sannomiya et al., 1998) and agarofurans (Gonzalez et al., 1993) with an interesting spectrum of biological activities have been found in plants belonging to the genus *Maytenus*. In addition to numerous terpenoids particularly sesquiterpenoids, various bioactive phenylalkylamines, maytansinoids, and flavonoids have been isolated. However, the bulk of the bioactive constituents of the Celastraceae are terpenoids. Different types of terpenoids are found in the extracts of *Maytenus ssp.* (Cordeiro et al., 1999; Gonzaleza et al., 2001; Gutierrez et al., 2007; Leite et al., 2001; Ohsaki et al., 2004; Queiroga et al., 2000; Spivey et al., 2002).

Maytenus procumbens (L.f.) Loes. is an indigenous native South African species, also known as 'Dune Koko tree' (*duinekokoboom* in Afrikaans) which characterizes a scrambling shrub or small tree. *M. procumbens* appears as a densely bushy plant with drooping branches that sometimes reach more than 6 m. Its bark is pale yellowish brown, which sometimes become fissured on old trees. The clusters of white to greenish-white flowers appear in winter and are replaced by spherical fruits containing bright orange seeds. *M. procumbens* occurs in south and east coast of South Africa in dune scrub and wooded areas up to an altitude about 150 m (Coates Palgrave, 2002). Due to the variety of biological activities of the genus most importantly being anti-cancer activity, we decided to focus our investigation on cytotoxic activity of *M. procumbens*. In the present study, the chemo-preventative (anticancer and cancer preventative) activity of *M. procumbens* has been determined. Meanwhile, the bioactive principles of the extract and the mechanism of action of selected samples were identified.

2. Materials and methods

2.1. Collection, identification and extraction of plant materials

The leaves of *M. procumbens* were collected from the Botanical Garden of the University of Pretoria during May 2007. The plant was identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria. Herbarium voucher specimens are stored in the herbarium of the University of Pretoria (PRU 094096). Seven hundred grams of the leaves of *M. procumbens* (shade dried) were ground to a soft powder (Junke & Kunkel grinder). *M. procumbens* leaves were exhaustively extracted with appropriate amount of acetone/ethanol (1:1) with constant stirring for 24 hours at 40ºC (three times). Subsequently, the total extract was filtered and concentrated under reduced pressure with a rotary evaporator (BUCHI, Rotavapor, R-200) to yield 55.0 g (L.M.P) of dry crude extract.

2.2. Isolation of bioactive compounds using bioassay-guided fractionation

A total of 55.0 g L.M.P was applied to a silica gel column chromatography (CC, size 10×80 cm). The column (**Ma**) was eluted with a solvent system of hexane/ethyl acetate in order of increasing polarity (100:0 to 0:100). The fractionation was continued with ethyl acetate/methanol (100:0 to 0:100). A total of 65 fractions were collected and pooled based on their thin layer chromatography (TLC) profiles (26 subfractions). Based on the cytotoxicity results, the subfractions 34-38**Ma** and 44-46**Ma** were selected for subsequent chromatographic purification. Combined subfractions of 34-38**Ma** (1198.0 mg) were subjected to a silica gel column (CC, size 5×60 cm) (**Mb**) eluted with hexane/ethyl acetate in order of (8:2 to 1:1). According to TLC profiles, subfractions of 16-37**Mb** (808.0 mg) were combined and subjected to different column chromatography to yield a white pure compound (HMO, 20.0 mg; 0.036%). Subfractions of 44-46**Ma** (634.0 mg) were applied on a Sepahdex column chromatography (LH-20, Sigma-Aldrich, South Africa) (**Mc**) using dichloromethane (100.0). Collected subfractions 17-25**Mc** were combined according to TLC profiles and a semi-pure powder precipitated. Thereafter, the powder was cleaned up with dichloromethane resulted in a pure compound (AA, 12.0 mg; 0.021%).

2.3. Identification of isolated compounds

The structural elucidations of the isolated compounds were identified by physical (mp. $[\alpha]_D$), nuclear magnetic resonance (NMR) and mass spectrometry. Proton (^{1}H NMR), carbon (^{13}C NMR) and (2D-NMR) spectra were compared with those already reported in literature (Ablise et al., 2004; Bruno et al., 1987; Duddeck et al., 1978; Johns et al., 1983; Muhammad et al., 2000; Shibata et al., 1987).

2.4. Cell culture

Cell lines of human cervical carcinoma (HeLa), human colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), and breast ductal carcinoma (T47D) were maintained as exponentially growing cultures in RPMI 1640 cell culture medium (PAA, Germany), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), for HT-29 cells and 15% FBS for Caco-2 and T47D cells. The Swiss mouse embryo fibroblast (NIH 3T3, non cancerous) cell line was kept in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Germany), supplemented with 10% FBS. 100 IU/ml penicillin and 100 µg/ml streptomycin (Roche, Germany) were added to the media. All cell lines were grown and maintained in a humidified atmosphere at 37°C and 5% CO_2.

2.5. *In vitro* cytotoxicity assay

Cytotoxicity was measured by the MTT method as described by Mosmann (1983) with some modifications. Briefly, cells (100 µl) were seeded (concentration $1x10^4$ cells/ml) into a microtitre plate and incubated for 24 h to allow the cells to attach. Samples were diluted (6.252-400 µg/ml for L.M.P, and 5-100 µg/ml for pure compound), added to the plates and incubated. The positive drug controls; methotrexate and cisplatin (final concentration of 100 µg/ml) were also included. Cells with no treatment were examined as negative control. After 72 hours of incubation for HT-29, HeLa and NIH 3T3 cells, 96 hours for T47D and Caco-2 cells, the plates were allowed to proliferate and grow. After appropriate incubation time for each cell line, the culture medium was removed without disturbing the cells. MTT was added at a final concentration of 5 mg/ml and incubated for 3-4 hours. Afterwards, the medium was removed and replaced with 100 µl of DMSO (dimethyl sulphoxide, culture grade) for each well. Finally, the plates were placed on a shaker for 20 minutes to dissolve formazan crystals. Absorbance of the developed color was

spectrophotometrically quantified using a Synergy4 BIOTEK multi-well plate reader (BIOTECK, USA), which measured the optical density at 570 nm with a reference wavelength of 690 nm. The samples were tested in triplicate. The inhibitory concentration of 50% of the cell population (IC_{50} values) were defined as the concentration of the sample at which absorbance was reduced by 50%.

2.6. Determination of induced apoptosis in HeLa cells by Flow Cytometry

Induction of apoptosis was detected by flow cytometry using Annexin-V-FITC and PI staining. HeLa cells (5×10^5) were seeded in each well of a 6 well culture plate which were incubated for 24 hours and exposed to HMO at its IC_{50} concentration (27.61 µg/ml). Cells without any treatment (only treated with RPMI) were considered as control group (4 wells). After the required incubation period, cells were trypsinized, centrifuged and washed with PBS. Cells were subsequently incubated for 10 minutes at 4^oC in dark with PI/AnV-FITC fluorescent dyes. Results were determined using Partec flow cytometer equipped with Argon laser. In total, 10000 cells were measured for fluorescent intensity in FL1 (FITC) and FL2 (PI) for each assay and were repeated three times. Living cells were Annexin-V-FITC and PI double negative (phase Q3), while late apoptotic and necrotic cells were double positive (phase Q2). Early apoptotic cells were only Annexin-V-FITC positive but early necrotic cells were only PI positive when quadrant analysis of collected data for FL1 vs. FL2 was utilized (Phase Q4). The X (FL-1) axis, represents the logarithm of Annexin V-FITC and Y (FL-2) represents the fluorescent of PI. FITC with blue light is excited at 493 nm and emits green fluorescence at 525 nm. PI with blue-green light is excited at 305-540 nm and emits red light at 620 nm.

2.7. Determination of genotoxicity in HeLa cells by comet assay

The alkaline comet assay for assessment of DNA damage was performed according to the method of Singh et al. (1988) with minor modifications. Exponentially growing HeLa cells were seeded at 5×10^5 cells per well of a culture plate (6 well). Cells were exposed to HMO at its IC_{50} concentration (27.61 µg/ml) and incubated for 72 h. The cells viability was assessed using trypan blue dye-exclusion staining. Afterward, the mixture of cells (2.4×10^5 cells/ml) and 0.5% LMP (Low Melting Point) agarose was added to the slides precoated with 1% NMP (Normal melting

point) agarose. The slides were immersed in lyses buffer (consisting of 2.5 M NaCl; 100 mM EDTA; 1% Triton X-100; 10 mM Tris-HCl; and pH 7.5) for 1 h at 4°C, followed by an alkaline solution (consisting of 300 mM NaOH, 1 mM EDTA, pH> 13) for 40 min at 4°C. The slides were kept at 4 ºC for 20 min at 300 mA in electrophoresis chamber. The slides were then neutralized with 0.4 M Tris-HCl, pH 7.5, stained with 10 µg/ml ethidium bromide and covered with cover slips. To prevent additional DNA damage, all the steps described above were conducted in the dark. The results were examined at 20X and 40X magnification by a fluorescence microscope (Olympus IX71) and 200 images were randomly analyzed with comet assay software (Casp software). The cells were evaluated with an image analysis system (CASP Comet assay Software Project; Comet Assay IV; Perceptive Instruments, United Kingdom). The results were expressed in terms of the percentage of DNA presented in the tail. Data from at least 100 cells were analyzed per sample with usually 3 slides per point.

2.8. Measurement of radical scavenging capacity (RSC)

The method of du Toit et al. (2001) was followed with some modifications. The radical scavenging capacities of the samples were determined by using a Synergy4 BIOTEK multi-well plate reader (BIOTEK, USA) after 15 and 30 minutes at 550 nm. The antioxidant activity of samples was reported as the percent inhibition of DPPH activity.

2.9. Preparation of cells for ferric-reducing antioxidant power (FRAP) and lipid peroxidation thiobarbituric acid reactive substance (TBARS) assays

HeLa cells (1×10^6) were seeded in 25-cm^2 cell culture flasks, until nearly confluent. After an overnight incubation, L.M.P (concentrations ranging from 12.5-400 µg/ml) and HMO (concentrations of 5-100 µg/ml) were added to the cells. The plates were incubated for 48 hours. Thereafter, the cells were trypisinized and centrifuged at 2000 rpm (Hettich, Germany) for five minutes and were resuspended in PBS, twice. The pellets were used for FRAP and TBARS.

2.10. Ferric-reducing antioxidant power assay

The procedure described by Benzie and Strain (1996) was followed with modifications. To report FRAP content; data were normalized by dividing the FRAP value on HeLa cells survival in related concentrations of samples.

2.11. Thiobarbituric acid reactive assay

Assay of TBARS is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The method has been set up in our lab and is used routinely. The details have been stated in our previous paper Sarkhail et al. (2007).

2.12. Measurement of intracellular reactive oxygen species

The theory behind using DCFH-DA is that nonfluorescent fluorescin derivatives will emit fluorescence after being oxidized by hydrogen peroxide. The emitted fluorescence is directly proportional to the concentration of hydrogen peroxide. When applied to intact cells, the nonionic, nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall ROS in cells.

HeLa cells (1×10^4) were cultured in 96-well clear bottom black walled fluorescent cell culture plates (IWAKI, Japan). After appropriate incubation time, the cells were exposed to different concentrations of samples (12.5-400 μg/ml for L.M.P and 3.1-100 μg/ml for pure compound). An hour later, medium was removed and cells were washed with Ca^{2+}, Mg^{2+} free HBSS (Hank's balanced salt solution) (Life Technologies, Inc.) twice. Thereafter, the cells were incubated with HBSS containing 10 μg/ml of DCFH-DA (2,7-dichlorofluorescin diacetate) for 15 minutes at 37°C. The fluorescence intensity of DCF was measured at 530 nm emission wavelength, after excitation at 480 nm, at 10-minute intervals, for up to 90 minutes using a Synergy4 BIOTEK multi-well plate reader (BIOTEK, USA). An increase in fluorescence (DCF) intensity was used to represent the generation of net intracellular ROS. The ROS levels were calculated by dividing the fluorescent intensity on the cells survival in related concentrations of samples. Non-treated

cells were used as negative control versus H_2O_2 (concentrations of 125-2000 mM) as positive control (Momtaz et al., 2010; Sun et al., 1999; Wang and Joseph, 1999).

2.13. Statistical Analysis

All data were expressed as mean±S.D. Statistical analysis was performed with one-way ANOVA followed by *Tukey posthoc test* for multiple comparisons. $P< 0.05$ was considered significant.

3. Results

Genus *Maytenus* has been studied intensively in different countries while astonishingly; literature reviews demonstrated a gap of biological index about *M. procumbens*. For clarification, we investigated new possible biological activities from these species with a particular emphasis on their antiproliferative properties.

3.1. Identification of compounds from L.M.P

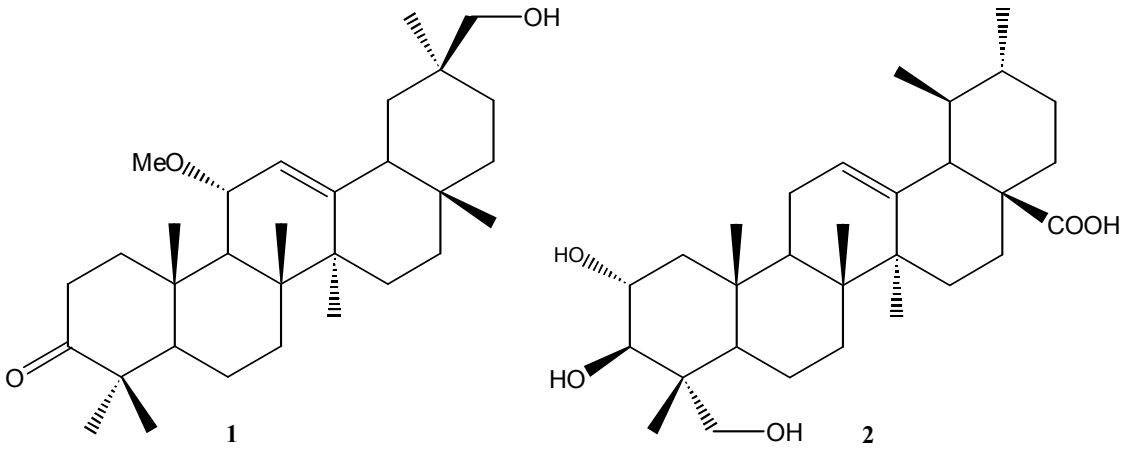


Figure 1. Chemical structures of the compounds isolated from the acetonic/ethanolic extract of the leaves of L.M.P: 1) '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) and 2) 'asiatic acid' (AA).

The biological activity guided fractionation of L.M.P which led to the purification of two triterpenes. A new compound had not been isolated before and was identified as; '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) (20.0 mg; 0.036%). The second pure compound

(AA) (12.0 mg; 0.021%) was known and identified as; 'asiatic acid'. The chemical structures of the isolated compounds are presented in **Fig. 1**.

3.1.1. Spectroscopic analysis of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO)

HMO was isolated as amorphous colorless powder, IR (KBr disc): 3420, 2950, 2925, 2870, 1727, 1702, 1464, and 1385 cm^{-1}; $[\alpha]_{589.3}^{25}$ +33.55 (*c* 0.21, $CHCl_3$). The HRFABMS of HMO displayed a pseudomolecular ion peak at m/z 493.3658 [M + Na]+, suggesting the molecular formula $C_{31}H_{50}O_3$ and eight degrees of unsaturation. ^1H NMR (500 MHz, $CDCl_3$) showed signals at δ_H 5.36 (1H, d, $J_{12,11\beta}$ = 3.1 Hz; H-12), 3.91 (1H, dd, $J_{11\beta,9\alpha}$ = 9.6 Hz, $J_{11\beta,12}$ = 3.1 Hz; H-11β), 3.59 (1H, d, $J_{30A,30B}$ = 11.0 Hz; H_A-30), 3.49 (1H, d, $J_{30B,30A}$ = 11.0 Hz; H_B-30), 3.24 (3H, s; OMe), 2.54 (1H, ddd, $J_{2\beta,2\alpha}$ = 15.9 Hz, $J_{2\beta,1\alpha}$ = 10.8 Hz, $J_{2\beta,1\alpha}$ = 7.5 Hz; H-2β), 2.39 (1H, ddd, $J_{2\alpha,2\beta}$ = 15.9 Hz, $J_{2\alpha,1\alpha}$ = 7.4 Hz, $J_{2\alpha,1\beta}$ = 3.9 Hz; H-2α), 2.29 (1H, ddd, $J_{1\beta,1\alpha}$ = 14.0 Hz, $J_{1\beta,2\alpha}$ = 3.9 Hz, $J_{1\beta,2\beta}$ = 7.5 Hz; H-1β), 2.04 (1H, td, $J_{16\alpha,16\beta} = J_{16\alpha,15\beta}$ = 13.5 Hz, $J_{16\alpha,15\alpha}$ = 4.4 Hz; H-16α), 1.96 (1H, br dd, $J_{18\beta,19\alpha}$ = 13.9 Hz, $J_{18\beta,19\beta}$ = 4.9 Hz; H-18β), 1.80 (1H, d, $J_{9\alpha,11\beta}$ = 9.6 Hz; H-9α), 1.24 (3H, s; Me-27), 1.15 (3H, s; Me-25), 1.11 (3H, s; Me-23), 1.07 (3H, s; Me-24), 1.05 (3H, s; Me-26), 0.91 (3H, s; Me-29), 0.84 (3H, s; Me-28); the remaining protons appeared as overlapped signals. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) showed signals at δ_C 40.3 (CH_2, C-1), 34.4 (CH_2, C-2), 218.0 (qC, C-3), 47.7 (qC, C-4), 55.5 (CH, C-5), 19.7 (CH_2, C-6), 32.9 (CH_2, C-7), 42.9 (qC, C-8), 50.4 (CH, C-9), 37.7 (qC, C-10), 76.3 (CH, C-11), 122.1 (CH, C-12), 148.7 (qC, C-13), 42.0 (qC, C-14), 26.1 (CH_2, C-15), 27.0 (CH_2, C-16), 32.4 (qC, C-17), 46.6 (CH, C-18), 41.6 (CH_2, C-19), 35.5 (qC, C-20), 29.5 (CH_2, C-21), 36.3 (CH_2, C-22), 26.7 (CH_3, C-23), 21.5 (CH_3, C-24), 16.4 (CH_3, C-25), 18.1 (CH_3, C-26), 25.2 (CH_3, C-27), 28.3 (CH_3, C-28), 27.4 (CH_3, C-29), 66.6 (CH_2, C-30), 53.9 (CH_3, O*C*H_3).

The ^1H and ^{13}C NMR spectra of HMO were consistent with an olean-12-ene (Chen et al., 1983; Nick et al., 1994; Nick et al., 1995). The ^{13}C NMR spectrum of HMO demonstrated the presence of a trisubstituted double bond (δ_C 122.1, 148.7), a ketone group (218.0), a hydroxylated methylene group (66.6), a methoxy group (53.9), and seven methyl groups (21.5, 26.7, 28.3, 27.4, 16.4, 18.1, 25.2) consistent with a 3-oxo-12-oleanene-30-ol carbon skeleton (Chen et al., 1983; Nick et al., 1994, 1995). The oxygenated doublet of doublets at δ 3.91, which correlated to the methine carbon at δ_C 76.6 in the HSQC spectrum, was assigned to H-11. This was based on

the observed COSY coupling with the olefinic proton doublet resonating at δ_H 5.36 (H-12) and the proton doublet absorbing at δ_H 1.80 (H-9). The orientation of H-11 was suggested by the high $J_{9,11}$ value (9.6 Hz), indicating diaxial coupling, as well as by NOESY, other spectroscopic data HSQC, HMBC and NOESY.

3.2. Cell viability

M. procumbens (L.M.P) exhibited the highest inhibition of cells growth with the IC_{50} value of 51.22 µg/ml in HeLa cells. The reduction of cell proliferation was followed in Caco-2, T47D and HT29 that represented the IC_{50} values of 68.79, 76.64, and 78.49 µg/ml. This extract showed cytotoxicity against non-tumor NIH3T3 cells with an IC_{50} of 76.59 µg/ml. HMO demonstrated the maximum cytotoxicity on HeLa and Caco-2 cells with IC_{50} values of 27.61 and 42.71 µg/ml respectively followed by T47D and HT29 (30.59 and 61.37 µg/ml). This compound appeared to be toxic to NIH3T3 cells with IC_{50} of 45.00 µg/ml. Methotrexate and cisplatin (positive controls) were significantly toxic to all cell lines tested in the experiment (**Table 1**).

Table 1. IC_{50} values (µg/ml) of L.M.P and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) against cancer and normal cells.

	IC_{50} (µg/ml)				
Cell lines	**Caco-2**	**HeLa**	**HT29**	**T47D**	**NIH3T3**
Samples					
L.M.P[a]	68.796±0.012	51.228±0.013	78.491±0.011	76.643±0.003	76.599±0.006
HMO[b]	42.712±0.005	27.613±0.022	61.375±0.003	30.593±0.004	45.977±0.001
Methotrexate[c]	0.23±0.02	0.071±0.8	0.23±0.02	0.16±0.09	0.24±0.013
Cisplatin[c]	3.869±0.245	2.820±0.320	11.430±0.682	16.891±1.533	6.751±0.38

Results are expressed as mean±SD.
[a] *M. procumbens* (acetone/ethanol extract of leaves).
[b] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one.
[c] positive control.

3.3. Apoptosis detection analysis by Flow Cytometry

The ability of HMO to induce apoptosis in HeLa cells at the concentration of its IC_{50} value (27.61 µg/ml) was assessed using flow cytometric method. Necrotic cells appeared in quadrant 1

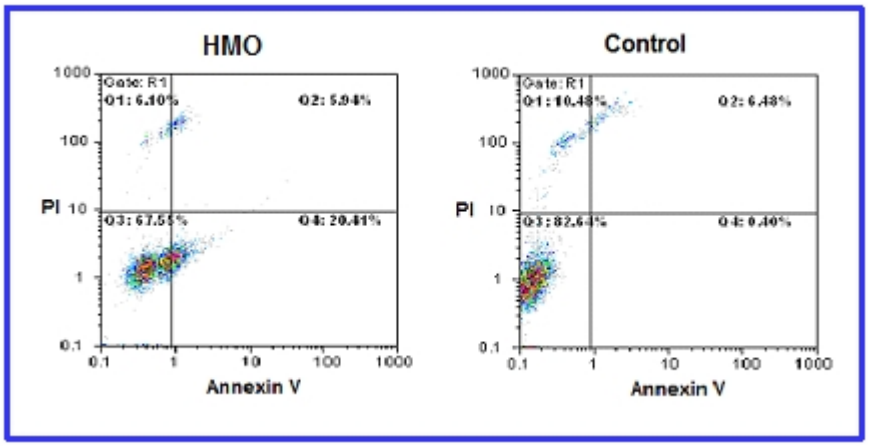
(Q1) (Annexin $^{-}$, PI $^{+}$), Q2 shows post-apoptosis cells (Annexin $^{+}$, PI $^{+}$), Q3 expresses alive cells (Annexin $^{-}$, PI $^{-}$), and Q4 describes apoptosis (Annexin $^{+}$, PI $^{-}$). Both compounds did not display differences in amount of necrotic cells compared to control. Assessment of quadrant Q2 (Annexin $^{+}$, PI $^{+}$) showed that HMO was unable to elevate the amount of post-apoptotic cells in comparison to control HeLa cells (6.48%).

Table 2. Percentage of alive, apoptotic, and necrotic HeLa cells treated with IC_{50} concentration of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) using flow cytometry method by Annexin V-FITC and PI as probes.

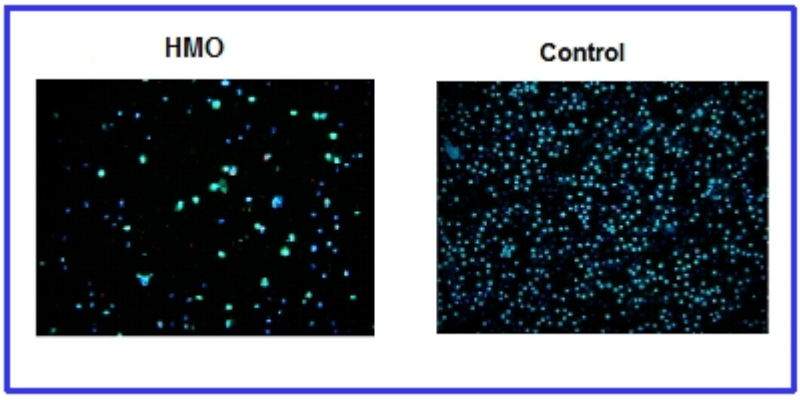
% of HeLa cells	%AnV^{+}/PI^{+} [a]	%AnV^{-}/PI^{-} [b]	%AnV^{+}/PI^{-} [c]	%AnV^{-}/PI^{+} [d]
Control	6.48	82.64	0.40	10.48
HMO [e]	5.94	67.55	20.41	6.10

[a] Annexin V $^{+}$/ PI $^{+}$= Post apoptotic cells.
[b] Annexin V $^{-}$/ PI $^{-}$= Live cells.
[c] Annexin V $^{+}$/ PI $^{-}$= Apoptotic cells.
[d] Annexin V $^{-}$/ PI $^{+}$= Necrotic cells.
[e] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one.

The percentages of apoptosis increased to 20.41% by HMO. Apoptosis was thus insignificantly induced in all samples tested. These results suggested that the anti-proliferation effect of the sample was mediated insignificantly by the induction of apoptosis. **Table 2** depicts the percentage of live, apoptotic, and necrotic cells detected by flow cytometry. In addition, induction of apoptosis in HeLa cells by this compound have been shown by flow cytometric quadrants in **Fig. 2**.



(a)



(b)

Figure 2. Flow cytometric graphs of induction of apoptosis in HeLa cells by '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) using Annexin V-FITC and PI as probes versus control cells. Quadrant 1 (Q1) represents necrosis (Annexin $^{-}$, PI $^{+}$), Q2 shows post-apoptosis cells (Annexin $^{+}$, PI $^{+}$), Q3 expresses alive cells (Annexin $^{-}$, PI $^{-}$), and Q4 describes apoptosis (Annexin $^{+}$, PI $^{-}$) (a); The Annexin V-FITC/PI staining pictures. Induction of apoptosis by '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO). Live cells are stained blue, apoptotic cells are stained green, and necrotic cells are stained red. The microscopic pictures were taken by fluorescence microscope, enlargement 200X (b).

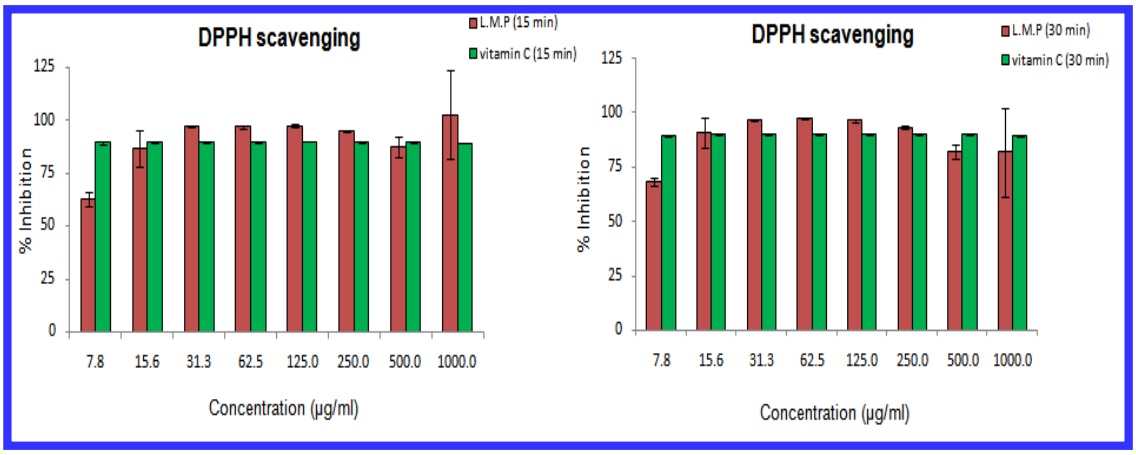
3.4. Comet assay

According to the results, HMO enhanced the percentage of DNA in the tail to 7.93% compared to control (3.74%) significantly ($P< 0.05$) (**Table 3**).

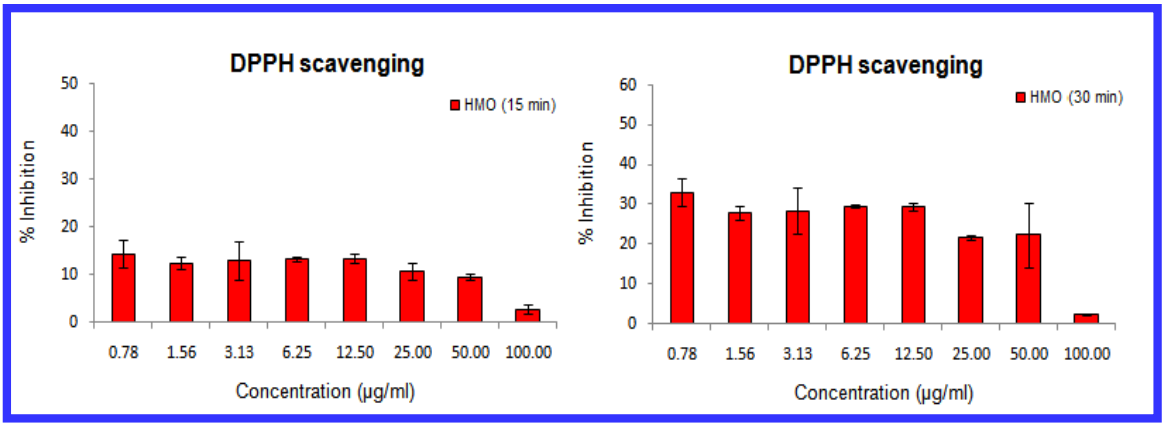
Table 3. Average median value (±SD) of the amount of DNA damage induced by '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO).

	% DNA in tail
Control	3.743±0.157
HMO[a]	7.939±2.358

[a] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one.



(a)



(b)

Figure 3. The percentage inhibition of DPPH activity after 15 and 30 minutes by; the ethanolic/acetonic extract of the leaves of *M. procumbens* (L.M.P), vitamin C (standard control) (a); and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO) (b). Each data point represents the mean of data from three wells (n= 3).

3.5. DPPH scavenging activities of experimental samples

A multiwell plate reader measured the intensities of the experimental samples with DPPH. Vitamin C (standard control) represented complete antioxidant activity (90% inhibition of $DPPH^{\bullet}$) at all concentrations tested ($P< 0.05$). L.M.P exhibited more than 60% DPPH scavenging activity at all the concentrations tested. The rate of DPPH discoloration was < 35% for HMO at all concentrations tested after 15 and 30 minutes (**Fig. 3**).

3.6. Effects of plant samples on HeLa cells FRAP and TBARS

The FRAP values in HeLa cells were promoted by L.M.P and HMO almost 9-fold and 12-fold of that of control, respectively. As a marker of lipid peroxidation, different concentrations of samples were incubated with HeLa cells and the variations in cell TBARS were assessed. Results showed that none of experimental samples could enhance the HeLa cells TBARS versus control cells.

3.7. Effects of plant samples on HeLa cells ROS level

The ROS intensity of HeLa cells was elevated about 1.5 and 5-fold by L.M.P and HMO as compared to control cells, respectively. HMO represented a time and concentration-dependent function of ROS formation *in vitro*. Generation of ROS happened while HeLa cells were exposed to H_2O_2 in all tested concentrations (ranging from 250-2000 mM) and significantly enhanced by time (**Fig. 4**).

4. Discussion

Plants engaged along history for treatment of cancer. Celastraceae family is known with majorly two types of compounds; quinonemethide triterpens (antitumor) and triterpene dimmers (cytotoxic) (Shirota et al., 1994), which composes the family promising for novel anticancer drug candidates.

The cytotoxicity of cyclic triterpenoids have been reported frequently that confirm our findings well. Kuo (1994) isolated two sesquiterpenes from *M. emarginata* namely ‘emarginatine F’ and

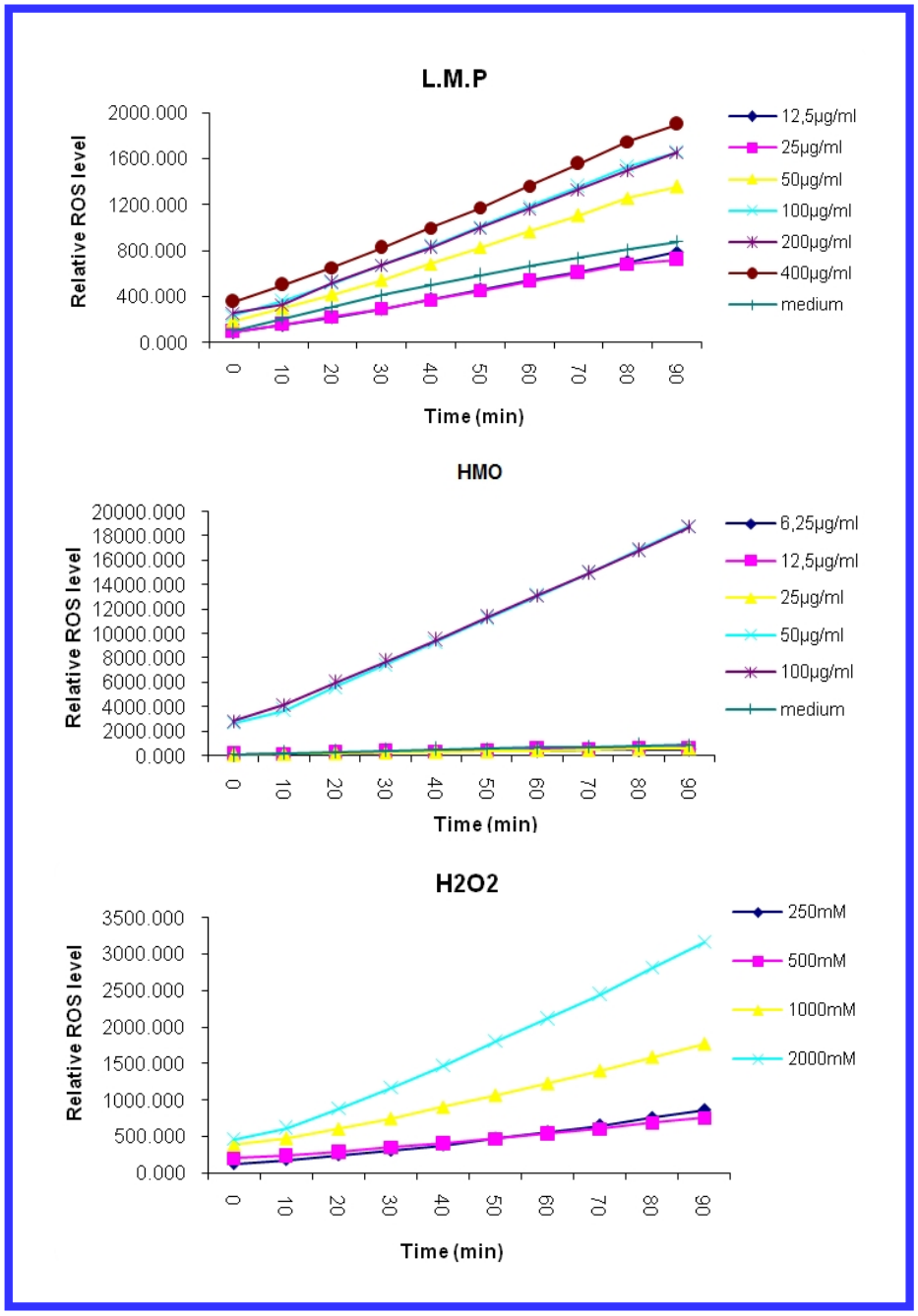


Figure 4. Time-response curve of increase of relative ROS level (DCF fluorescence) in HeLa cells after 90 minutes exposure to various concentrations of L.M.P and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO). Each data point represents the mean of data from three wells (n = 3).

'emarginatine G'. Interestingly, 'emarginatine F' exhibited cytotoxicity on KB (nasal pharyngeal carcinoma) and A-549 (human lung carcinoma) cells with IC_{50} values of 0.5 and 5.05 µg/ml, respectively while the other one was inactive. It was also found 'Butulin' isolated from the chloroformic extract of *M. forsskaoliana* afforded cytotoxic activity on two cancerous cell lines; HeLa and Hep-2 (hepatoma carcinoma) (IC_{50}= 40 µg/ml) (Deeb et al., 2003). 'Pristimerin' isolated from the ethanol extract of root bark of *M. ilicifolia* was tested on HL-60 (leukemia) cells and demonstrated the IC_{50} of 0.61 µg/ml (Costa et al., 2008).

Literatures confirmed that pentacyclic triterpenoids trigger apoptosis in cancer cells. Annexin V-FITC assay revealed that 'maslinic acid [(2α, 3β)-2,3-dihydroxyolean-12-en-28-oic acid]', an isolated triterpene from *Olea europaea* induced apoptosis in HT29 cells up to 25% while its IC_{50} concentration was 28.8 µg/ml (Zurita et al., 2009). Several studies have been reported that 'asiatic acid' induces apoptosis in human hepatoma, colon cancer, breast cancer, and melanoma cells (Cho et al., 2006). They reported that 'asiatic acid' induces cell death via both apoptosis and necrosis in U-87MGhuman glioblastoma.

The antioxidant abilities of the triterpenoids are mainly correlated with the structural properties of the molecules of which the presence of an aromatic ring bearing hydroxyl groups and the number of hydroxyl groups are important (Cefarelli et al., 2006; Yang et al., 2007). Radical scavenging activity of the ethanol extract of the root bark of *M. aquifolium* was found 35.5% at 40 µg/ml (Vellosa et al., 2007). Our findings are in agreement with those of Cefarelli et al. (2006) which reported 'betulinic acid' and 'betulinic aldehyde' (belonging to lupane triterpenoids) isolated from the fruits of *Malus domestica* reduced free radical absorbance by 58.4% and 55.4% at 250 µg/ml. They also found 'oleanolic aldehyde' and 'oleanolic acid' (belonging to oleane triterpenoids) inhibited DPPH radical by 42.7% and 32.2%, respectively.

Various plant extracts possess the ability in triggering the apoptotic pathway via ROS generation (Liu et al., 2000; Liu et al., 2001). Park et al. (2004) reported that 'asiatic acid' isolated from *Centella asiatica* increased intracellular ROS in human melanoma cancer cells. A large number of terpenoids modulate neuronal signal transduction by interfering with ion channels, ion pumps,

neuroreceptors, choline esterase, monoamine oxidase and other enzymes related to signal transduction pathways. ROS-mediated DNA damage has long been thought to play a role in inhibition of carcinogenesis and malignant transformation (Fruehauf and Meyskens, 2007; Valko et al., 2006).

L.M.P exhibited antioxidant properties in both extra and intracellular experiments which clarifies its slight oxidation action. In contrast, HMO exhibited marginal antioxidant activity (< 40%). Its weak antioxidant potential might be a logical explanation for enhancement of ROS levels at higher concentrations *in vitro*. Therefore, generation of ROS might be a part of the mechanisms by which this compound induces apoptosis in HeLa cells.

However, L.M.P and HMO induced ROS generation in the HeLa cellular environment. Thus, the active components in L.M.P might serve as a mediator of the ROS scavenging system and have the potential to act as a prooxidant and an antioxidant, depending on the biological environment of the cells. Such a dual-property role for antioxidants has been reported previously (Turley et al., 1997; Yang et al., 2006; Zou et al., 2001).

Overall, HMO was capable to induce apoptosis at its IC_{50} concentration in HeLa cells as evidenced by DNA staining (PI), and plasma membrane permeability (Annexin V binding assay). In addition to genetical changes (as proved by comet assay), and the participation of ROS in mediating apoptosis induced by this compound, other pathways may also be involved. The elucidation of these mechanisms by which this sample induces apoptosis in different cancer cells will be helpful for better understanding new apoptotic signaling pathways and will benefit the clinical application of them in the prevention and treatment of cancer.

The present study revealed a new biological index of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens*. Additionally, a new triterpene was isolated from L.M.P for the first time. There is no report until date on the anticancer, and antioxidant properties of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens* (L.M.P), and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (HMO).

As HMO was found to be novel, there is limited information about its medicinal properties except from those reported in this study. Wide spectrum of biological activities of triterpenoids have been recognized such as; bactericidal, fungicidal, antiviral, cytotoxic, analgetic, anticancer, spermicidal, cardiovascular, antiallergic, and so on. Regarding oxidant/antioxidant effects of this herb in various cell lines, steps should be put forward to examine its efficacy in some relevant diseases (Hassani-Ranjbar et al., 2009; Rahimi et al., 2005; Rezaie et al., 2007).

Additionally, the other possible biological activities of this genus and new cyclic terpenes should be discovered. The biological relevancy of LMB signaling events in particular cells treated with this compound should be examined in further studies. A proposed mechanism to explain the anticancer actions of these compounds might be mitochondrial swelling, which together with changes in the mitochondrial potential and release of proapoptogenic proteins leads to the death of transformed cells. Further studies are required to understand the effect of different functional group substitutions and the mode of inhibition of cell proliferation by the purified compounds. These compounds might be worthy as new anticancer agents alone or in combination with other antiproliferative drugs.

Limitations of the study: A major limitation of the study is the isolation of insufficient amount of compounds. In addition, considering a single limit dose for comet assay could be a drawback.

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