CHAPTER 3

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

3.1. 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. L.M.P showed IC_{50} values of 68.79, 51.22, 78.49, 76.59 and 76.64 µg/ml on Caco-2, HeLa, HT29, NIH3T3 and T47D cells by use of MTT cytotoxicity assay. Bioassay guided fractionation led to the isolation and identification of two new triterpenes: '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'. In addition, a known terpenoid: 'asiatic acid **4**' was purified. Due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested. Pure compounds **3** and **5** exhibited the most cytotoxicity against HeLa cells and were further investigated for their abilities for induction of apoptosis (at the concentration of their IC_{50}) in HeLa cells using flow cytometric method. Both compounds induced apoptosis up to 73.20%, (compound **3**) and 20.40% (compound **5**) in HeLa cells versus control group (0.40%). The anti-tyrosinase activity of L.M.P was tested using monophenolase and diphenolase as substrates of which L.M.P did not show significant inhibitory activity (Due to the unavailability of sufficient amount of the isolated pure compounds, these compounds were not tested).

Antioxidant/oxidative properties of L.M.P and its isolated compounds were investigated using extracellular (DPPH) and intracellular reactive oxygen species (ROS) assays. L.M.P and the isolated compounds exhibited marginal DPPH discoloration. Experimental samples represented a time and concentration-dependent function of ROS formation in HeLa cells. ROS generation might be a part of the mechanisms by which compounds **3** and **5** induced apoptosis in HeLa cells. It can be concluded that the active components in L.M.P might serve as a mediator of the reactive oxygen scavenging system and have the potential to act as a prooxidant and an antioxidant, depending on the biological environment of the cells.

3.2. 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 1,300 species of plants. The great majority of the genera are tropical, with only *Celastrus* (the staff vines), *Euonymus* (the spindles) and *Maytenus* widespread in temperate climates. The member of genus *Maytenus* are 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 sub polar. Celastraceae generally grow as small trees, bushes, or lianas and have resinous stems and leaves (Spivery *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, antitumor, anti-leukemic, antibacterial, insecticidal, and insect repellent activities (Costa *et al.,* 2008; Ghazanfar, 1994; Spivery *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 (Cargg and Newman, 2005; Gonzalez *et al.,* 2000; Hong,

2000; Hui *et al.,* 2009; Lee *et al.,* 1982; Martucciello *et al.,* 2010; Spivery *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 stated before (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 an 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; Spivery *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 meters. 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 meters (Coates Palgrave, 2002).

Oxidative stress has defined as 'a disturbance in the pro-oxidant/antioxidant balance in favour of the former'. Thus oxidative stress is essentially an imbalance between the production of various reactive species and the ability of the organism's natural protective mechanisms to cope with these reactive compounds and prevent adverse effects (Sies, 1985). The oxidative stress concept was redefined as 'an imbalance between oxidants and antioxidants in favor of the oxidants leading to a disruption of redox signaling and control and/or molecular damage' (Veskoukis *et al.,* 2012). Versus, an antioxidant can be defined as a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly prevents or delays a pro-oxidant initiated oxidation of the substrate (Abdollahi *et al.,* 2004). A pro-oxidant is a toxic substance that can cause oxidative damage to lipids, proteins and nucleic acids, resulting in various pathologic events and/or diseases. Pro-oxidant is a synonym for reactive species (Prior and Cao, 1999).

In biological systems, ROS are constantly generated through a variety of pathways, including both enzyme-catalyzed reactions and non-enzyme reactions (Pelicano *et al.,* 2004). Oxidative modification of nucleic acids by reactive oxygen species is of remarkable biological importance, as it results in the transformation of non malignant cells into malignant ones (Guyton and Kensler, 1993). Malignant cells have been reported to show decreased susceptibility to lipid peroxidation compared to normal cells. The low availability of polyunsaturated fatty acid has been suggested to be a limiting factor for peroxidation in tumors (Kolanjiappan *et al.,* 2003).

ROS are increased in malignant cells in part because of oncogene signaling via the NADPH oxidase complex and by hypoxia-related mitochondrial ROS. Increased oxidant levels contribute to enhanced cell proliferation and apoptosis suppression. Two independent therapeutic strategies targeting these pathways are possible. One point of attack would be to increase ROS scavenging, thereby dampening H_2O_2 signaling and

depressing tumor growth. An opposite approach would be to treat cells with agents that interfere with ROS scavenging, resulting in excess ROS that would trigger apoptosis. In opposition, there are therapeutic maneuvers that interfere with ROS removal, leading to an accumulation of excess ROS. High levels of ROS can cause apoptosis by triggering mitochondrial permeability transition pore opening and release of proapoptotic factors (Fruehauf and Meyskens, 2007).

The role of ROS in mediating apoptosis induced by a variety of agents, including tumor necrosis factor-α, anti-Fas antibodies, some chemotherapeutic agents, and radiation has been well established (Sun *et al.,* 1999). This study aimed to investigate whether the experimental samples would increase the scavenging of free radicals, so suppress the growth of tumors or excess the level of oxidants and lead to cell death (apoptosis)? The pro-oxidant/antioxidant activity of samples was measured using:

a) Measurement of radical scavenging capacity (RSC)
b) Measurement of intracellular ferric reducing/antioxidant power (FRAP)
c) Measurement of intracellular thiobarbituric acid reactive substances (TBARS)
d) Measurement of intracellular reactive oxygen species (ROS)

Due to the variety of biological activity of the genus most importantly being anti-cancer activity, it was decided to focus investigations on *M. procumbens* for its cytotoxic activity. In addition, this investigation intended to determine the chemo-preventative (anti-cancer and cancer preventative) activity of *M. procumbens*. The bioactive principles of the extract were also identified and the mechanism of action of selected samples was investigated.

3.3. MATERIALS AND METHODS

3.3.1. Collection, identification and extraction of plant materials

The leaves of *M. procumbens* were collected from the Botanical Garden of the University of Pretoria (South Africa) during May 2007. The plant was identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria. Herbarium voucher specimens have been submitted in the herbarium of the University of Pretoria (PRU 094096). Different articles have cited hexane, acetone, ethanol, methanol and water; alone or in combination were frequently used to extract various compounds from *Maytenus spp.* (Orabi *et al.*, 2001; Queiroga *et al.,* 2000; Salazar *et al.,* 1997; Tambekar and Khante, 2010). Acetone/ethanol (v:v; 1:1) were used to extract the medium and polar constituents of the leaves of *M. procumbens.*

Seven hundred grams of the leaves of *M. procumbens* (shade dried) were ground to a soft powder (Junke & Kunkel grinder, UK). *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.

3.3.2. 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) (Appendices B.1.a). TLC plate was developed using (hexane: ethyl acetate; 8:2) as eluent. Acidic vanillin was used as a detecting agent. A pure powder was crystallized from fractions 51-52**Ma** (compound **3**, 43.0 mg; 0.078%).

Based on the cytotoxicity results, subfractions 34-38**Ma** and 44-46**Ma**, were selected for subsequent chromatographic purification. Combined subfractions 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 16-37**Mb** (808.0 mg) were combined and subjected to a sephadex column chromatography (**Md**) eluted with ethanol (100.0). Thereafter, according to TLC profiles subfractions 6-10**Md** (390.0 mg) were combined and subjected to a sephadex column (**Me**) eluted with dichloromethane (100.0). Subfractions 14-40**Me** were combined to precipitate a pure compound (compound **5**, 20.0 mg; 0.036%) (Appendices B.1.b).

Subfractions 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 (compound **4**, 12.0 mg; 0.021%) (Appendices B.1.c).The schematic presentation of the isolation steps are shown in Appendices B.2.

3.3.3. *Identification of isolated compounds*

The structural elucidations of the isolated compounds were identified by physical (mp. $[α]_D$), 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; Dela *et al.,* 1978; Duddeck *et al.,* 1978; Johns *et al.,* 1983; Muhammad *et al.,* 2000; Shibata *et al.,* 1987).

3.3.4. *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. Penicillin (100 IU/ml) 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 (Appendices C.1.1-C.1.2).

3.3.5. *In vitro cytotoxicity assay*

Cytotoxicity was measured by the MTT method as described by Mosmann, (1983) and O'Brien *et al.,* (2000) with some modifications. Briefly, cells (100 µl) were seeded (concentration $1x10^4$ cells/ml) into a microtitre plate and incubated for 24h 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 compounds), added to the plates and incubated (due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested). 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 their exponential phase of growth.

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, Vermont, 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%. The results were statistically analyzed with Sigmaplot software.

3.3.6. 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 the isolated compounds at their IC_{50} concentrations (compound **3**= 43.99 µg/ml; and compound **5**= 27.61 µg/ml (Due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested) (Appendices C.2). 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 $4^{o}C$ in dark with PI/AnV-FITC fluorescent dyes; results were determined using Partec flow cytometer equipped with Argon laser.

In total, 10,000 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.

3.3.7. 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 the isolated compounds at their IC_{50} concentrations (compound **3**= 43.99 µg/ml; and compound **5**= 27.61 µg/ml), then incubated for 72 h. The cells viabilities were assessed using trypan blue dye-exclusion staining (Appendices C.1.4). Afterward, the mixture of cells (2.4×10^5 cells/ml) and 0.5% LMP (Low Melting Point) agarose were 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 (Appendices C.3.1-C.3.4). 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). The results were expressed in terms of TM (tail moment), OTM (Olive Tail Moment), comet length and tail length. Data from at least 100 cells were analyzed per sample with usually 3 slides per point.

3.3.8. 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, Vermont, USA) after 15 and 30 minutes at 550 nm. The antioxidant activity of samples was reported as the percent inhibition of DPPH activity (Appendices C.4.1-C.4.2).

3.3.9. 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 pure compounds **3** and **5** (concentrations of 5-100 μg/ml) were added to the cells (Due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested). 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.

3.3.10. Ferric-reducing antioxidant power assay (FRAP)

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 (Appendices C.5).

2.3.11. Thiobarbituric acid reactive substance assay (TBARS)

Assay of TBARS is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. This assay was performed following the method of Sarkhail *et al.,* (2007) with minor modifications (Appendices C.6).

3.3.12. 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 compounds (due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested). 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 dichlorofluorescein 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, Vermont, USA). An increase in fluorescence (DCF) intensity

was used to represent the generation of net intracellular ROS. 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).

3.3.13. Antibacterial activity

The microorganisms used in this study included *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538p, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404 were stocks of the Department of Drug and Food Control, School of Pharmacy, Tehran University of Medical Sciences, Iran. The assay was performed by means of the agar-based cup–plate method (Ahmed and Beg, 2001) (due to insignificant activity shown by L.M.P and the isolated compounds, these compounds were not tested against *Mycobacterium smegmatis*) (Appendices C.7.1-C.7.3). The results were expressed as MIC, which regarded the lowest concentration of the samples that did not permit visible growth when compared that of the controls.

3.3.14. 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.

3.4. RESULTS AND DISCUSSIONS

One of the most unique aspects of planet earth definitely would be described with plants. A variety of plant species have been identified traditionally as well as in scientific literatures for their medicinal properties. Convenience and affordability of the medicinal herbs offer health care to many people's life all over the world. Genus *Maytenus* has studied intensively in different countries while astonishingly; literature reviews demonstrated a gap of biological index about *M. procumbens* in between. Thus, we

were encouraged for continuation of research on new possible biological activities from these species with a particular emphasis on their antiproliferative properties.

3.4.1. Identification of compounds from L.M.P

The biological activity guided fractionation of L.M.P which led to the purification of two triterpenes. Two new compounds that had not been isolated before were identified as; '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' (43.0 mg; 0.078%) and '30-Hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' (20.0 mg; 0.036%). 'Compound **4**' (12.0 mg; 0.021%) was known and identified as; 'asiatic acid' (Appendices B.3-B.4). The chemical structures of the isolated compounds are presented in Figure 3.1.

3.4.1.1. Spectroscopic analysis of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one 3'

The ^{13}C and ^{1}H NMR spectra of 'compound **3**' are similar to 'compound **5**' and consistent with an olean-12-ene. The ^{13}C NMR spectrum of 'compound **3**' demonstrated the presence of a trisubstituted double bond [$δ_C$ 120.9, 146.9, $δ_H$ 5.56 (1H, d, $J_{12,11β}$ = 3.1 Hz; H-12)], a ketone group (217.8), a hydroxylated methylene group [$δ_C$ 66.6, $δ_H$ 3.60 (1H, d, $J_{30A,30B}$ = 11.0 Hz; H_A-30), 3.51 (1H, d, $J_{30B,30A}$ = 11.0 Hz; H_B-30)], and seven methyl groups 1.27 (3H, s; Me-27), 1.13 (3H, s; Me-25), 1.12 (3H, s; Me-23), 1.09 (3H, s; Me-24), 1.03 (3H, s; Me-26), 0.91 (6H, s; Me-29, 28). The structure is consistent with a 3-oxo-12-oleanene-30-ol carbon skeleton (Chen *et al.,* 1983; Nick *et al.,* 1994; Nick *et al.,* 1995) (Fig 3.2.-3.7). The only difference between compounds **3** and **5** is the existence of a methoxy group at position C-12 of 'compound **5**', the proton and carbon-13 data of both compounds almost identical (Appendices B.5). The instability of the 'compound **5**' did not permit a complete assignment and measuring of 2D spectra, the proposed structure of this compound is new.
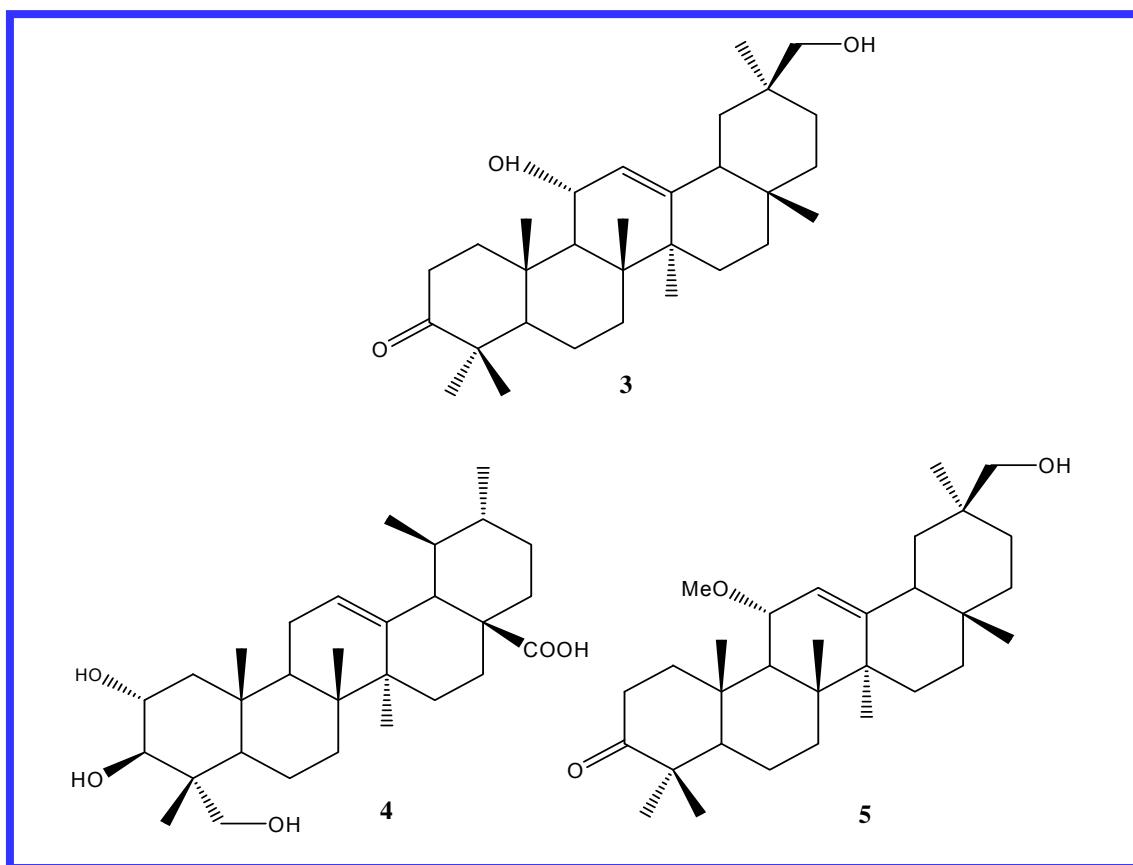
Figure 3.1: Chemical structures of the isolated compounds from the acetonic/ethanolic extract of the leaves of *M. procumbens* (L.M.P): '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**', 'asiatic acid **4**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

'Compound **5**' was isolated as amorphous colorless powder, IR (KBr disc): 3420, 2950, 2925, 2870, 1727, 1702, 1464, and 1385 cm^{-1}; [α] $_{589.3}$^{25} +33.55 (*c* 0.21, CHCl$_3$). The HRFABMS of 'compound **5**' 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. ^{1}H 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 ^{1}H and ^{13}C NMR spectra of 'compound **5**' 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 'compound **5**' 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; Nick *et al.,* 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 (Fig 3.8-3.13).

3.4.2. 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. 'Compound **3**' showed the IC_{50} values of 45.49, 43.99, 62.78 and 66.08 µg/ml on Caco-2, HeLa, HT29, and T47D cells, respectively. 'Compound **5**' 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) (Appendices B.6.-B.8). These compounds 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 3.1).

The cytotoxicity of cyclic triterpenoids have been reported frequently thus confirm our findings well. Kuo, (1994) isolated two sesquiterpenes from *M. emarginata* namely 'emarginatine F' and '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).
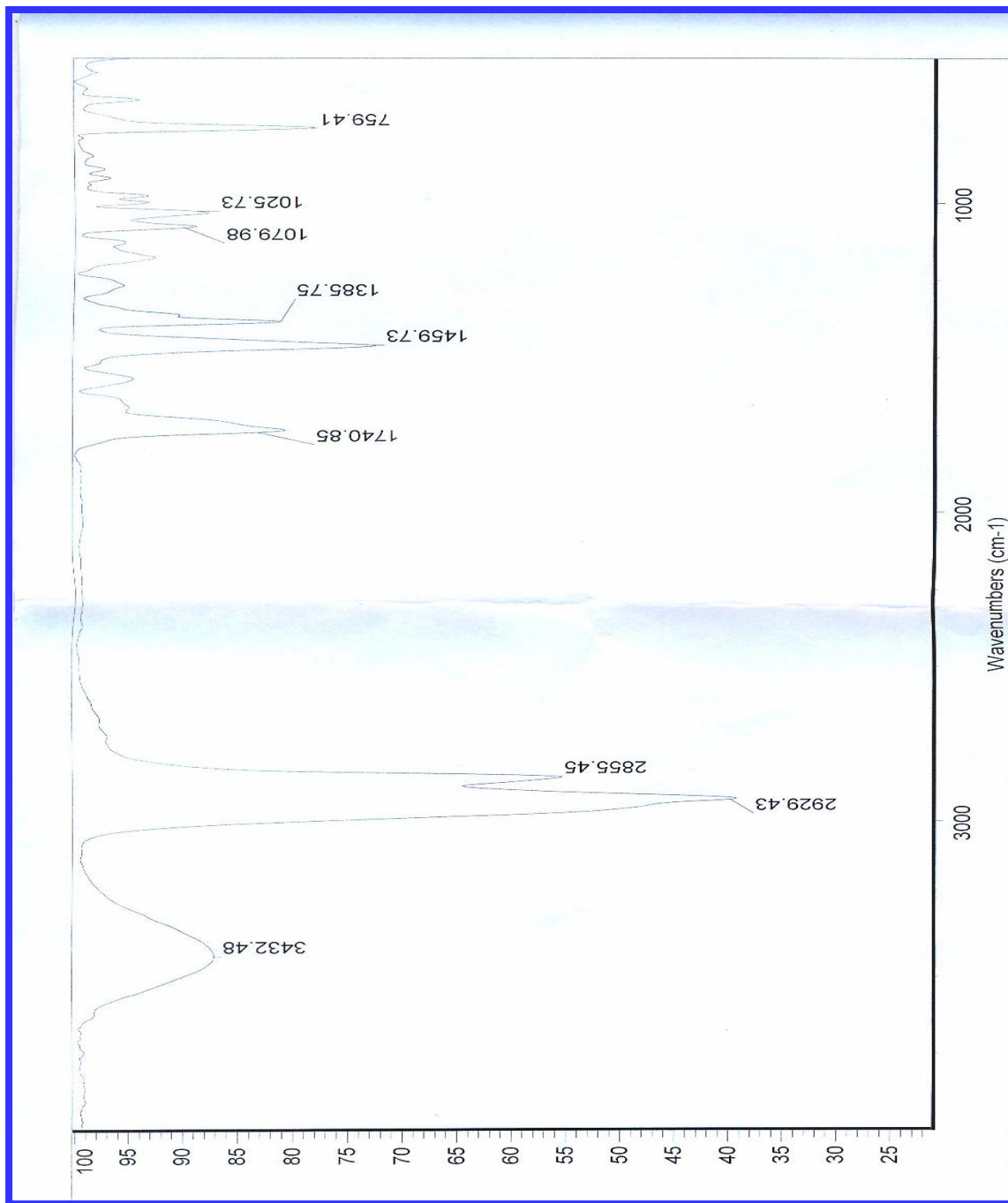
Figure 3.2: The Infra red (IR) spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.
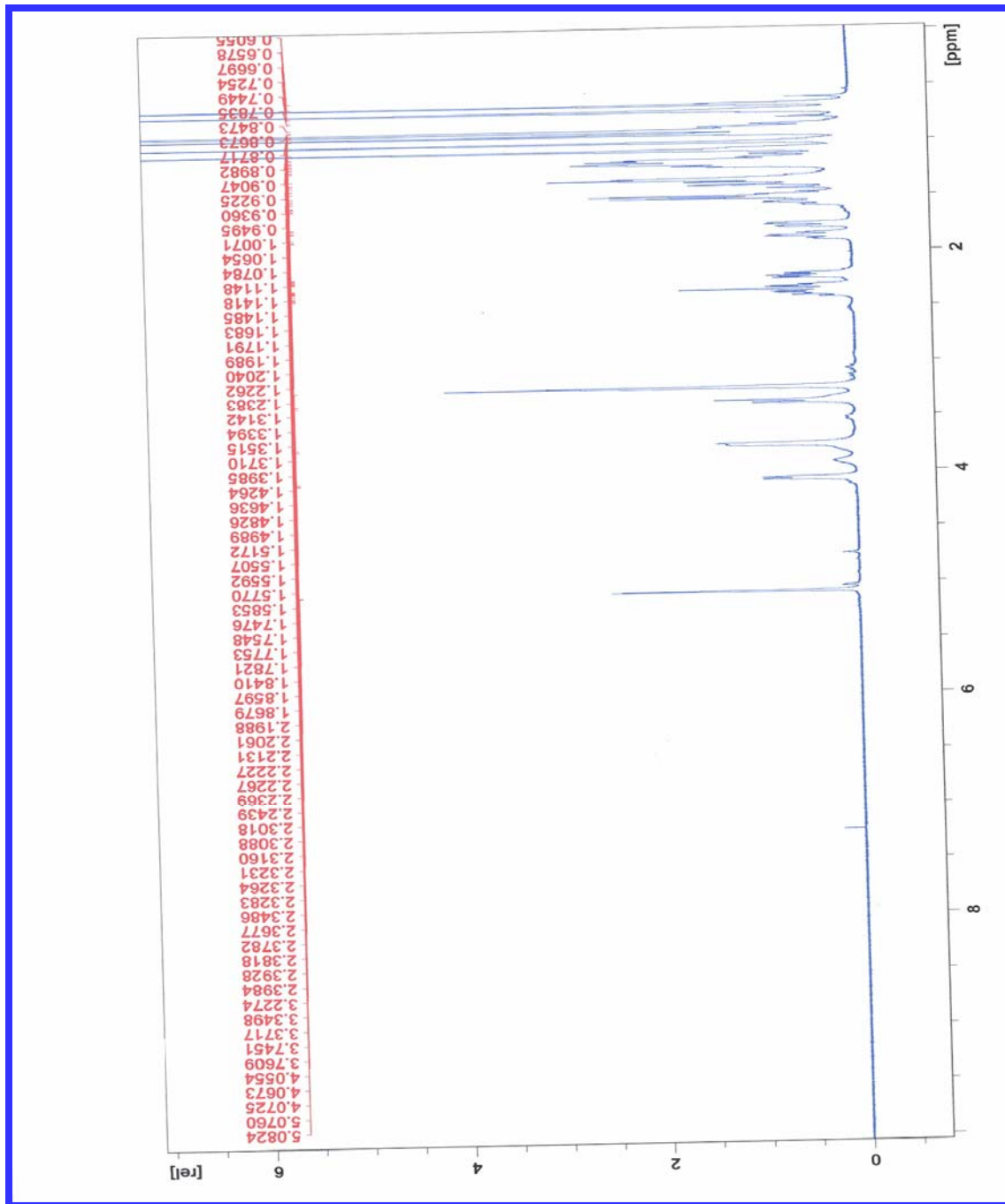
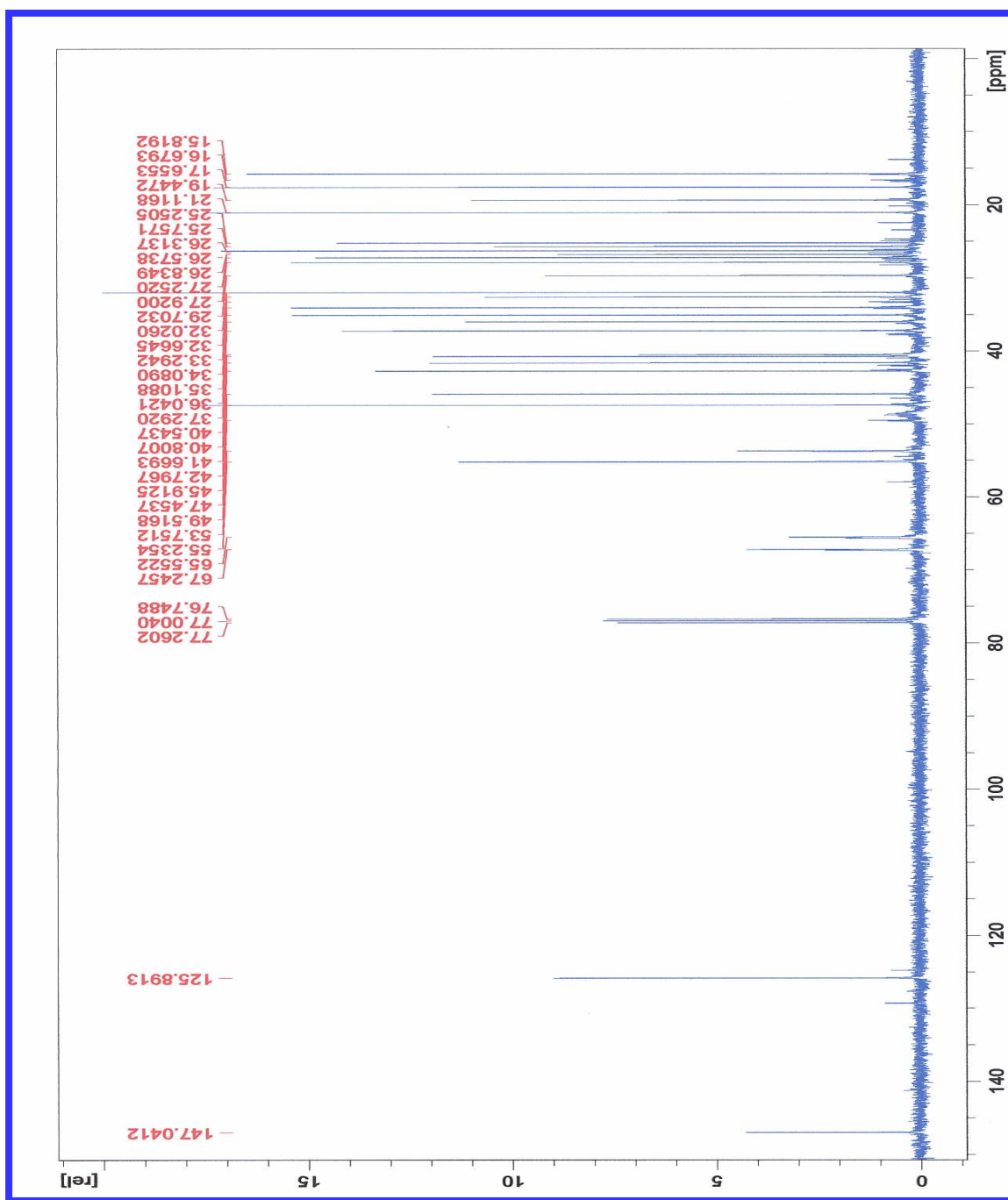**Figure 3.3:** The ^{1}H NMR spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one 3'.

Figure 3.4: The ^{13}C NMR spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.
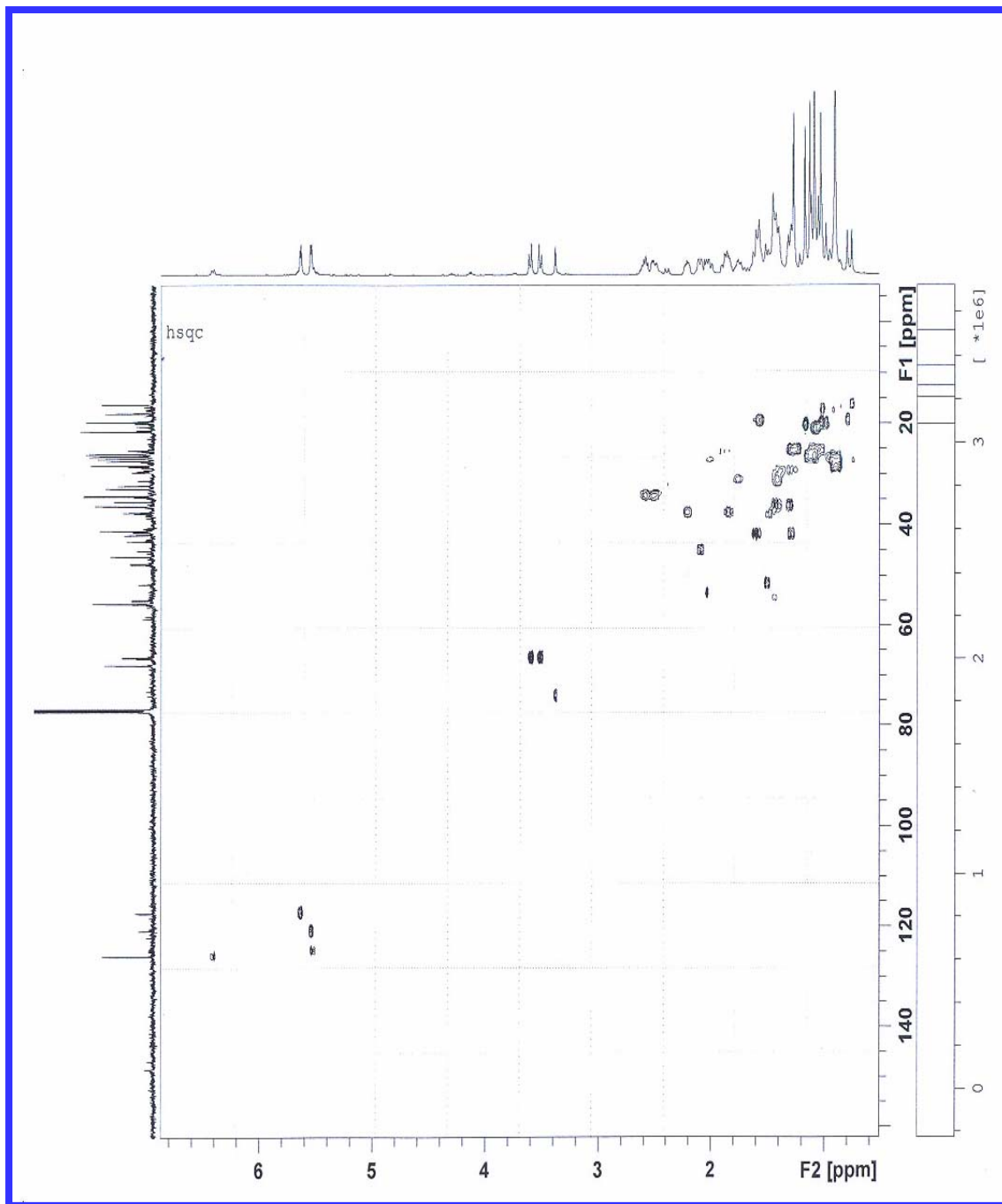
Figure 3.5: The HSQC spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.
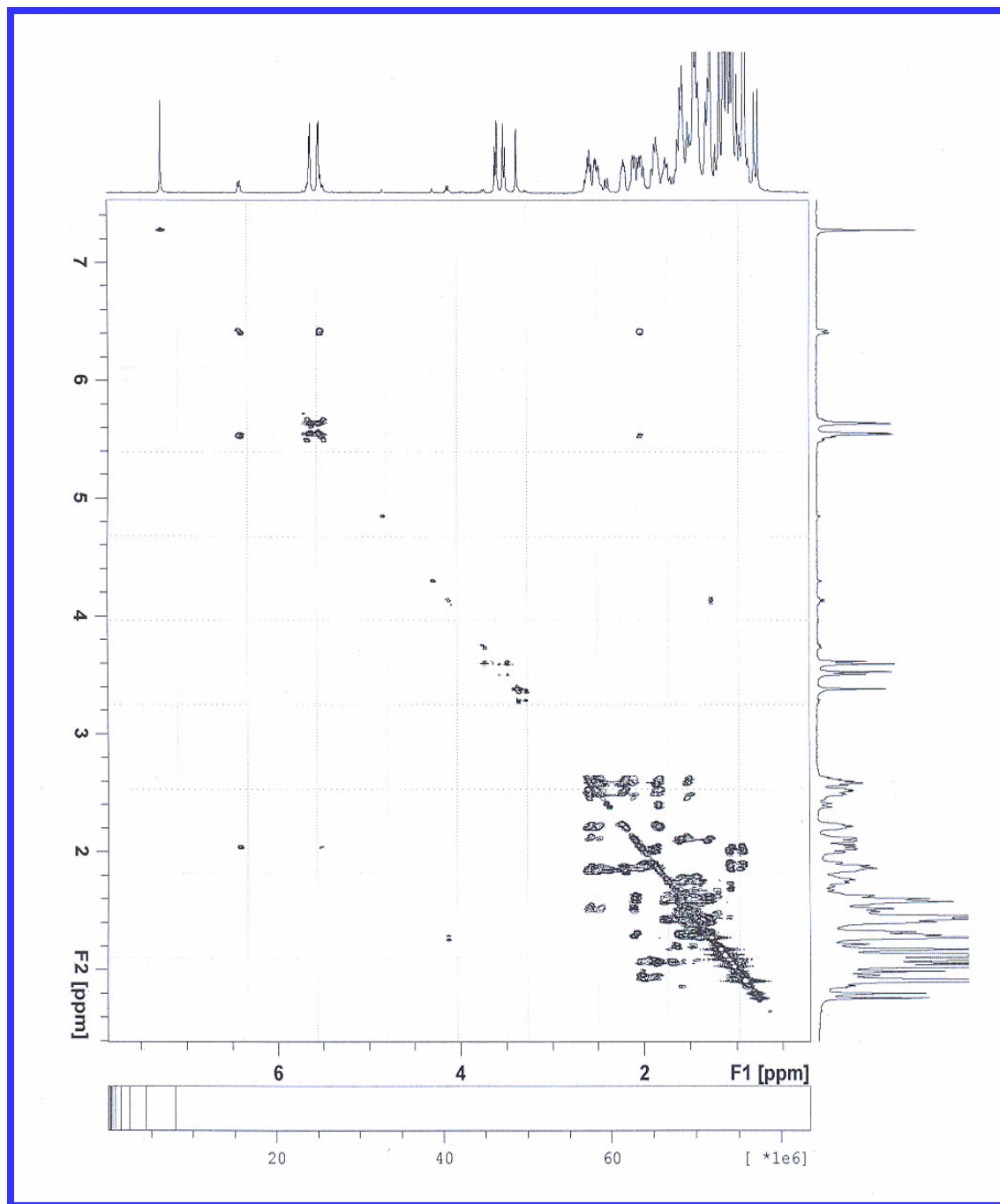
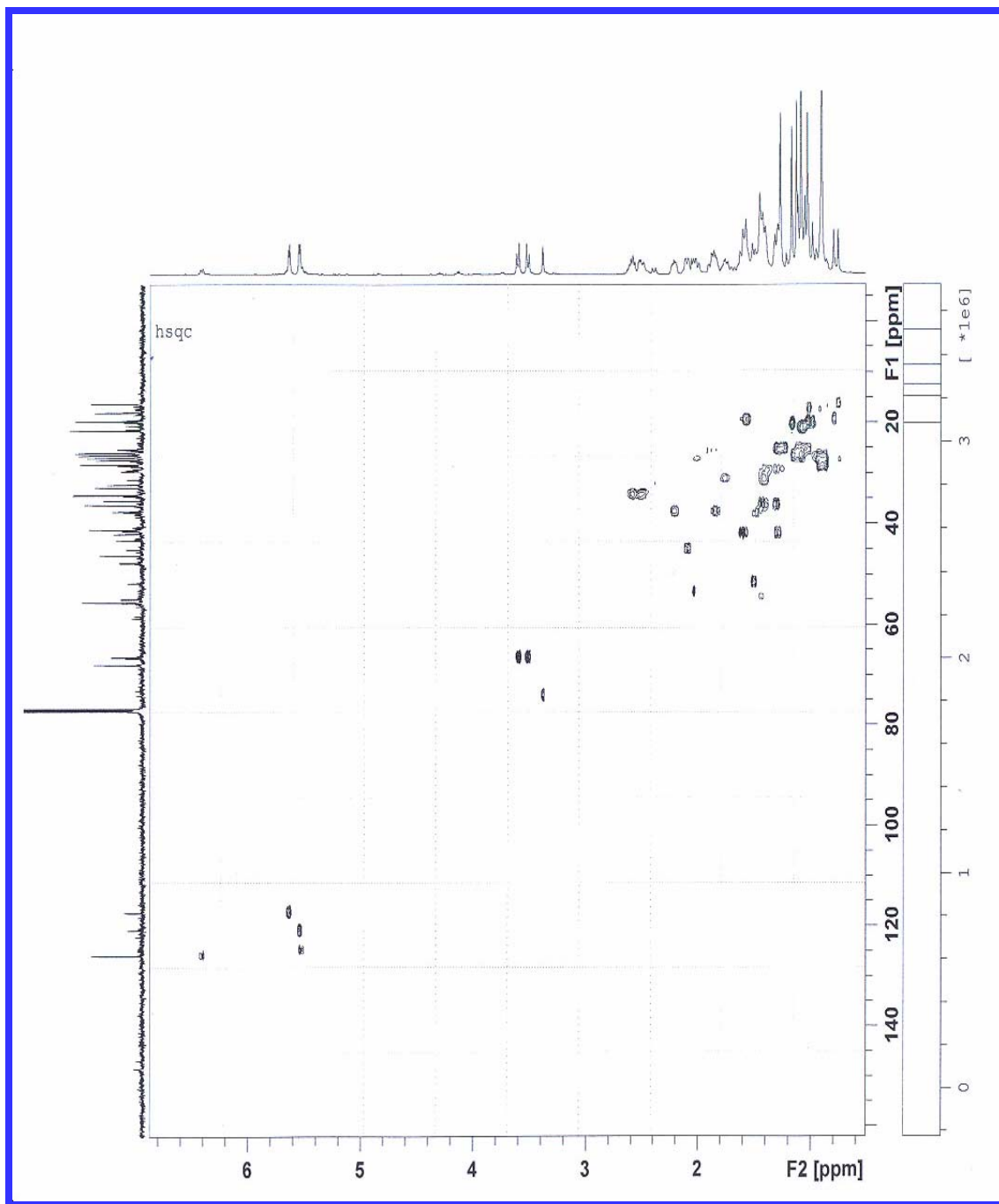**Figure 3.6:** The COSY spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

Figure 3.7: The HMBC spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.
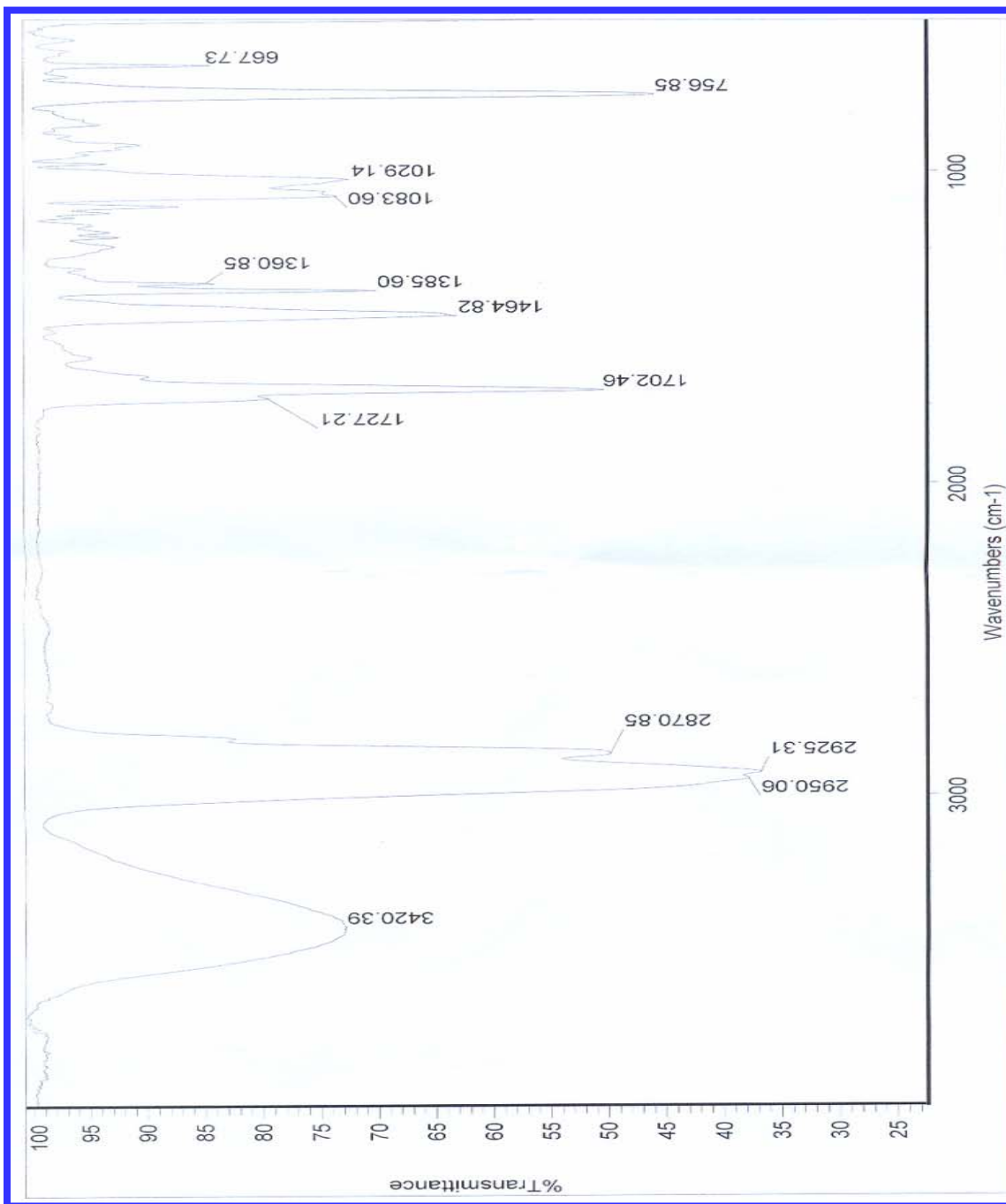
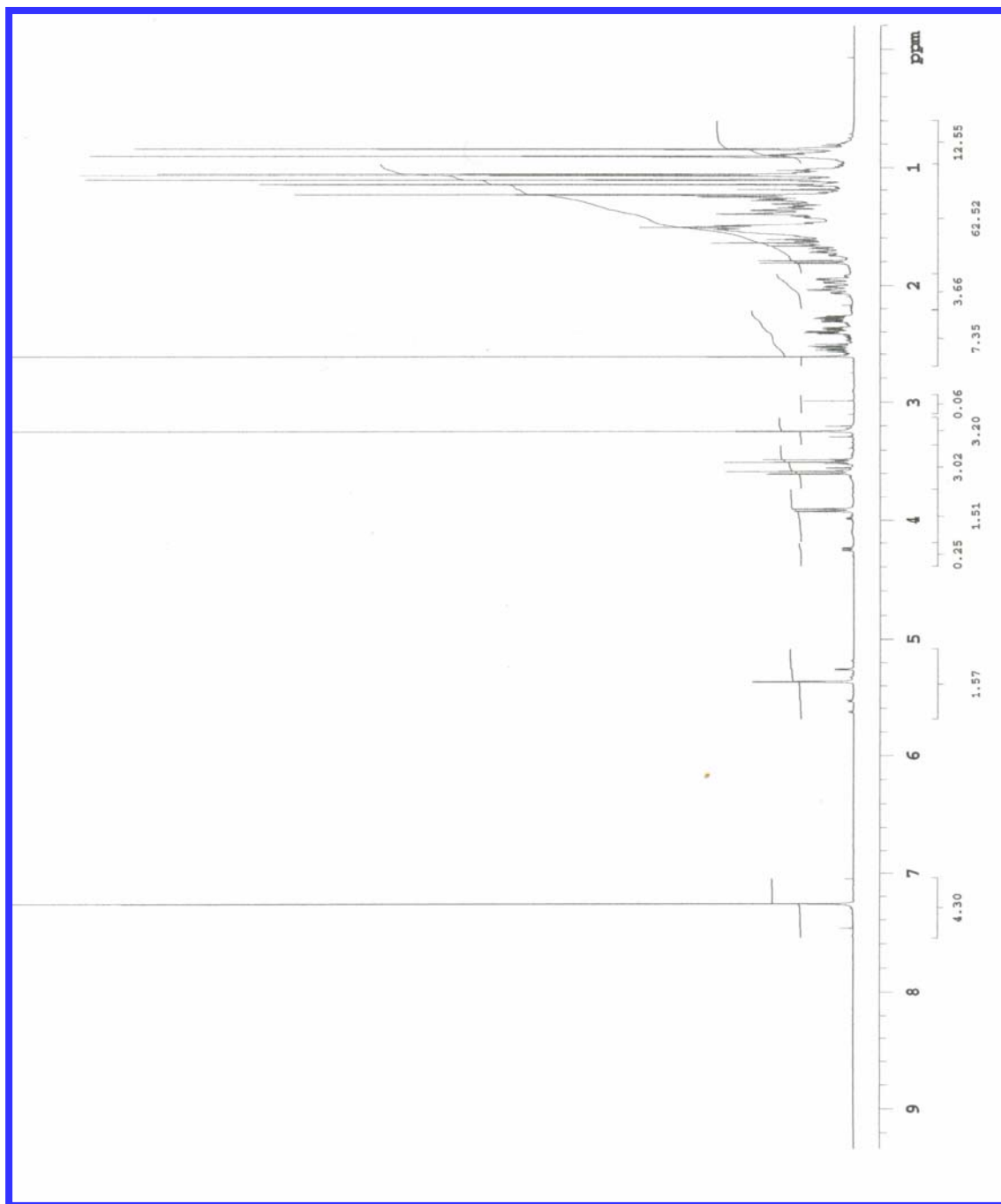**Figure 3.8:** The Infra red (IR) spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-

one **5**'.

Figure 3.9: The ^{1}H NMR spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.
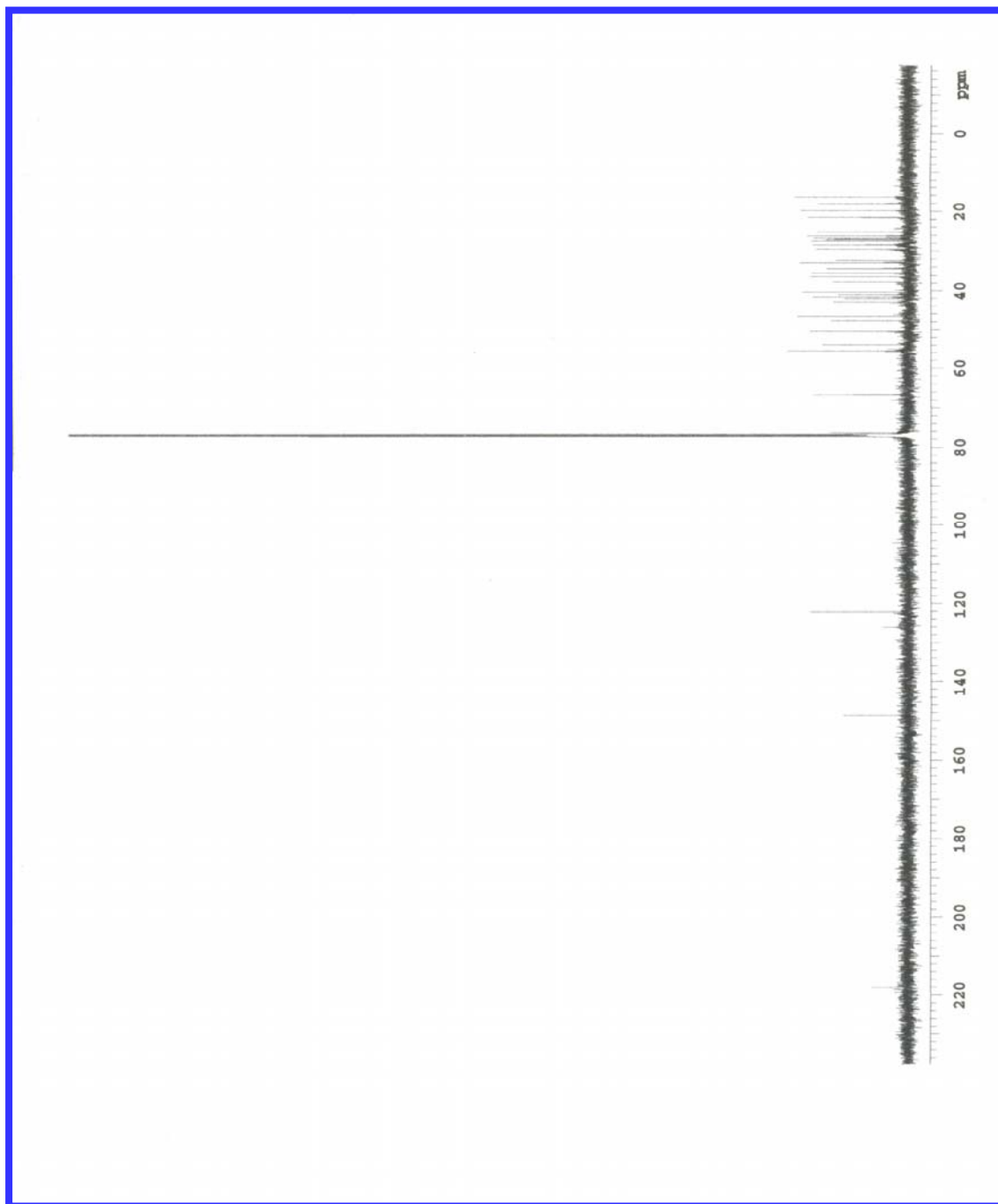
Figure 3.10: The ^{13}C NMR spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one
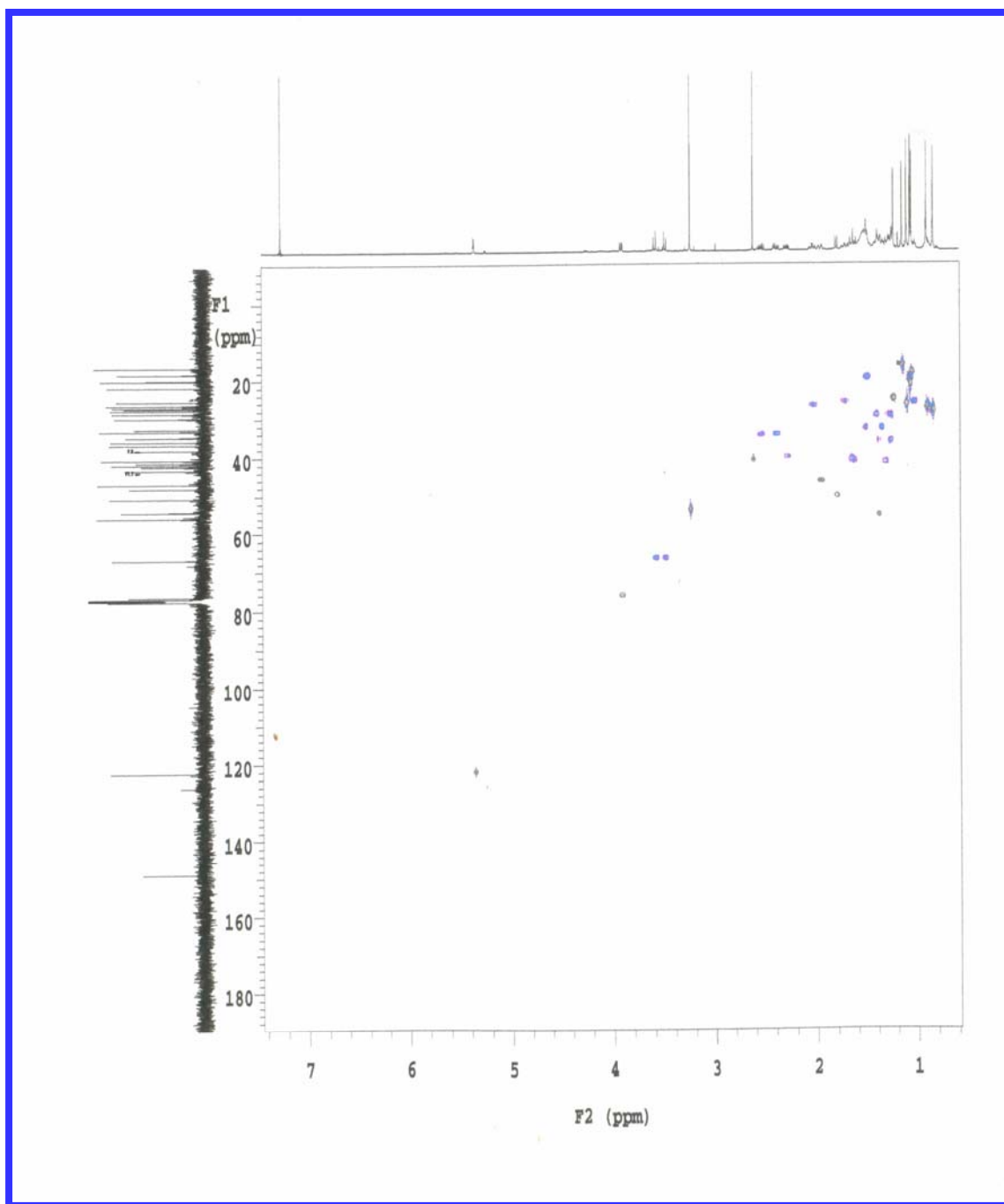
5'.

Figure 3.11: The HSQC spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.
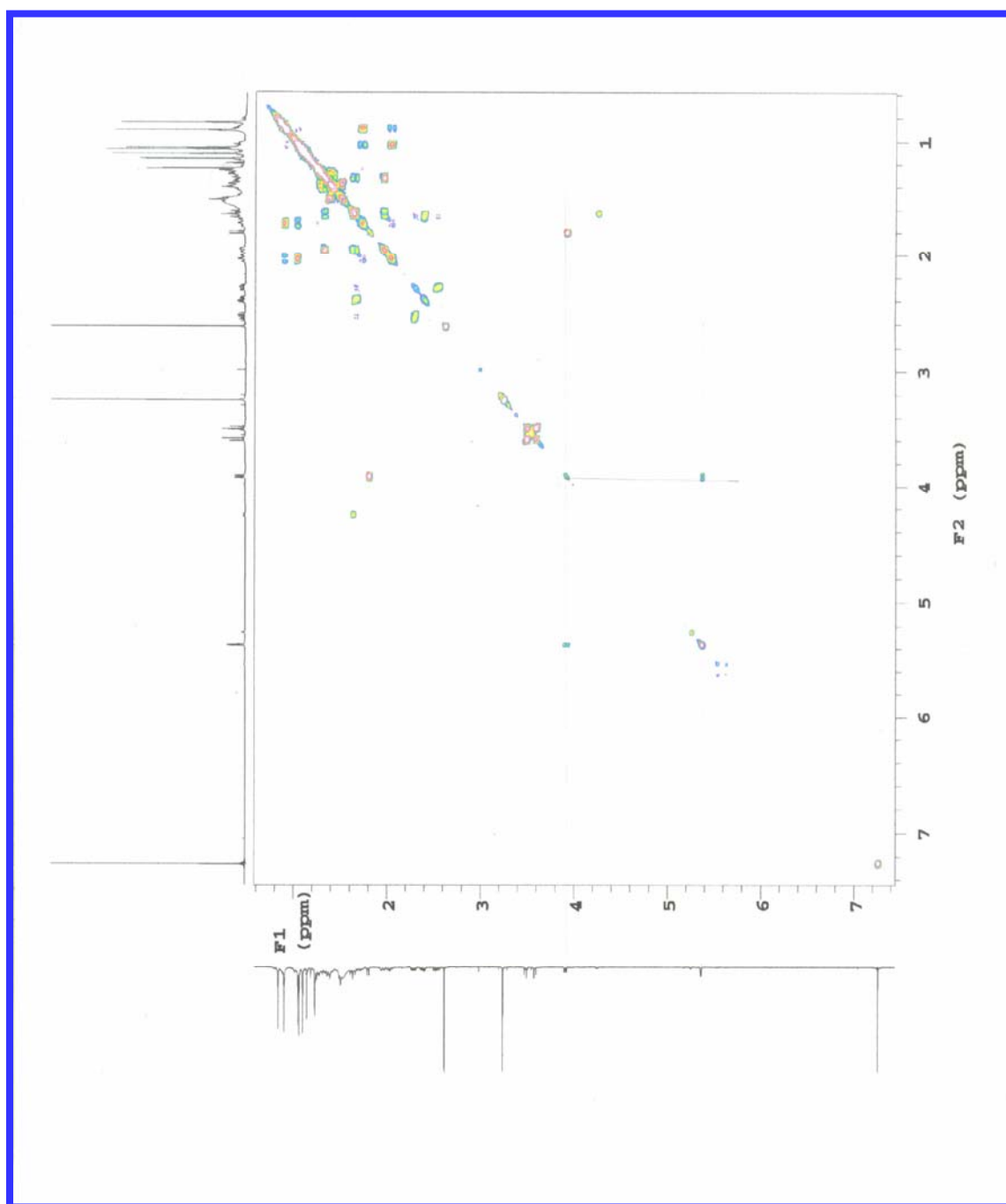
Figure 3.12: The COSY spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.
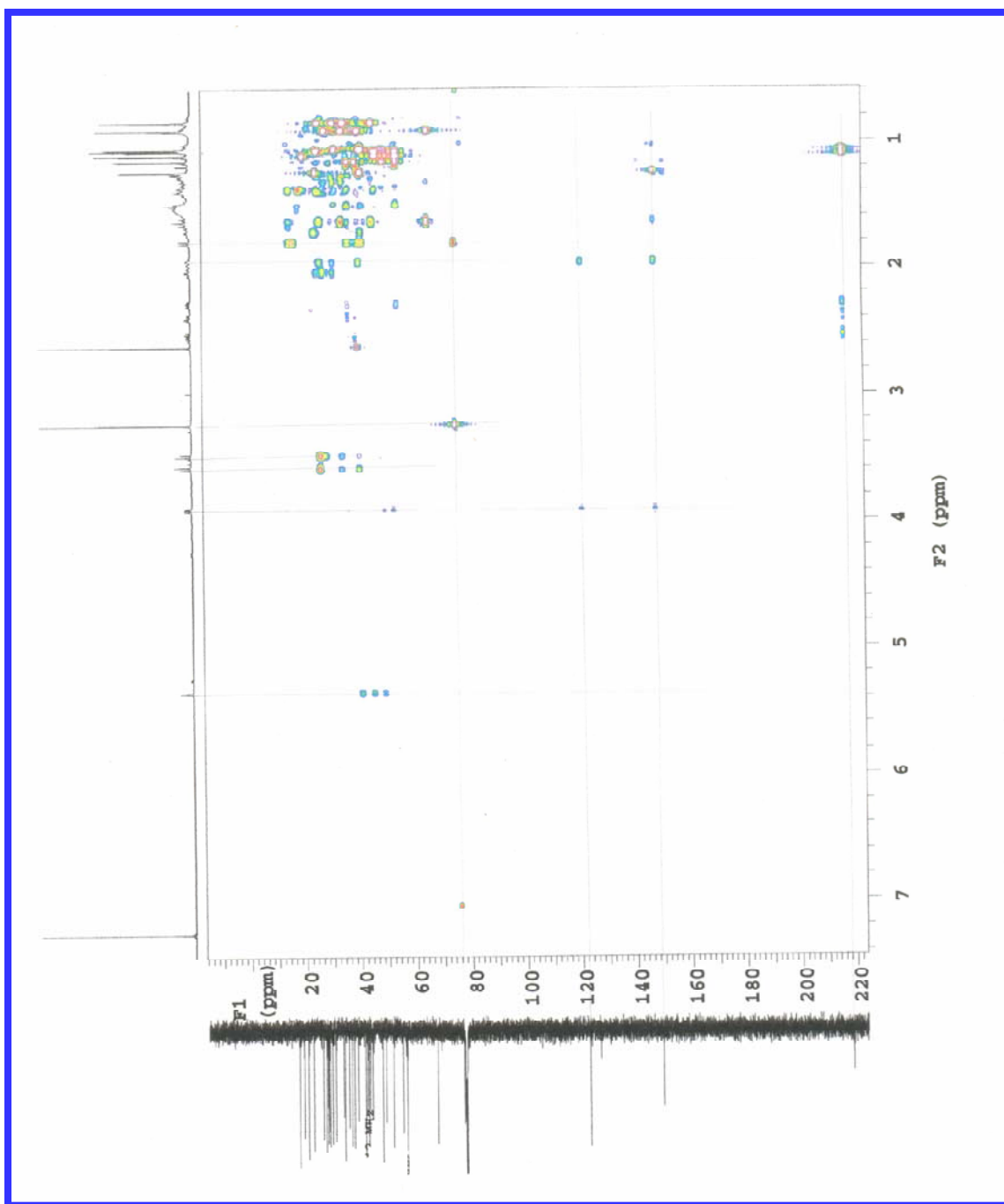
Figure 3.13: The HMBC spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one

5'.

Table 3.1: IC_{50} values (μg/ml) of the ethanolic/acetonic extract of the leaves of *M. procumbens* (L.M.P), '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' 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
3[b]	45.490±0.002	43.993±0.014	62.786±0.004	66.086±0.004	45.747±0.002
5[c]	42.712±0.005	27.613±0.022	61.375±0.003	30.593±0.004	45.977±0.001
Methotrexate[d]	0.23±0.02	0.071±0.8	0.23±0.02	0.16±0.09	0.24±0.013
Cisplatin[d]	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-hydroxy-18β-olean-12-en-3-one **3**.
[c] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**.
[d] positive control.

3.4.3. Apoptosis detection analysis by flow cytometry

The ability of pure compounds to induce apoptosis in HeLa cells at the concentration of their IC_{50} values (compound **3**= 43.99 μg/ml and compound **5**= 27.61 μg/ml) were 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 compare to control. Assessment of quadrant Q2 (Annexin [+], PI [+]) showed 'compound **3**' promoted the amount of post-apoptotic cells to 20.18% whereas 'compound **5**' was unable to elevate the amount of post-apoptotic cells as compared to control HeLa cells (6.48%).

The percentages of apoptosis increased to 73.16% and 20.41% by compounds **3** and **5**, respectively. The percentage of live cells reduced while treated with 'compound **3**' to 1.93% as compared to control cells (82.64%). Apoptosis was thus insignificantly

induced in all samples tested. These results suggested that the anti-proliferation effect of the samples were mediated insignificantly by the induction of apoptosis. Table 3.2 depicts the percentage of live, apoptotic, and necrotic cells detected by flow cytometry. In addition, induction of apoptosis in HeLa cells by these compounds have been shown via flow cytometric quadrants in Figure 3.14 (a&b).

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 published 'asiatic acid' induces cell death via both apoptosis and necrosis in U-87MGhuman glioblastoma.

Table 3.2: Percentage of alive, apoptotic, and necrotic HeLa cells treated with IC_{50} concentration of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' using flow cytometry method by Annexin V-fluorescein isothiocyanate and propidium iodide (Annexin V-FITC/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
Compound 3[e]	20.18	1.93	73.16	4.73
Compound 5[f]	5.94	67.55	20.41	6.10

[a] Annexin V $^+$/ PI $^+$= Post apoptotic cells.
[b] Annexin V $^-$/ PI $^-$= Live cells.
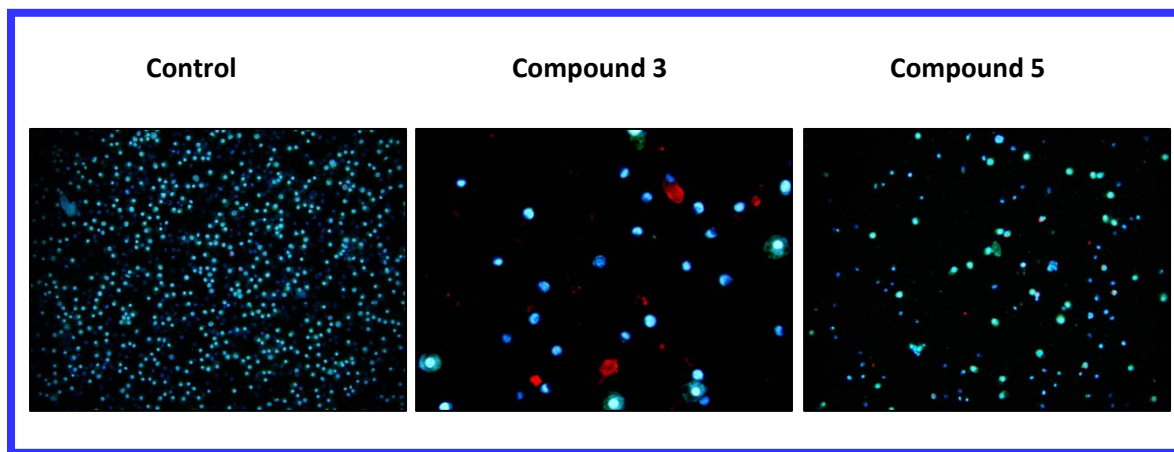[c] Annexin V $^+$/ PI $^-$= Apoptotic cells.
[d] Annexin V $^-$/ PI $^+$= Necrotic cells.
[e] 30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**.
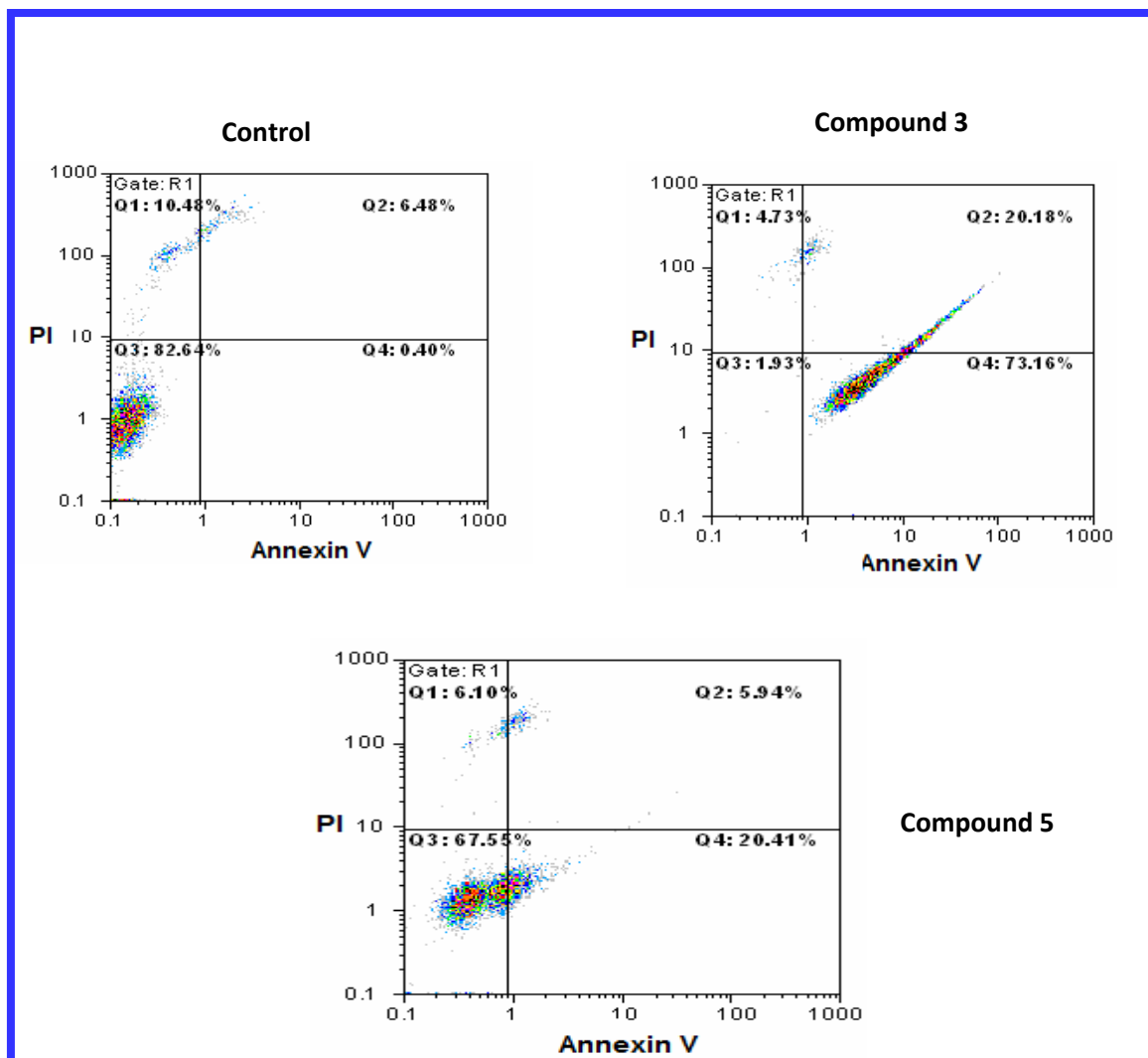[f] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**.

3.4.4. Comet assay

According to the results, 'compound **3**' significantly increased tail length, comet length, tail moment (TM), and Olive tail moment (OTM) to 12.81%, 30.36%, 4.86%, and 3.00% respectively when exposed to HeLa cells at its IC_{50} concentration (43.99 µg/ml) (*P< 0.05*). In contrast, there were not significant differences between 'compound **5**' and control group in concept of tail length, comet length, and OTM but this compound enhanced the value of TM to 0.54% significantly (*P< 0.05*) (Table 3.3). Microscopic snap shots of HeLa cells assessed by alkaline comet assay after exposure to pure compounds are pictured in Figure 3.15.

(a)

Figure 3.14.a: The Annexin V-fluorescein isothiocyanate and propidium iodide (Annexin V-FITC/PI) staining pictures. Induction of apoptosis by compounds **3** and **5**. 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)

Figure 3.14.b: Flow cytometric graphs of induction of apoptosis in HeLa cells by '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' using Annexin V-fluorescein isothiocyanate and propidium iodide (Annexin V-FITC/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 [-]).
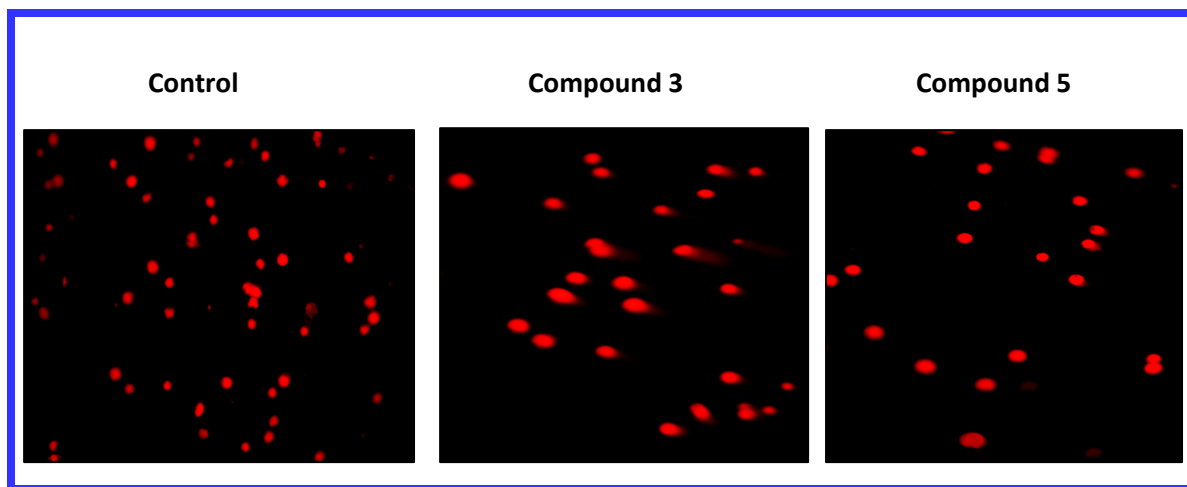
Figure 3.15: Microscopic analysis of HeLa cells 72 h after treatment with '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' with alkaline comet assay. The microscopic pictures were taken by fluorescence microscope, enlargement 200X.

3.4.5. 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˙) at all the 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 < 40% for compounds **3** and **5** at all the concentrations tested after 15 and 30 minutes (Fig 3.16).

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 to be 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' (belong 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' (belong to oleane triterpenoids) inhibited DPPH radical by 42.7% and 32.2%, respectively.

Table 3.3: Average median values (±SD) of the amount of DNA damage induced by '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

	L tail (%)[a]	L comet (%)[b]	TM (%)[c]	OTM (%)[d]
Control	3.090±0.032	25.650±0.996	0.189±0.121	0.394±0.043
Compound 3[e]	12.814±1.603	30.363±1.656	4.864±1.067	3.000±0.417
Compound 5[f]	4.433±0.301	25.962±.558	0.544±0.083	0.810±0.065

[a] L Tail = the length of tail.
[b] L comet = the length of entire comet (from head to the end of tail).
[c] TM (tail moment) = [tail length] × [percentage of DNA in tail].
[d] OTM (Olive tail moment) = [The horizontal distance between the center of DNA in tail to the center of DNA in head] × [percentage of DNA in tail].
[e] 30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**.
[f] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**.

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

The FRAP values were promoted by L.M.P, compounds **3** and **5** as almost 9-fold, 6-fold and 12-fold, respectively in HeLa cells as compared to control group. As a marker of lipid peroxidation, different concentrations of samples were incubated with HeLa cells, consequently variation in cell TBARS were assessed. As results showed, none of experimental samples could enhance the HeLa cells TBARS versus control cells significantly.
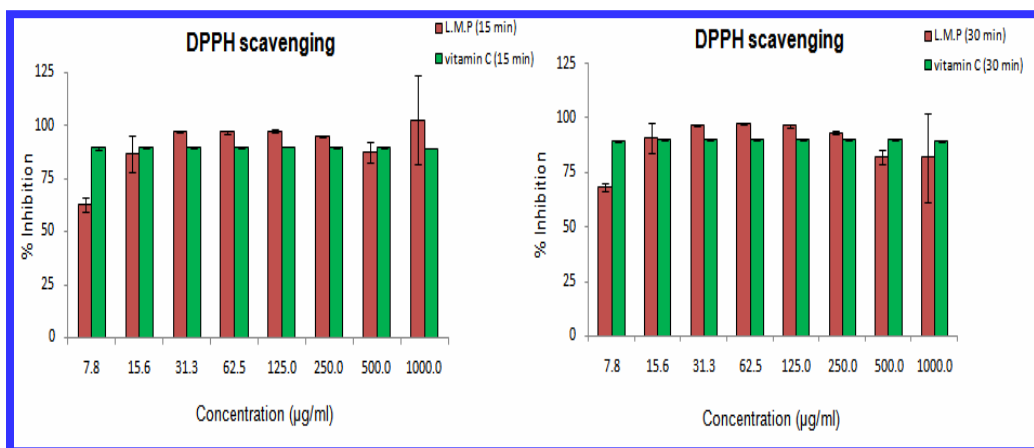
(a)
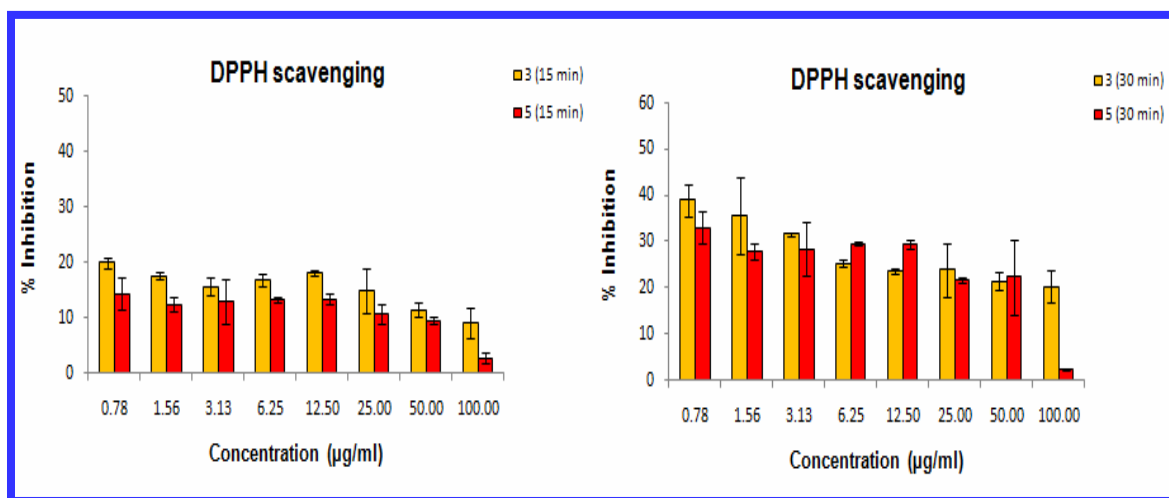
(b)

Figure 3.16: The percentage inhibition of 1,2-diphenyl-2-picrylhydrazyl (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); '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' (b). Each data point represents the mean of data from three wells (n= 3).

3.4.7. Effects of plant samples on HeLa cells ROS level

The ROS intensity of HeLa cells was elevated about 1.5, 21 and 5-fold by L.M.P, compounds **3** and **5** compared to control cells, respectively. Presence of an additional OH group in the chemical structure of 'compound **3**' explains its highly prooxidant activity.

'Compound **5**' represented a time and concentration-dependent function of ROS formation *in vitro*. ROS generation was happened while HeLa cells were exposed to H_2O_2 in all concentrations tested in this experiment (ranging from 250-2000 mM) and enhanced by time significantly (Fig 3.17). 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 '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 carcinogenesis initiation and malignant transformation (Fruehauf and Meyskens Jr, 2007; Valko *et al.,* 2006).

3.4.8. Antibacterial activity of plant samples

Crude extract of *M. procumbens* exhibited the MICs of 2 and 8 mg/ml against *S. aureus* and *P. aeruginosa*, respectively. Compounds **3** and **5** represented negligible inhibition of the growth of microorganisms tested in present study. The reference control (streptomycin sulfate), inhibited the growth of all bacteria tested in this study at 10 µg/ml except *P. aeruginosa* which exhibited the MIC of 50 µg/ml (Table 3.4). Our results are confirming the findings of Bruni *et al.,* (2006). They investigated the antimicrobial activity of the hydroalcoholic extract of *M. krukovii* bark of which it was completely inactive at 1000 mg/ml against both Gram (+) and Gram (−) bacteria. In their experiment, the efficacy of the extract on fungal strains was shown weak. It seems there are some variations in antimicrobial activity of genus *Maytenus*. In a very recent investigation, among the triterpenes isolated from *M. blepharodes*, the MIC value of 'zeylasterone'

showed a higher activity against the two *S. aureus* strains evaluated, while 'demethylzeylasterone' was inactive (MIC> 40 µg/ml) (de. Leon *et al.,* 2010). It was found the methanol extract of the leaves of *M. ilicifolia* was inactive against *B. subtilis*, *P. aeruginosa* and *S. aureus* (Oliveira *et al.,* 2007). '3α-Hydroxyolean-12-en-27-oic acid' and '3β-hydroxyolean-12-en-27-oic acid' isolated from the root of *Aceriphyllum rossii* inhibited the growth of *S. aureus*, with MIC value of 128 µg/ml (Zheng *et al.,* 2008).

L.M.P exhibited antioxidant properties both extra and intracellular which clarifies its slight oxidation action. In contrast, compounds **3** and **5** exhibited marginal antioxidant activity (< 40%). Their weak antioxidant potential might be a logical explanation for enhancement of ROS levels at higher concentrations *in vitro*. Therefore, ROS generation might be a part of the mechanisms by which these compounds induce apoptosis in HeLa cells.

However, L.M.P and pure compounds 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 also been reported previously (Turley *et al.,* 1997; Yang *et al.,* 2006; Zou *et al.,* 2001).

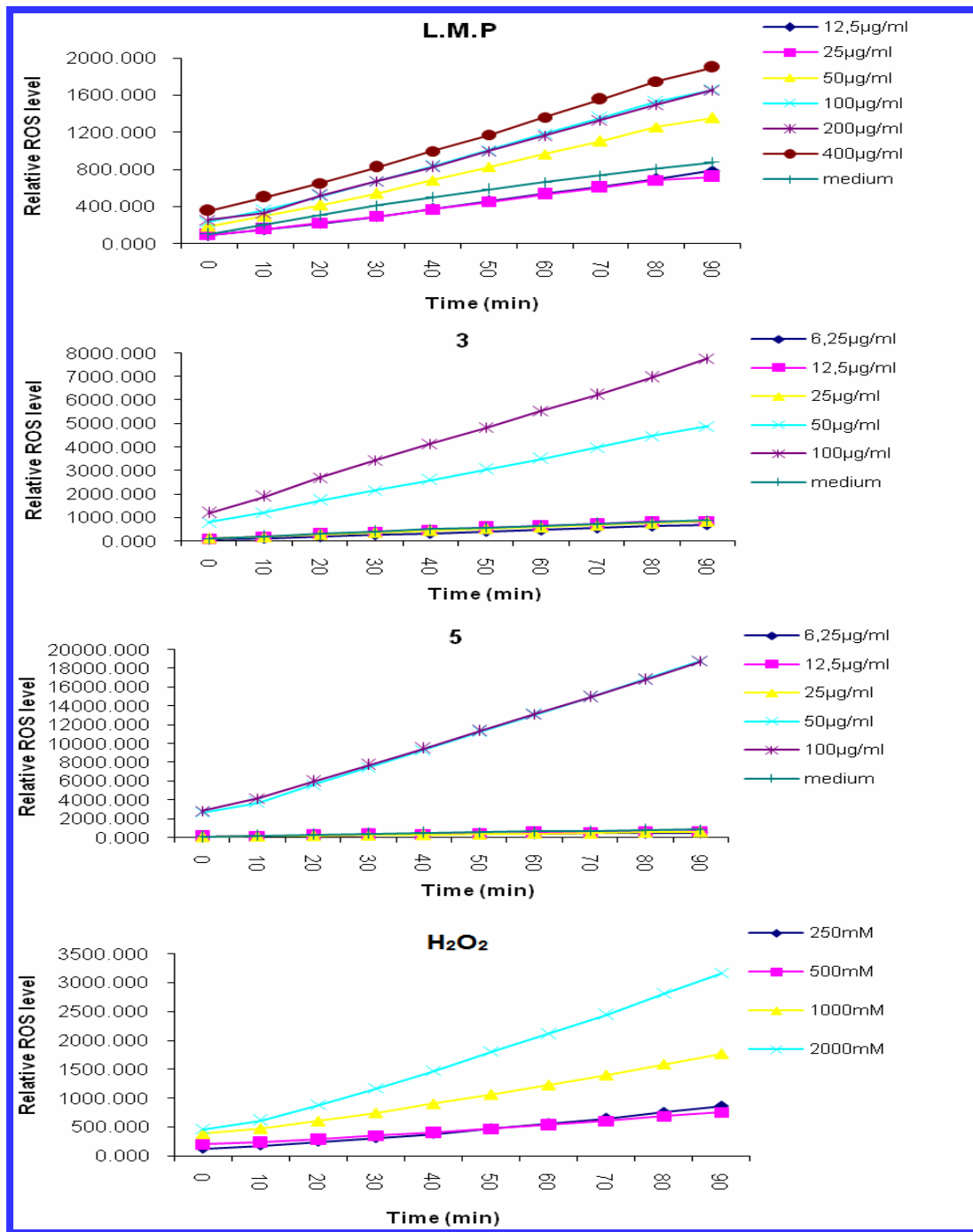**Figure 3.17:** Time-response curve of increase of relative ROS level (DCF fluorescence) in HeLa cells after 90 minutes exposure to various concentrations of the ethanolic/acetonic extract of the leaves of *M. procumbens* (L.M.P), '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'. Each data point represents the mean of data from three wells (n = 3).

Table 3.4: Minimum inhibitory concentrations (MICs) of the ethanolic/acetonic extract of the leaves of *M. procumbens* (L.M.P) and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' against selected bacteria and fungi.

	MIC[a] (mg/ml)				
Samples	**L.M.P.[b]**	**3[c]**	**5[d]**	**e**	**f**
Bacterium					
B. subtilis (+)	-	-	-	0.01	N
S. aureus (+)	2	0.4	0.2	0.01	N
E. coli (-)	-	0.4	-	0.01	N
P. aeruginosa (-)	8	-	-	0.05	N
C. albicans	-	-	-	N	0.001
A. niger	-	-	-	N	0.0002

[a] minimum inhibitory concentration.
[b] *M. procumbens* (acetone/ethanol extract of leaves).
[c] 30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**.
[d] 30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**.
[e] Streptomycin sulfate.
[f] Amphotricin B.
[-] MICs were more than the highest concentration tested.
[N] not tested.
[±] Gram statues.

Overall, compounds **3** and **5** were 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 their clinical application 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, two new triterpenes were isolated from L.M.P for the first time.

There is no report until date on the anticancer, antioxidant, and antibacterial properties of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens* (L.M.P), '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

As compounds **3** and **5** were found to be novel, there is limited information about their 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. The results indicated that *M. procumbens* and its isolated constituent possess potential therapeutic properties. As triterpenoids play their anticancer roles through various mechanisms, the other possible mechanism/s of action should still be determined in future studies. Regarding oxidant/antioxidant effects of this herb in various cell lines, steps should be put forward to examine its efficacy in some relevant disease (Hassani-Ranjbar *et al.,* 2009). Additionally, the other possible biological activities of this genus and new cyclic terpenes should be discovered.

3.5. CONCLUSION

Ovarian, epidermoid carcinoma, melanoma, and leukemic cell lines could be considered as next candidates for future cytotoxic assays. Several triterpenoids, including ursolic acid, oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol, and avicins possess antitumor property and are evaluated for their cytotoxicity in mammalian cancer models *in vivo.*

Therefore, the elucidation of the mechanisms by which the crude extract and pure compounds induce apoptosis in different cancer cells will be helpful for better

understanding new apoptotic signaling pathways and will benefit clinical application in the prevention and treatment of cancer. 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 purified compounds. These compounds might be worth considering as new anticancer agents alone or in combination with other antiproliferative drugs.

Despite the large number of molecules exhibiting anti-cancer properties *in vitro*, only some of them are able to induce an effective antiproliferation effect measurable in clinical trials. This gap between *in vitro* and *in vivo* studies suggests that new strategies are needed for discovering new anticancer drugs and validating their efficacy and safety. For instance, set up better easier *in vivo* models can be very much helpful. The combination of two or more agents acting on different mechanisms to produce a synergistic anticancer effect should be considered.

3.6. REFERENCES

Abdollahi, M., Mostafalou, S., Pournourmohammadi, S. and Shadnia, S., 2004. Oxidative stress and cholinesterase inhibition in saliva and plasma of rats following sub-chronic exposure to malathion. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology., 137: 29-34.

Ablise, M., Leininger-Muller, B., Wong, C.D., Siest, G., Loppinet, V. and Visvikis, S., 2004. Synthesis and *in vitro* antioxidant activity of glycyrrhetinic acid derivatives tested with the cytochrome P450/NADPH system. Chemical and Pharmaceutical Bulletin., 52: 1436-1439.

Ahmad, I. and Beg, A.Z., 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology., 74: 113-133.

Avilla, J., Teixido, A., Velazquez, C., Alvarenga, N., Ferro, S. and Canela, R., 2000. Insecticidal Activity of Maytenus Species (Celastraceae) Nortriterpene Quinone Methides against Codling Moth, Cydia pomonella (L.) (Lepidoptera: Tortricidae). Journal of Agricultural and Food Chemistry., 48: 88-92.

Benzie, I.F. and Strain, J.J., 1996. Ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Analytical Biochemistry., 239: 70-76.

Bruni, R., Rossi, D., Muzzoli, M., Romagnoli, D., Paganetto, G., Besco, E., Choquecillo, F., Peralta, K., Lora, W.S. and Sacchetti, G., 2006. Antimutagenic, antioxidant and antimicrobial properties of *Maytenus krukovii* bark. Fitoterapia., 77: 538-545.

Bruno, M., Savona, G., Hueso-Rodriguez, J.A., Pascual, C. and Rodriguez, B., 1987. Ursane and oleanane triterpenoids from *Salvia argentea*. Phytochemistry., 26: 497-501.

Cefarelli, G., D'Abrosca, B., Fiorentino, A., Izzo, A., Mastellone, C., Pacifico, S. and Piscopo, V., 2006. Free-Radical-Scavenging and Antioxidant Activities of Secondary Metabolites from Reddened Cv. Annurca Apple Fruits. Journal of Agricultural and Food Chemistry., 54: 803-809.

Chen, T.K., Ales, D.C., Baenziger, N.C. and Wiemer, D.F., 1983. Ant-repellent triterpenoids from *Cordia alliodora*. Journal of Organic Chemistry., 48: 3525-3531.

Cho, C.W., Choi, D.S., Cardone, M.H., Kim, C.W., Sinskey, A.G. and Rha, C., 2006. Glioblastoma cell death induced by asiatic acid. Cell Biology and Toxicology., 22: 393-408.

Coates Palgrave, K., 2002. Trees of Southern Africa, Third ed. Struik Publishers (Pty) Ltd, Cape Town, South Africa.

Cordeiro, P.J.M., Vilegas, J.H.Y. and Lanças, F.M., 1999. HRGC-MS Analysis of Terpenoids from *Maytenus ilicifolia* and *Maytenus aquifolium* ("Espinheira Santa"). Journal of Brazilian Chemical Society., 6: 523-526.

Corsino, J., Furlam, M., Bolzani, V.D.A.S., Pereira, A.M.S. and Franca, S.E., 1998. Further sesquiterpene pyridine alkaloids from *Maytenus aquifolium*. Phytochemistry., 49: 2181-2183.

Costa, P.M., Ferreira, P.M.P., Bolzani, V.S., Furlan, M., dos Santos, V.A.F.M., Corsino, J., de Moraes, M.O., Costa-Lotufo, L.V., Montenegro, R.C. and Pessoa, C., 2008. Antiproliferative activity of pristimerin isolated from *Maytenus ilicifolia* (Celastraceae) in human HL-60 cells. Toxicology *In Vitro*., 22: 854-863.

Cragg, G.M. and Newman D.J., 2005. Plants as a source of anti-cancer agents. Perspective paper. Journal of Ethnopharmacology., 100: 72-79.

da Silva, G., Serrano, R. and Silva, O., 2011. *Maytenus heterophylla* and *Maytenus senegalensis*, two traditional herbal medicines. Journal of Natural Science, Biology and Medicine., 2: 59-65.

De la Torre, M.C., Bruno, M., Piozzi, F., Savona, G., Rodriguez, B. and Arnold, N.A., 1990. Terpenoids from *Salvia willeana* and *S. virgata*. Phytochemistry., 29: 668-670.

Deeb, K.H.E., Al-Haidari, R.A., Mossa, J.S. and Ateya, A.M., 2004. Phytochemical and pharmacological studies of *Mytenus forsskaoliana*. Saudi Pharmaceutical Journal., 11: 184-191.

deLeon, L., Lopez, M.R. and Moujir, L., 2010. Antibacterial properties of zeylasterone, a triterpenoid isolated from *Maytenus blepharodes*, against *Staphylococcus aureus*. Microbiology Research., 165: 617-626.

du Toit, R., Volsteedt, Y. and Apostolides, Z., 2001. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology., 166: 63-69.

Duddeck, H., Elgamal, M.H.A., Ricca, G.S., Danieli, B. and Palmisano, G., 1978. Carbon-13 nuclear magnetic resonance spectra. VIII-18α- and 18β-glycyrrhetic acid derivatives. Organic Magnetic Resonance., 11: 130-132.

Fruehauf, J.P. and Meyskens, F.L., 2007. Reactive oxygen species: a breath of life or death? Clinical Cancer Research., 13: 789-794.

Guyton K.Z. and Kensler, T.W., 1993. Oxidative mechanisms in carcinogenesis. British Medical Bulletin., 49: 523-544.

Ghazanfar, S.A., 1994. Handbook of Arabian medicinal plants, CRC Press, Boca Raton., p. 83.

Gonzalez, A.G., Jimenez, I.A., Ravelo, A.G., Sazatornil, G. and Bazzocchi, I.L., 1993. New sesquiterpene with antifeedent activity from *Maytenus canariensis* (Celastraceae). Tetrahedron., 49: 697-702.

Gonzalez, A.G., Tincusi, B.M., Bazzocchi, I.L., Tokuda, H., Nishino, H., Konoshima, T., Jimenez, I.A. and Ravelo, A.G., 2000. Anti-tumor promoting effects of sesquiterpenes from *Maytenus cuzcoina* (celastraceae). Bioorganic and Medicinal Chemistry., 7: 1773-1778.

Gonzaleza, A.G., Kennedya, M.L., Rodrigueza, F.M., Bazzocchi, I.L., Jimeneza, I.A., Raveloa, A.G. and Moujirb, L., 2001. Absolute configuration of triterpene dimmers from *Maytenus* species (Celastraceae). Tetrahedron., 57: 1283-1287.

Gutierrez, F., Estevez-Braun, A., Ravelo, A.G., Astudillo, L. and Zarate, R., 2007. Terpenoids from the medicinal plant *Maytenus ilicifolia*. Journal of Natural Products., 6: 1049-1052.

Hasani-Ranjbar, S., Larijani, B. and Abdollahi, M., 2009. A systematic review of the potential herbal sources of future drugs effective in oxidant-related diseases. Inflamm. Allergy Drug Targets., 8: 2-10.

Hong, S., 2000. New Taxa of *Maytenus molina* in China. Bulletin of Botanical Research (BBR).

Hui, T., Feng, L., Xiao, W., Hong, L. and Xi-Yang, H., 2009. Advance of Researches on medicinal plants of *Maytenus*. Hubei Agricultural Sciences.

Johns, S.R., Lamberton, J.A., Morton, T.C., Suares, H. and Willing, R.I., 1983. Triterpenes of *Lantana tiliaefolia*. 23-Hydroxy--3-oxours-12-en-28-oic acid, a new triterpene. Australian Journal of Chemistry., 36: 2537-2547.

Kloucek, P., Polesny, Z., Svobodova, B., Vlkova, E. and Kokoska, L., 2005. Antibacterial screening of some Peruvian medicinal plants used in Calleria District. Journal of Ethnopharmacology., 2: 309-312.

Kolanjiappan, K., Ramachandran, C.R. and Manoharan, S., 2003. Biochemical changes in tumor tissues of oral cancer patients. Clinical Biochemistry., 36: 61-65.

Kuo, Y.H., King, M.L., Chen, C.F., Chen, C.H., Chen, K. and Lee, K.H., 1994.Two new macrolide sesquiterpene pyridine alkaloids from *Maytenus emarginata*: emarginatine G and the cytotoxic emarginatine F. Journal of Natural Products., 57: 263-269.

Lee, K.H., Nozaki, H., Hall, I.H., Kasai, R., Hirayama, T.; Suzuki, H., Wu, R.Y. and Huang, H.C., 1982. Antitumor agents 60. Maytansine, an antileukemic principle from *Maytenus diversifolia*. Journal of Natural Products., 45: 509-510.

Leite, G.V.P., Rastrelli, L., Romussi, G., Oliveira, A.b., Vilegas, J.H.Y., Vilegas, W. and Pizza, C., 2001. Isolation and HPLC quantitative analysis of flavonoid glycosides from Brazilian beverages (*Maytenus ilicifolia* and *M. aquifolium*). Journal of Agricultural and Food Chemistry., 8: 3796-3801.

Lindsey, K.L., Matu, E.N. and van Staden, J., 2006. Antibacterial activity of extracts from in vitro grown *Maytenus senegalensis* root cultures. South African Journal of Botany., 72: 310-312.

Liu, J., Shen, H.M. and Ong, C.N., 2001. Role of intracellular thiol depletion, mitochondrial dysfunction and reactive oxygen species in *Salvia Miltiorrhiza*-induced apoptosis in human hepatoma HepG2 cells. Life Science., 69: 1833-1850.

Liu, J., Shen, H.M. and Ong, C.N., 2000. *Salvia Miltiorrhiza* inhibits cell growth and induces apoptosis in human hepatoma HepG2 cells. Cancer Letters., 153: 85-93.

Martuccielloa, S., Balestrierib, M.L., Feliceb, F., Estevamc, C.S., Sant'Anac, A.E.G., Pizzad, C. and Piacente, S., 2010. Effects of triterpene derivatives from *Maytenus rigida* on VEGF-induced Kaposi's sarcoma cell proliferation. Chemico-Biological Interactions., 183: 450-454.

Matu, E.N. and van Staden, J., 2003. Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. Journal of Ethnopharmacology., 87: 35-41.

Momtaz, S., Lall, N., Hussein, A., Ostad, S.N. and Abdollahi. M., 2010. Investigation of the possible biological activities of a poisonous South African plant; *Hyaenanche globosa* (Euphorbiaceae). Pharmacognosy Magazine., 6: 34-41.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods., 65: 55-63.

Muhammad, I., El Sayed, K.A., Mossa, J.S., Al-Said, M.S., El-Feraly, F.S., Clark, A.M., Hufford, C.D., Oh, S. and Mayer, A.M.S., 2000. Bioactive 12-oleanene triterpene and secotriterpene acids from *Maytenus undata*. Journal of Natural Products., 63: 605-610.

Muregi, F.W., Ishih, A., Suzuki, T., Kino, H., Amano, T., Mkoji, G.M., Miyate, T. and Terada, M., 2007. *In vivo* antimalarial activity of aqueous extracts from Kenyan medicinal plants and their chloroquine (CQ) potentiation effects against a blood-induced CQ-resistant rodent parasite in mice. Phytotherapy Research., 21: 337-43.

Nick, A., Wright, A.D., Rali, T. and Sticher, O., 1995. Antibacterial triterpenoids from *Dillenia papuana* and their structure-activity relationships. Phytochemistry., 40: 1691-1695.

Nick, A., Wright, A.D., Sticher, O. and Rali, T., 1994. Antibacterial triterpenoid acids from *Dillenia papuana*. Journal of Natural Products., 57: 1245-1250.

O'Brien, J., Wilson, I., Orton, T. and Pognan, T., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Journal of Biochemistry., 267: 5421-5426.

Ohsaki, A., Imai, Y., Naruse, M., Ayabe, S., Komiyama, K. and Takashima, J., 2004. Four new triterpenoids from *Maytenus ilicifolia*. Journal of Natural Products., 67: 469-471.

Oliveira, D.F., Pereira, A.C., Figueiredo, H.C.P., Carvalho, D.A., Silva, G., Nunes, A.S., Alves, D.S. and Carvalho, H.W.P., 2007. Antibacterial activity of plant extracts from Brazilian southeast region. Fitoterapia., 78: 142-145.

Orabi, K.Y., Al-Qasoumi, S.I., El-Olemy, M.M., Mossa, J.S. and Muhammad, L., 2001. Dihydroagarofuran alkaloid and triterpenes from *Maytenus heterophylla* and *Maytenus arbutifolia*. Phytochemistry., 58: 475-480.

Park, B.C., Bosire, K.O., Lee, E.S., Lee, Y.S. and Kim, J.A., 2004. Asiatic acid induces apoptosis in SK-MEL-2 human melanoma cells. Cancer Letters., 218: 81-90.

Pelicano, H., Carney, D. and Huang, P., 2004. ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates., 7: 97-110.

Prior, R.L. and Cao, G., 1999. *In vivo* total antioxidant capacity: comparison of different analytical methods. Free Radical Biology & Medicine., 27: 1173-1181.

Queiroga, C.L., Silvaa, G.F., Diasb, P.C., Possentib, A. and Carvalho, G.M., 2000. Evaluation of the antiulcerogenic activity of friedelan-3β-ol and friedelin isolated from *Maytenus ilicifolia* (Celastraceae). Short Communication. Journal of Ethnopharmacology., 72: 465-468.

Salazar, G.D.C.M., Silva, G.D.F. and de Sousa, J.R., 1997. Chemical constituents from bark wood and leaves of *Maytenus floribunda* (Reiss). International Society for Horticultural Science., ISHS Acta Horticulturae 501: II WOCMAP Congress Medicinal and Aromatic Plants, Part 2: Pharmacognosy, Pharmacology, Phytomedicine, Toxicology.

Sannomiya, M., Vilegas, W., Rastrelli, L. and Pizza, C.A., 1998. Flavonoid glycoside from *Maytenus aquifolium*. Phytochemistry., 49: 237-239.

Sarkhail, P., Rahmanipour, S., Fadyevatan, S., Mohammadirad, A., Dehghan, G., Amin, G., Shafiee, A. and Abdollahi, M., 2007. Antidiabetic effect of *Phlomis anisodonta*: Effects on hepatic cells lipid peroxidation and antioxidant enzymes in experimental diabetes. Pharmacology Research., 569: 261-6.

Shibata, S., Takahashi, K., Yano, S., Harada, M., Saito, H., Tamura, Y., Kumagai, A., Hirabayashi, K., Yamamoto, M. and Nagata, N., 1987. Chemical modification of glycyrrhetinic acid in relation to the biological activities. Chemical and Pharmaceutical Bulletin., 35: 1910-1918.

Shirota, O., Tamemura, T., Morita, H., Takeya, K. and Itokawa, H., 1996. Triterpenes from Brazilian medicinal plant chuchuhausi (*Maytenus krukovii*). Journal of Natural Products., 59: 1072-1075.

Sies, H., 1985. Oxidative stress. Introductory remarks. In: Oxidative Stress (Sies, H., ed.), pp. 1-8. Academic Press, London, U.K.

Singh, N. P., McCoy, M.T., Tice, R.R. and Schneider, E.L.A., 1988. Simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research., 175: 184-191.

Souza-Formigonia, M.L.O., Oliveira, M.G.M., Monteiroa, M.G., de Silveira-Filhoa, N.G., Braza, S. and Carlinia, E.A., 1991. Antiulcerogenic effects of two *Maytenus* species in laboratory animals. Journal of Ethnopharmacology., 34: 21-27.

Spivey, A.C., Weston, M. and Woodhead, M., 2002. Celastraceae sesquiterpenoids: biological activity and synthesis. Chemical Society Reviews., 31: 43-59.

Sun, S.Y., Li, W., Yue, P., Lippman, S.M., Hong, W.K. and Lotan, R., 1999. Mediation of N-(4-Hydroxyphenyl)retinamide-induced apoptosis in human cancer cells by different mechanisms. Cancer Research., 59: 2493-2498.

Tambekar, D.H. and Khante, B.S., 2010. Antibacterial properties of traditionally used plants for enteric infections by Adivasi,s (Bhumka) in Melghat forest (AMRAVATI DISTRICT). International Journal of Pharmaceutical Sciences and Research., 1: 120-128.

Turley, J.M., Fu, T., Ruscetti, F.W., Mikovits, J.A., Bertolette III, D.C. and Birchenall-Roberts, M.C., 1997. Vitamin E succinate induces Fas-mediated apoptosis in estrogen receptor-negative human breast cancer cells. Cancer Research., 57: 881-890.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions., 160: 1-40.

Vellosa, J.C.R., Barbosa, V.F., Khalil, N.M., Santos, V.A.F.M.M., Furlan, M., Brunetti, I.L. and Oliveira, O.M.M.F., 2007. Profile of *Maytenus aquifolium* action over free radicals and reactive oxygen species. Brazilian Journal of Pharmaceutical Sciences., 43: 447-453.

Veskoukis, A.S., Tsatsakis, A.M. and Kouretas, D., 2012. Dietary oxidative stress and antioxidant defense with an emphasis on plant extract administration. Cell Stress Chaperones.,17: 11-21.

Wang, H. and Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology and Medicine., 27: 612-616.

Yang, H.L., Chen, C.H., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T. and Hseu, Y.C., 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by Antrodia camphorate. Cancer Letters., 231: 215-227.

Yang, Z.G., Li, H.R., Wang, L.Y., Li, Y.H., Lu, S.G., Wen, X.F., Wang, J., Daikonya, A. and Kitanaka, S., 2007. Triterpenoids from *Hippophae rhamnoides* L. and their nitric oxide. Chemical and Pharmaceutical Bulletin., 55: 15-18.

Zheng, C.J., Sohn, M.J., Kim, K.Y., Yu, H.E. and Kim, W.G., 2008. Olean-27-carboxylic acid-type triterpenes with potent antibacterial activity from *Aceriphyllum rossii*. Journal of Agricultural and Food Chemistry., 56: 11752-11756.

Zou, C., Liebert, M., Zou, C., Grossman, H.B. and Lotan, R., 2001. Identification of effective retinoids for inhibiting growth and inducing apoptosis in bladder cancer cells. Journal of Urology., 165: 986-992.

Zurita, F.J., Palmares, E.E.F., Lupianez, J.A. and Cascante, M., 2009. Maslinic acid, a natural triterpene from *Olea europaea* L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. Cancer Letters., 273: 44-54.