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LIST OF ABBREVIATIONS

Annexin V-FITC	Annexin V-fluorescein isothiocyanate
ATP	Adenosine triphosphate
^{13}C-NMR	Carbon nuclear magnetic resonance
CoQ	Coenzyme Q
COSY	Correlation Spectroscopy
Da	Dalton
DAPI	4′-6-Diamidino-2-phenylindole
DCF-DA	2,7-Dichlorofluorescin diacetate
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,2-Diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
FasL	Fas ligand
FBS	Fetal bovine serum
FRAP	Ferric-reducing antioxidant power

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FSC ..... Forward scatter

HBSS ..... Hanks' balanced salt solution

H. globosa ..... *Hyaenanche globosa*

1H-NMR ..... Proton nuclear magnetic resonance

HMBC ..... Heteronuclear Multiple Bond Coherence

HSQC ..... Heteronuclear Single Quantum Coherence Spectroscopy

IC_{50} Concentration of an inhibitor that is required for 50% for inhibition of its target

IR ..... Infra red

LMPA ..... Low Melting Point Agarose

MDA ..... Malondialdehyde

MH ..... Mueller Hinton

MIC ..... Minimum inhibitory concentration

MLL ..... Myeloid/lymphoid or mixed-lineage leukemia

M. procumbens ..... *Maytenus procumbens*

MTT ..... 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

NCBI ..... National Cell Bank of Iran

NCHS ..... National Cancer for Health Statistics

NCI ..... National Cancer Institute

NMA ..... Normal Melting Agarose

NMR ..... Nuclear Magnetic Resonance Spectroscopy

NOESY ..... Nuclear Overhauser Effect Spectroscopy

NSAIDs ..... Non-steroidal anti-inflammatory drugs

OA ..... Oleanolic acid

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OTM ..... Olive tail moment

PI ..... Propidium iodide

PS ..... Phosphatidyl serine

RSC ..... Radical scavenging capacity

ROS ..... Reactive oxygen species

RPMI ..... Roswell park memorial institute

SA ..... South Africa

SCGE ..... Single cell gel electrophoresis

SD ..... Sabouraud dextrose

SERMs ..... Selective estrogen receptor modulators

SO ..... Synthetic oleanane triterpenoids

SSC ..... Side scatter

TBA ..... 2-Thiobarbituric acid

TBARS ..... Thiobarbituric acid reactive substance

TCA ..... Trichloroacetic acid

TGF-â ..... Transforming growth factor

TLC ..... Thin layer chromatography

TM ..... Tail moment

TNF ..... Tumor necrosis factor

TRAIL ..... TNF-related apoptosis inducing ligand

TPTZ 2,4,6-Tripyridyl-s-triazine

UA ..... Ursolic acid

UV ..... Ultra violet

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US	.....	United State
VCR	.....	Vincristine
VDS	.....	Vindesine
VLB	.....	Vinblastine
VRLB	.....	Vinorelbine
WHO	.....	World Health Organization

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SUMMARY

AN EVALUATION OF ANTI-CANCER ACTIVITIES OF *HYAENANCHE GLOBOSA* LAMB. (EUPHORBIACEAE) AND *MAYTENUS PROCUMBENS (L.F.) LOES.* (CELASTRACEAE)

BY

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Written records about medicinal plants date back at least 5,000 years to the Sumerians. The objected plants for present investigation were indigenous to South Africa and as explored, only a few biological studies were found on the previous studies on *Hyaenanche globosa* and *Maytenus procumbens*.

Phytochemical studies of the ethanol extract of the fruits of *H. globosa* (F.E) resulted in isolation of two known pure sesquiterpene lactones; 'tutin **1**' and 'hyenanchin **2**'. The crude extract and its isolated constituents were tested on four cancerous and a

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normal cell lines. F.E exhibited the highest antiproliferative activity on HeLa cells which followed by Caco-2 cells. None of the isolated compounds were found to be toxic to the cells tested in this experiment. F.E demonstrated potent inhibition of DPPH radical activity similar to vitamin C. 'Tutin **1**' and 'hyenanchin **2**' were found with marginal antioxidant activity of which 'compound **1**' presented more potent activity than 'compound **2**'. The amounts of ROS radicals formed by pure compounds (**1** and **2**) were not significantly higher than those of controls. This is the first report on phytochemical index, anticancer, antioxidant and antibacterial properties of F.E and its purified compounds.

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%). 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 therefore be concluded that the active components in L.M.P might serve as a

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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. There is no report until date on phytochemical index, anticancer, antioxidant and antibacterial properties of L.M.P and its isolated compounds.

Keywords: *Hyaenanche globosa*; *Maytenus procumbens*; Cytotoxicity; Antioxidant

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ABSTRACT

ABSTRACT

A variety of plant species have been identified traditionally as well as in scientific literatures for their cytotoxicity against cancer cells. According to statistics, cancer is the second leading cause of death after cardiovascular diseases worldwide. The inadequacy of current therapies to treat cancer as well as high toxicity, expenses, and mutagenicity of existing anticancer drugs prompted to seek new agents from plants. The purpose of present study was to determine whether *Hyaenanche globosa* Lamb. (Euphorbiaceae) and *Maytenus procumbens* (L.F.) Loes. (Celastraceae) contain constituents that can inhibit the growth of human cancer cells, and therefore, might eventually be useful in the prevention or treatment of cancer.

Ethanol extract of *H. globosa* (fruits) (F.E) and the ethanolic/acetonic extract of *M. procumbens* (leaves) (L.M.P) were evaluated for growth inhibitory activity using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) MTT cytotoxicity assay against different cancer cell lines. F.E showed 50% inhibitory concentration (IC_{50}) values of 50.10, 37.80, 94.30, and 96.80 µg/ml on cancerous cell lines; Human Colorectal adenocarcinoma (Caco-2), Human Cervical adenocarcinoma (HeLa), Human epithelial-like Colon carcinoma (HT29) and Human Breast ductal-carcinoma (T47D), respectively. Besides, F.E reduced growth rate in non-cancerous NIH3T3 (Swiss mouse embryo fibroblast) cells with IC_{50} of 91.80 µg/ml.

F.E exhibited the IC_{50} of 37.80 µg/ml on the viability of HeLa cells, thus subsequently was fractionated using phase-partitioning with *n*-hexane, ethyl acetate, and *n*-butanol. The *n*-hexane fraction demonstrated the highest inhibition of cell growth/proliferation (IC_{50}; 56.10 µg/ml) in the HeLa cells. Therefore, this fraction was subjected to further

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separation by chromatographic methods. Two pure compounds belonging to sesquiterpene class of compounds known as: 'tutin' (compound **1**) and 'hyenanchin' (compound **2**), were isolated and their structures were determined by NMR spectroscopic methods. Unpredictably, none of them showed significant ($p< 0.05$) inhibition on cell viability/proliferation at the highest concentration (100 µg/ml) that were used.

Antioxidant/oxidant activities of *H. globosa* (F.E) and its isolated compounds was determined extracellularly (1,2-diphenyl-2-picrylhydrazyl) (DPPH) antioxidant assay, and intracellularly (in cultured HeLa cells) by three methods; ferric reducing/antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and measurement of intracellular reactive oxygen species (ROS) assays.

H. globosa (F.E) demonstrated potent inhibition of DPPH radical activity similar to vitamin C (positive control). Almost 90% at concentrations ranging from 7.8 to 1000 µg/ml. Compounds **1** and **2** were found with marginal antioxidant activity of which 'compound **1**' showed more potent activity than 'compound **2**'. In the present study, it was found that, F.E enhanced the FRAP content in HeLa cells almost 4-fold to that of control group at concentrations of 50-400 µg/ml ($P< 0.05$). Compounds **1** and **2** exhibited the highest FRAP values of 3.60 and 3.00 mM at 100 µg/ml versus 1.20 mM in control cells ($P< 0.05$).

As a marker of lipid peroxidation, different concentrations of compounds **1** and **2** were incubated with HeLa cells, consequently variation in cell TBARS were assessed. According to the results obtained, none of experimental samples could enhance the HeLa cells TBARS versus control cells significantly. The level of reactive oxygen species enhanced by F.E was only at 400 µg/ml (approximately 1-fold), whereas the amount of ROS radicals formed by compounds **1** and **2** were not significantly higher than those of controls.

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The antibacterial activities of the extracts of *H. globosa* (ethanol extract) and purified compounds **1** and **2** were assessed using Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), and Gram-negative bacteria (*Escherichia coli* & *Pseudomonas aeruginosa*). Their antifungal activities were assayed using *Candida albicans* and *Aspergillus niger*.

The minimum inhibitory concentration (MIC) of samples values of ethanol extract of F.E was found to be 1, 1, 8 and 2 mg/ml against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginos*, respectively. Amongst pure compounds, only 'compound **1**' showed inhibitory activity exhibiting MICs of 400 and 800 µg/ml for *S. aureus* and *P. aeruginosa,* respectively. None of samples inhibited the growth of fungi tested at the highest concentrations (8 mg/ml for crude extracts and 400 µg/ml for pure compounds) when tested in present study.

The present study reports for the first time the anticancer, anti/prooxidant, and antibacterial activity of the ethanol extract of *Hyaenanche globosa* and its purified compounds; 'tutin **1**' and 'hyenanchin **2**'.

Secondly, the acetone/ethanol extract of *M. procumbens* (leaves) (L.M.P) was assessed for growth inhibitory activity using MTT cytotoxicity assay against different cancer cell lines. L.M.P exhibited IC_{50} values of 68.80, 51.20, 78.50, 76.60 and 76.65 µg/ml on experimental cell lines; Caco-2, HeLa, HT29, NIH3T3 and T47D, respectively.

L.M.P showed the IC_{50} of 51.20 µg/ml on the viability/proliferation of HeLa cells and later bioassay guided fractionation led to the isolation and identification of two new triterpenes: '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one' (compound **3**) and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one' (compound **5**). In addition, a known terpene: 'asiatic acid' (compound **4**) was purified. Due to the insufficient amount of 'asiatic acid **4**', this compound was not tested for cytotoxicity and mechanistic studies.

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'Compound **3**' showed IC_{50} values of 45.50, 44.00, 62.80, 45.75, and 66.10 µg/ml on experimental cell lines; Caco-2, HeLa, HT29, NIH3T3 and T47D, respectively. Newly isolated 'compound **5**' exhibited the IC_{50} (µg/ml) values of Caco-2 (42.70), HeLa (27.60), HT29 (61.40), NIH3T3 (46.00), and T47D (30.60). Both compounds were found to be toxic to the non-cancerous fibroblast NIH3T3 cells. Compounds **3** and **5** have not been isolated before from any plant species and this is a first report of their antiproliferation activities.

Following the MTT assays, the induction of apoptosis by compounds **3** and **5** (at the concentration of their IC_{50}) were investigated in HeLa cells. The affinity of compounds **3** and **5** for Annexin V and PI were determined through microscopic and flow cytometric analysis. Compounds **3** and **5** induced apoptosis in HeLa cells at their IC_{50} concentrations. The percentage of apoptosis elevated up to 73.20% and 20.40% by compounds **3** and **5** in HeLa cells, respectively versus control group (0.40%).

Single gel electrophoresis (comet) method was utilized to highlight the percentage of DNA damaged caused by compounds **3** and **5** *in vitro*. As data exerted, significant elevation of DNA damage in concept of tail moment (TM) were detected in cultured human HeLa cells by compounds **3** and **5**. Additionally, 'compound **3**' significantly increased tail length, comet length, TM and OTM (Olive tail moment) to 12.80%, 30.40%, 4.90%, and 3.00%, respectively when exposed to HeLa cells at its IC_{50} concentration (44.00 µg/ml) ($P< 0.05$). The percentage of tail length, comet length, TM and OTM were found to be 3.10%, 25.65%, 0.20% and 0.40% in control group. 'Compound **3**' appeared to be more genetoxic than 'compound **5**'.

Antioxidant/pro-oxidant activity of *M. procumbens* (L.M.P) and their isolated compounds was determined in the same manners as F.E; extracellularly (DPPH antioxidant assay) and intracellularly (in cultured HeLa cells) by three methods; FRAP, TBARS and ROS assays. The rate of DPPH discoloration was < 40% for 'compound **3**' while 'compound **5**' exhibited less than 35% antioxidant activity at all

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the concentrations tested after 15 and 30 minutes. None of pure compounds showed activity similar to vitamin C (positive control) with regard to DPPH inhibition. 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 results showed L.M.P, compounds **3** and **5** were not able to elevate the HeLa cells TBARS versus control cells significantly.

The ROS intensity of HeLa cells was elevated by L.M.P (1.5-2 fold) at the concentrations ranging from 50 to 400 µg/ml during 0-90 minutes ($P< 0.05$). 'Compound **3**' elevated the ROS level up to 5-fold and 8-fold compared to that of control at 50 and 100 µg/ml, concentrations respectively. The ROS contents rose up by 'compound **5**' to 21-fold to that of control cells at the same concentrations (50-100 µg/ml). L.M.P, compounds **3** and **5** showed a time and concentration-dependent function of ROS formation *in vitro*.

The antibacterial activities of the *M. procumbens* (acetone/ethanol extract) and compounds **3** and **5** were assessed using Gram-positive bacteria (*Bacillus subtilis* & *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* & *Pseudomonas aeruginosa*). Their antifungal activities were assayed using *Candida albicans* and *Aspergillus niger*.

L.M.P exhibited the MICs of 2 and 8 mg/ml against *S. aureus* and *P. aeruginosa*, respectively. None of L.M.P or its isolated compounds inhibited the growth of fungi tested at the highest concentration tested in present study (0.5-8 mg/ml for crude extract and 5-400 µg/ml for pure compounds). The antibacterial/fungal activities of the leaves of *M. procumbens* (acetone/ethanol extract), compounds **3** and **5** have not been reported previously.

The gradual reduction of antioxidant potential of L.M.P, compounds **3** and **5** might be a logical explanation for enhancement of ROS levels at higher concentrations *in vitro*.

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Therefore, ROS generation might be a part of the mechanisms by which compounds **3** and **5** induce apoptosis in HeLa cells. Thus, 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. Such a dual-property role for antioxidants has also been reported previously. In addition to genetical changes (as proved by comet assay) and the participation of ROS in mediating apoptosis induced by compounds **3** and **5**, other pathways may also be involved.

This is the first report on the isolation and identification of the chemical structures of two new triterpenes: 'compound **3**', 'compound **5**' and well known 'compound **4**' (asiatic acid) from the acetone/ethanoilc extract of the leaves of *M. procumbens* (L.M.P). Indeed, the current study has also reports for the first time on the biological activities (anticancer, anti/prooxidant activity, antibacterial) of L.M.P and its new isolated triterpenes. According to the positive antiproliferation activity of pure compounds isolated from *M. procumbens* found in this study, these compounds are worth considering for further studies due to their potential as anticancer agents in pre-clinical studies.

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CHAPTER 1

1.1. INTRODUCTION

1.1.1. Cancer

According to World Health Organization (WHO), 'cancer' is defined as a generic term for a group of more than 100 diseases that can affect any part of the body. The other synonyms that are used include malignant tumors and neoplasms. Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion that intrudes upon and destroys adjacent tissues, and sometimes metastasis, or spreading to other locations in the body via lymph or blood. Also known as a multifactor, multifaceted and multi-mechanistic disease enquiring a multidimensional approach for its treatment, control and prevention (Murthy *et al.,* 1990).

It is now widely recognized that cancer results from a series of genetic alterations causing a loss of normal growth controls, resulting in unregulated growth, lack of differentiation, apoptosis, genomic instability, and metastasis. Cancer knows no boundaries and can develop in any tissue of any organ at any age. However, one of the hallmarks of tumor development is a long latent period with no obvious clinical evidence of disease (Baudino, 2004).

The causes of cancer have been divided to two major categories: those with an environmental cause and those with a hereditary genetic cause. Cancer is primarily an environmental disease, though genetics influence the risk of some cancers. Generally, 90-95% of cases attributed to environmental factors and 5-10% due to genetics. Environmental, as used by cancer researchers, means any cause that is not genetic. Common environmental factors that contribute to cancer death include: tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation (both ionizing and non ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants

(Anand *et al.,* 2008). These environmental factors cause or enhance abnormalities in the genetic material of cells.

Roukos, (2009) reported the vast majority of cancers are non-hereditary, also are called 'sporadic cancers'. Hereditary cancers are primarily caused by an inherited genetic defect. Less than 0.3% of the populations are carriers of a genetic mutation which has a large effect on cancer risk. They cause less than 3-10% of all cancer. Some of the syndromes leading to hereditary cancer include:

- Inherited mutations in the genes BRCA1 and BRCA2 have more than 75% risk of breast cancer and ovarian cancer
- Li-Fraumeni syndrome (various tumors such as osteosarcoma, breast cancer, soft tissue sarcoma, brain tumors) due to mutations of p53
- Turcot syndrome (brain tumors and colonic polyposis)
- Familial adenomatous polyposis; an inherited mutation of the APC gene that leads to early onset of colon carcinoma.
- Hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch syndrome) can include familial cases of colon cancer, uterine cancer, gastric cancer, and ovarian cancer, without a preponderance of colon polyps.
- Retinoblastoma, when occurring in young children, is due to a hereditary mutation in the retinoblastoma gene.
- Down syndrome patients, who have an extra chromosome 21, are known to develop malignancies such as leukemia and testicular cancer, though the reasons for this difference are not well understood (Roukos, 2009).

Cancer is fundamentally a disease of failure to regulate tissue growth. In order for a normal cell to transform into a cancer cell, the genes, which regulate cell growth and differentiation, must be altered (Croce, 2008). Cell proliferation is an extremely complex process, which is normally regulated by several classes of genes including; 'oncogenes' and 'tumor suppressor genes'. The genes activated during the carcinogenesis process were termed oncogenes because they are capable of transforming certain cells by DNA transfection. Oncogenes may be normal genes, which are expressed at inappropriately

high levels, or altered genes with novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. The genes inactivated or lost in tumor cells were called 'anti-oncogenes' or 'tumor suppressor genes' because the normal unchanged genes were able to suppress tumorigenicity when reintroduced into tumor cells (Fusenig and Boukamp, 1998) (Fig 1.1). Tumor suppressor genes inhibit cell division, survival, or other properties of cancer cells. Malignant transformation can occur through the formation of novel oncogenes, the inappropriate over-expression of normal oncogenes, or by the under-expression or disabling of tumor suppressor genes. Typically, changes in many genes are required to transform a normal cell into a cancer cell (Knudson, 2001).

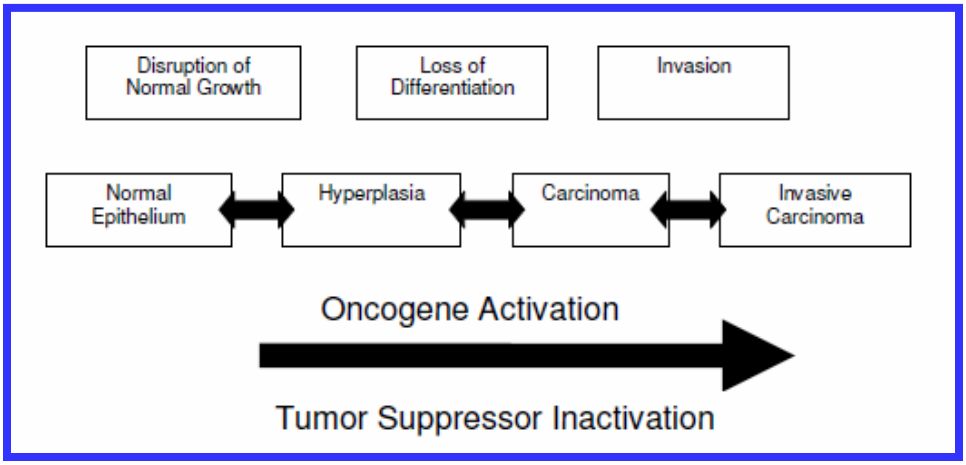

Figure 1.1: Carcinoma development and invasion. The upper row represents disturbances in growth, differentiation and tissue integrity that lead to the phenotypes that characterize the different stages of cancer shown in the lower row (Baudino, 2004).

1.1.1.1. Types of cancer

Cancers are classified by the type of cells that the tumor resembles and is therefore presumed to be the origin of the tumor. The main categories of cancer include:

- Carcinoma; cancer that begins in the skin or in tissues that line or cover internal organs.
- Sarcoma; cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

- Leukemia; cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- Lymphoma and myeloma; cancers that begin in the cells of the immune system.
- Central nervous system cancers; cancers that begin in the tissues of the brain and spinal cord (National Cancer Institute (NCI, 2010)).

1.1.1.2. Cancer stages

Staging describes the extent or spread of the disease at the time of diagnosis. Proper staging is essential in determining the choice of therapy and in assessing prognosis. A cancer's stage is based on the primary tumor's size and whether it has spread to other areas of the body (Cancer Facts & Figures, 2011). Along development in molecular properties of cancer, prognostic models have been developed for some cancer sites that incorporate biological markers and genetic features in addition to anatomical characteristics. A number of different staging systems are used to classify tumors:

- The TNM (Tumor, Node, Metastasis) staging system assesses tumors in three ways: extent of the primary tumor (T), absence or presence of regional lymph node involvement (N), and absence or presence of distant metastases (M).
- Once the T, N, and M are determined, a stage of I, II, III, or IV is assigned, with stage I being early and stage IV being advanced disease (Cancer Facts & Figures, 2011).
- Different systems of summary staging (in situ, local, regional, and distant) are used for descriptive and statistical analysis of tumor registry data. If cancer cells are present only in the layer of cells where they developed and have not spread, the stage is "in situ". If cancer cells have penetrated the original layer of tissue, the stage is "invasive". If an invasive malignant cancer confined entirely to the organ of origin, the stage is "local". If malignant cancer (1) has extended beyond the limits of the origin directly into surrounding organs or tissues; (2) involves regional lymph nodes by way of lymphatic system; or (3) has both regional extension and involvement of regional lymph nodes; the stage is "regional". If malignant cancer spreads to parts of the body remote from the primary tumor either by direct extension or by discontinuous metastasis to distant organs and tissues, or via the

lymphatic system to distant lymph nodes the stage is “distant” (Bagchi and Preuss, 2005; Cancer Facts & Figures, 2011).

Staging systems are specific for each type of cancer (e.g., breast cancer and lung cancer). Some cancers, however, do not have a staging system. Systems of staging may differ between diseases or specific manifestations of a disease. Cancer staging can be divided into a ‘clinical stage’ and a ‘pathologic stage’. Clinical stage is based on all of the available information obtained before a surgery to remove the tumor. Thus, it may include information about the tumor obtained by physical examination, radiologic examination and endoscopy.

Pathologic stage adds additional information gained by examination of the tumor microscopically by a pathologist (http://en.wikipedia.org/wiki/Cancer_staging). The staging system of the Gleason system according to Bagchi and Preuss, (2005) is as follows:

Stage 0 (Carcinoma in situ): very early cancer. The abnormal cells are found only in the first layer of the primary site and do not invade deeper into the tissue.

Stage I: Cancer involves the primary site but did not spread to nearby tissue.

Stage IA: A very small amount of cancer was found to be visible under the microscope and is found deeper in the tissue.

Stage IB: Here larger numbers of cancer cells were found in the tissue.

Stage II: The cancer has spread to the nearby tissue but is still found inside the primary site.

Stage IIA: Cancer has spread beyond the primary site.

Stage IIB: Cancer has spread to other tissues around the primary site.

Stage III: Cancer has spread throughout the nearby area.

Stage IV: Cancer has spread to other parts of body.

Stage IVA: Cancer has spread to organs close to pelvic area.

Stage IVB: cancer has spread to distant organs, such as the lungs.

- Recurrent: Cancer has recurred at the same location were the original tumor was or at a different location after it has been treated and supposedly eliminated.

1.1.2. Carcinogenesis

'Carcinogenesis' or 'oncogenesis' is literally the creation of cancer. It is a process by which normal cells are transformed into cancer cells. It is characterized by a progression of changes on cellular and genetic level that ultimately reprogram a cell to undergo uncontrolled cell division, thus forming a malignant mass. As a general definition, carcinogenesis is a complex multistage processes including initiation, promotion, and progression steps (Tanaka *et al.,* 2004).

Under normal circumstances, cell division is maintained by regulating normal balance between proliferation and programmed cell death (apoptosis) to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer (only certain mutations can lead to cancer and the majority of potential mutations will have no bearing) disrupt these orderly processes by disrupting the programming regulating the processes.

Carcinogenesis is caused by mutation of the genetic material (DNA) of normal cells, which upsets both processes of proliferation and death. This results in uncontrolled cell division and the evolution of those cells by natural selection in the body. The uncontrolled and often rapid proliferation of cells can lead to benign tumors; some types may turn into malignant tumors (cancer). Benign tumors do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active, for instance, producing a hormone. Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening. More than one mutation is necessary for carcinogenesis. In fact, a series of mutations to certain classes of genes is usually required before a normal cell transforms into a cancer cell (Fearon and Vogelstein, 1990). Only mutations in those certain types of genes which play vital roles in cell

division, apoptosis (cell death), and DNA repair will cause a cell to lose control of its cell proliferation.

1.1.3. Metastasis

'Metastasis' is the spread of a disease from one organ to another non-adjacent organ (Chiang, 2008; Klein, 2008). In another word, the process by which cancer spreads from the place at which it first arose as a primary tumor to distant locations in the body is defined as 'metastasis'. Metastasis is the major cause of death by cancer, which depends on the cancer types acquire two separate abilities; increased motility and invasiveness. Metastasis occurs by four routes:

- Spread into body cavities. This occurs by the seeding surface of the peritoneal, pleural, pericardial or subarachnoid spaces. For example, ovarian tumors spread transperitoneally to the surface of the liver. Mesotheliomas can spread through the pleural cavity.
- Invasion of lymphatics. This is followed by the transport of tumor cells to regional nodes and ultimately to other parts of the body; it is common in initial spread of carcinomas.
- Hematogenous spread. This is typical of all sarcomas but it is the favored route in certain carcinomas (e.g. those originating in kidneys). Because of their thinner walls veins are more frequently invaded than arteries and metastasis follows the pattern of the venous flows.
- Transplantation. Mechanical carriage of fragments of tumor cells by surgical instruments during operation or the use of needles during diagnostic procedures (Bacac and Stamenkovic, 2008).

Metastasis is a complex series of steps in which cancer cells leave the original tumor site and migrate to other parts of the body via bloodstream or lymphatic system. Thereafter, malignant cells break away from the primary tumor and are attached to the proteins in surrounding extracellular matrix (ECM). As a result, the tumor is separated from adjoining tissues. By degrading these proteins, cancer cells are able to breach the ECM and escape. When oral cancers metastasize, they commonly travel through the

lymph system to the lymph nodes in the neck. The body resists metastasis by a variety of mechanisms through the actions of a class of proteins known as metastasis suppressors (Yoshida *et al.,* 2000). One of the critical events required for cancer metastasis is the growth of a new network of blood vessels, called 'tumor angiogenesis' (Weidner *et al.,* 1991). It has been found that angiogenesis inhibitors would therefore prevent the growth of metastases (Kumar *et al.,* 2005).

1.1.4. Cell death

Dying cells can engage in a process that is reversible until a first irreversible step or 'point-of-no-return' is trespassed. The Nomenclature Committee on Cell Death (NCCD) suggests that a cell should be considered dead when any of the following molecular or morphological criteria occur: (1) the cell has lost the integrity of the plasma membrane, as defined by vital dyes *in vitro*, (2) the cell including its nucleus has undergone complete fragmentation into discrete bodies (which are frequently referred to as 'apoptotic bodies') and/or (3) its corpse (or its fragments) have been engulfed by an adjacent cell *in vivo*. Thus, 'dead cells' would be different from 'dying cells' that are in the process of cell death, which can occur through a variety of different pathways. Moreover, cells whose cell cycle is arrested (as it occurs in senescence) would be considered as alive and the expression 'replicative cell death' (which alludes to the loss of the clonogenic capacity) should be avoided (Kroemer *et al.,* 2005). A complex cascade of biological processes, also normally part of a cells life (pathways, enzyme systems, functioning of organelles, plasma membrane structure and function, modulation of transcriptional and translational activities etc) was activated in preparation for and during cell death (Darzynkiewicz *et al.,* 1997). It is also vital to understand that every cell is programmed to die subsequent to a suitable stimulus (Trump *et al.,* 1997).

There are several forms of cell death; apoptosis, autophagy, necrosis, oncosis. Cell death is generally defined into two different mechanisms that are mutually exclusive and stand in sharp contrast, apoptosis (programmed cell death) and necrosis (accidental cell death) which are the two fundamental types of cell death (Darzynkiewicz *et al.,* 1997).

1.1.4.1. Apoptosis

The word 'apoptosis' has been coined by Kerr *et al.,* (1972) describing a particular morphological aspect of cell death. Apoptosis has come to be used synonymously with the phrase ‘programmed cell death’ as it is a cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signaling pathways. For cell death to be classified as apoptotic, rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), nuclear condensation and fragmentation (karyorhexis), cleavage of chromosomal DNA into internucleosomal fragments and packaging of the deceased cell into apoptotic bodies without plasma membrane breakdown must be observed (Edinger and Thompson, 2004; Kroemer *et al.,* 2005). Apoptotic bodies are recognized and removed by phagocytic cells and thus apoptosis is notable for the absence of inflammation around the dying cell. The morphologic features of apoptosis result from the activation of caspases (cysteine proteases) by either death receptor ligation or the release of apoptotic mediators from the mitochondria. Apoptosis as a tidy regulated cell death requires energy in the form of ATP (Edinger and Thompson, 2004).

Apoptosis is widespread during development and it is often in situation that actually represents a prelethal phase of programmed reaction to injury on a schedule set by various hormonal, nutritional and micro/macro-environmental factors. Apoptosis also occurs following a variety of chemical and microbiologic injuries in many different organ systems (Trump *et al.,* 1997).

1.1.4.2. Necrosis

Necrosis generally defines as nonapoptotic, accidental cell death (Fink and Cookson, 2005). Necrosis is the end result of a bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival and was thought to be initiated mainly by cellular ‘accidents’ such as toxic insults or physical damage (Edinger and Thompson, 2004). In pathology, necrosis is used to designate the presence of dead tissues or cells and is the sum of changes that have occurred in cells after they have died, regardless of the prelethal processes (Fink and Cookson, 2005). Necrosis is characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane and an induction of inflammation around the dying cell attributable to the release of

cellular contents and pro-inflammatory molecules. Cells that die by necrosis frequently exhibit changes in nuclear morphology but not the organized chromatin condensation and fragmentation of DNA into 200 bp fragments that is characteristic of apoptotic cell death (Edinger and Thompson, 2004) (Fig 1.2). The most common microscopic settings of necrosis are: 1) cells that died singly displaying the morphological changes of apoptosis, for which we have suggested the term apoptotic necrosis, and 2) groups of cells that died of ischemia, which we can call ischemic necrosis or massive necrosis when the mechanism is not known (Majno and Joris, 1995).

Necrosis, therefore, refers to morphological stigmata seen after a cell has already died and reached equilibrium with its surroundings. Thus, in the absence of phagocytosis, apoptotic bodies may lose their integrity and proceed to secondary or apoptotic necrosis. The term apoptotic necrosis describes dead cells that have reached this state via the apoptotic program. The presence of necrosis tells us that a cell has died but not necessarily, how death occurred (Fink and Cookson, 2005).

1.1.4.3. Oncosis

The term ‘oncosis’ (from ‘onkos’, meaning swelling) was coined by von Recklinghausen almost 100 years ago, precisely with the meaning of cell death with swelling. In a monograph on rickets and osteomalacia, published posthumously in 1910, von Recklinghausen described death with swelling primarily in bone cells (Majno and Joris, 1995) (Fig 1.2). The expression 'oncosis' defines a cell death morphology with cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation (Kroemer *et al.,* 2005). The term ‘oncosis’ has been accepted by many investigators as a counterpoint to apoptosis. The process of ‘oncosis’ ultimately leads to depletion of cellular energy stores and failure of the ionic pumps in the plasma membrane. Oncosis may result from toxic agents that interfere with ATP generation or processes that cause uncontrolled cellular energy consumption. The changes accompanying oncosis may result from active enzyme-catalyzed biochemical processes (Fink and Cookson, 2005).

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In vivo, oncosis typically affects broad areas or zones of cells, e.g., the early changes of cells following total ischemia or the cells in a particular region of the liver or kidney tubule following chemical toxins, such as CCl_4 or $HgCl_2$, respectively. When broad zones of cells are involved followed by death of the cells, a pronounced inflammatory reaction typically occurs at the periphery of the zone, although oncosis is not typical of programmed cell death in development, it has been described in some situations (Trump *et al.,* 1997).

1.1.4.4. Autophagy

Apoptotic bodies and the cellular debris released during lysis of oncotic cells can both be phagocytized and degraded by neighboring viable cells *in vivo*. Another form of cell death, autophagy or type II cell death, features degradation of cellular components within the dying cell in autophagic vacuoles. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation (Fig 1.1). Studies on autophagy suggest that it proceeds through a sequence of morphological changes in a highly regulated process. Briefly, the autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes. The sequestration process is under the control of GTPases and phosphatidylinositol kinases and involves novel ubiquitin-like conjugation systems.

Autophagosomes then fuse with lysosomes in a process depending on microtubules, and the contents are degraded (Fink and Cookson, 2005). It is important to consider that necrosis occurs in cells that are undergoing severe bioenergetic stress, the same conditions that would stimulate autophagy as a mechanism for boosting ATP levels. Thus, it is likely that autophagy and necrosis often occur in parallel, initiated in response to the same stimuli but with completely opposite objectives (Edinger and Thompson, 2004). *In vivo*, cells undergoing autophagy can be phagocytized by neighboring cells (Fink and Cookson, 2005).

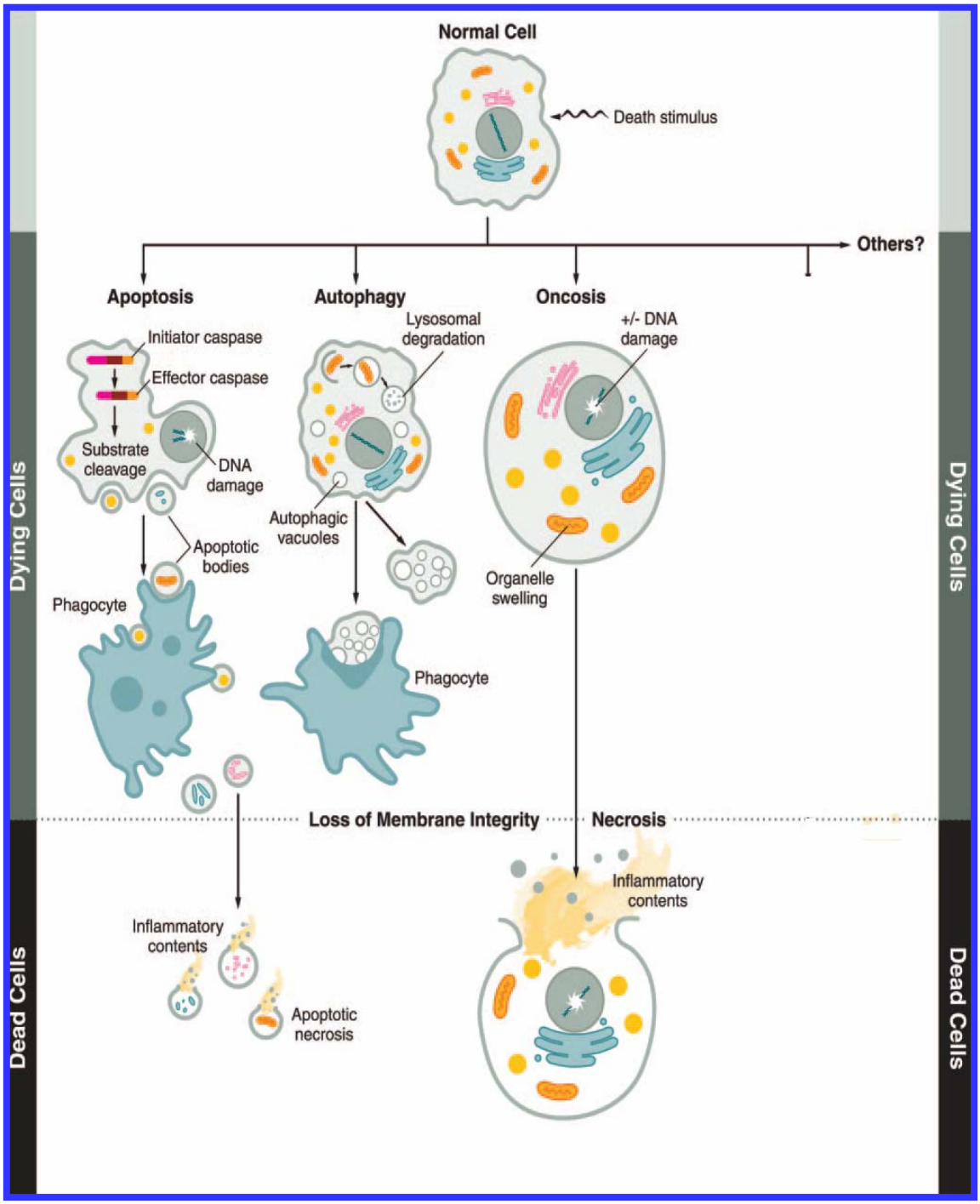

Figure 1.2: Pathways leading to cell death (Fink and Cookson, 2005).

1.1.5. Cancer statistics

The United State (US) National Institutes of Health estimates overall costs of cancer in 2010 to be about $263.8 billion in total of which, $102.8 billion has been spent for direct medical costs (total of all health expenditures); $20.9 billion for indirect morbidity costs (cost of lost productivity due to illness); and $140.1 billion for indirect mortality costs (cost of lost productivity due to premature death) (Cancer Facts & Figures, 2011). In 2008, approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) and 7.6 million people died of cancer worldwide (Jemal *et al.,* 2011). Cancer approximately causes 13% of all deaths each year with the most common being: lung cancer (1.3 million deaths), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths), and breast cancer (519,000 deaths) (WHO, 2011). This makes invasive cancer the leading cause of death in the developed world and the second leading cause of death in the developing world. Over half of cases occur in the developing world (Jemal *et al.,* 2011).

Each year, the American Cancer Society estimates the number of new cancer cases and death expected in the United States in the current year, and compiles the most recent data on cancer incidence, mortality, and survival by using incidence data from the National Cancer Institute (NCI) and mortality data from National Cancer for Health Statistics (NCHS). In 2012, 1,638,910 new cancer cases and 577,190 deaths from cancer are reported in the United States (Cancer Facts & Figures, 2012). They estimated 1.44 million new cases of invasive cancer expected among men and women in the United States in 2008 (Jemal *et al.,* 2008).

1.2. LITERATURE REVIEW

1.2.1. Medicinal plants and human health care

Accessibility and affordability of the medicinal herbs have made them as a fundamental part of many people's life all over the world. The selection of medicinal plants is a conscious process, which has led to an enormous number of medicinal plants being

used by plentiful cultures in the world (Heinrich *et al.,* 2004). Surveys conducted in Australia and United State (US) indicates that almost 48.5% and 34% of individuals had used at least one form of unconventional therapy. According to WHO, about 65-80% of the world's population in developing countries, due to poverty and lack of access to modern medicine, depends essentially on plants for their primary health care (Calixto, 2005). Indeed, evaluation of effective plants to cure certain diseases has been recommended by WHO due to the lack of safe modern drugs.

In recent years, efficacy of herbal medicines in diseases like inflammatory bowel disease (Achike and Kwan, 2003; Calixto, 2005; Rahimi *et al.,* 2009; Rezaie *et al.,* 2007), obesity (Hasani-Ranjbar *et al.,* 2009a; Heber, 2003), diabetes (Edzard, 2005; Rahimi *et al.,* 2005), pancreatitis (Ara Tachjan *et al.,* 2010; Mohseni-Salehi-Monfared *et al.,* 2009), cancers (Angelo and Edzard, 2009; Boon and Wong, 2004; Calixto, 2000; Paduch *et al.,* 2007) and inflammatory and oxidant-related diseases (Hasani-Ranjbar *et al.,* 2009b; Rahimi *et al.,* 2010) has been systematically reviewed. Even at dawn of 21th centuray, 11% of 252 drugs considered as basic and essential by WHO was exclusively of flowering plant origin (Rates, 2001). It is estimated that close to 25% of the active compounds in currently prescribed synthetic drugs were first identified in plant resources (Halberstein, 2005) and 20,000 plants have been used for medicinal proposes of which, 4000 have been used commonly and 10% of those are commercial. Out of the 250,000–500,000 plant species on earth, only 1-10% have been studied chemically and pharmacologically for their potential medicinal value (Verpoorte, 2000).

1.2.2. Medicinal plants in South Africa

South Africa, which comprises of less than 1% of the world's land surface, contains 8% of its plant species. This rich plant biodiversity, with over 20,000 different species, is a great source of interest to the scientific community (Fouche *et al.,* 2006). About 70% of these species are endemic to South Africa (SA). It is estimated that there are more than 100,000 practicing traditional healers in the country, with a liable industry worth about R500 million (56,715,000 $) per annum (Mander and Le Breton, 2005). As estimated 27 million indigenous medicine consumers live in South Africa with a large supporting industry (Mander, 1998). Annually up to 700,000 tons of plant materials are consumed,

of which most of them are collected in the wild for local use (Cunningham, 1988) and international trade (Lange, 1997).

Plants harvested from a diverse range of vegetation types, including Valley Thicket, Afromontane Forest, Coastal Forest and Moist Upland Grassland. The Forest Biome was the vegetation type found to be most threatened by over-harvesting (Dold and Cocks, 2002). South Africa is a land with great possibility for discovering novel natural-based products. In an intensive investigation in South Africa, Fouche *et al.,* (2006) reported that among 7,500 randomly selected plant extracts representing 700 taxa, 32 extracts demonstrated potent anticancer activity, represent 24 different plant taxa, which is a hit rate of 3.4% based on the number of taxa screened. The current demand for numerous plant species resulting in intensive harvesting of indigenous plant stocks (this is in association with the lack of main resource management and plant production) has resulted in the rareness of various indigenous medicinal plants.

1.2.3. Plant-derived anticancer agents

Plants engaged along history for treatment of cancer. It was estimated that about 67% of pharmaceutical products approved between 1974 and 1994, for human cancer therapy were derived from natural sources (Richard *et al.,* 2005) of which plants have been a main source of several clinically convenient anticancer agents. Approximately five decades longed till several natural and synthetic antineoplastic were discovered. Cragg and Newman, (2005) revealed that there are remarkable numbers of plant-sourced agents in clinical trials for the treatment of cancer. Some are being investigated as direct cytotoxins, whereas others are being studied from the aspect of their potential role as inhibitors of particular cell cycle enzymes, proteins, or pathways. The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, ‘vinblastine’ (VLB) and ‘vincristine’ (VCR), and the isolation of the cytotoxic ‘podophyllotoxins’ (Cragg and Newman, 2005). The most important plant derived anticancer agents are depicted in Figure 1.3.

1.2.3.1. Alkaloids and anticancer properties

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. In addition, some synthetic compounds of similar structure are attributed to alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine, and phosphorus. Plant alkaloids make up a group of chemotherapy medications used to treat cancer. Alkaloids block cell division by preventing microtubule function which is vital for cell division. Alkaloids are divided into three major subgroups based on the type of plant origin;

- "Vinca alkaloids" bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules (M phase of the cell cycle). The first agents to advance into clinical use were (VLB) and (VCR), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae) which was used by various cultures for the treatment of diabetes (Gueritte and Fahy, 2005). Recently, 'vinorelbine' (VRLB) and 'vindesine' (VDS) have been developed as the semi-synthetic analogs of these agents (Lee and Xiao, 2005).

- "Podophyllotoxin" is a plant-derived compound, which help with digestion as well as used to produce two other cytostatic drugs, 'etoposide' and 'teniposide'. These compounds prevent the cell from entering the G1 phase (the start of DNA replication) and the replication of DNA (the S phase). Etoposide and teniposide; semi-synthetic derivatives of the natural product, 'epipodophyllotoxin' (an isomer of podophyllotoxin), could be considered as links to a plant originally used for the treatment of cancer (Lee and Xiao, 2005).

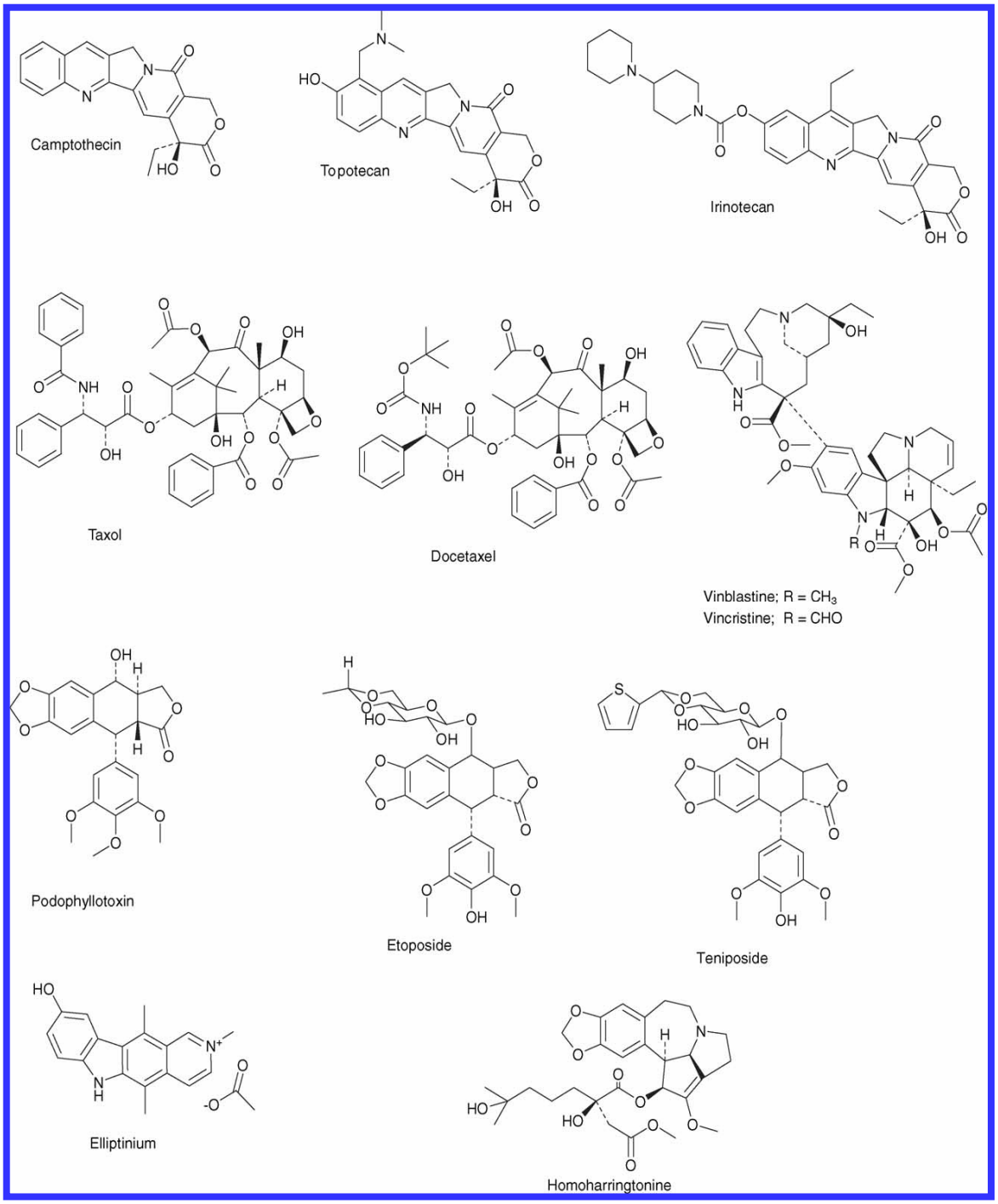

Figure 1.3: Plant-derived anticancer agents in clinical use (Cragg and Newman, 2005).

- "Taxanes" enhance stability of microtubules, preventing the separation of chromosomes during anaphase. The taxane group of plant alkaloids includes paclitaxel, protein-bound paclitaxel and docetaxel. 'Taxanes' and its derivatives (paclitaxel, docetaxel and taxotere) isolated from various Taxus species; active

agents derived from 'camptothecin' (topotecan and irinotecan) isolated from Chinese ornamental tree [*Camptotheca acuminata* Decne (Nyssaceae)] (Rahier *et al.,* 2005); 'homoharringtonine' isolated from the Chinese tree [*Cephalotaxus harringtonia* var. drupacea (Sieb and Zucc.) (Cephalotaxaceae)] (Itokawa *et al.,* 2005); and 'elliptinium' a derivative of ellipticine isolated from species of several genera of the Apocynaceae family etc.

The 'combretastatins' (tubulin polymerization inhibitor) (colchicin related alkaloid), derived from South African bush *Combretum caffrum*, are a family of stilbenes which act as anti-angiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis, and a water-soluble analog, combretastatin A-4 phosphate, has shown promise in clinical trials Phase II (Cragg and Newman, 2005).

1.2.3.2. Coumarins and anticancer properties

Plant coumarins are structurally distinct, non-anticoagulant compounds that have significant medicinal activity (Yarnell and Abascal, 2009). Coumarins, also known as benzopyrones, are present in remarkable amounts in plants, although their presence has also been detected in microorganisms and animal sources (Borges *et al.,* 2005). The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution. Coumarins are aromatic compounds with a specific ring structure (Kostova *et al.,* 2006).

The structural diversity found in this family of compounds led to the division into different categories, from simple coumarins to many other kinds of policyclic coumarins, such as furocoumarins and pyranocoumarins (Fig 1.4). Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. The coumarins have been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Kostova *et al.,* 2006).

'Coumarin' is a natural substance that has shown anti-tumor activity in vivo, with the effect believed to be due to its metabolites (e.g. 7-hydroxycoumarin). A recent study has

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shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. 'Esculetin' (6,7-dihydroxycoumarin) inhibits growth and cell cycle progression by inducing arrest of the G1 phase in HL-60 leukemia cells, resulting from the inhibition of retinoblastoma protein phosphorylation (Aoife and O'Kennedy, 2004).

1.2.3.3. Flavonoids and anticancer properties

Flavonoids (or bioflavonoids) (from the Latin word flavus meaning yellow), are a class of plant secondary metabolites or yellow pigments having a structure similar to that of flavones. Flavonoids are polyphenolic compounds that are ubiquitously in plants. Flavonoids can be classified into: flavonoids, isoflavonoids, neoflavonoids, which are all ketone-containing compounds. Over 5000 naturally occurring flavonoids have been characterized from various plants. They have been shown to possess a variety of biological activities at nontoxic concentrations in organisms (IUPAC Compendium of Chemical Terminology, 1997).

The role of dietary flavonoids in cancer prevention is widely discussed. Compelling data from laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and chemotherapy. Many mechanisms of action have been identified, including carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms (Ren *et al.,* 2003).

Flavonoids were found to be strong topoisomerase inhibitors and induce DNA mutations in the (myeloid/lymphoid or mixed-lineage leukemia) MLL gene, which are common findings in neonatal acute leukemia (Strick *et al.,* 2000). The DNA changes were increased by treatment with flavonoids in cultured blood stem cells (Van Doorn-Khosrovani *et al.,* 2007). A high flavonoid-content diet in mothers is suspected to increase risk particularly of acute myeloid leukemia in neonates (Ross, 1998; Ross, 2000; Spector *et al.,* 2005).

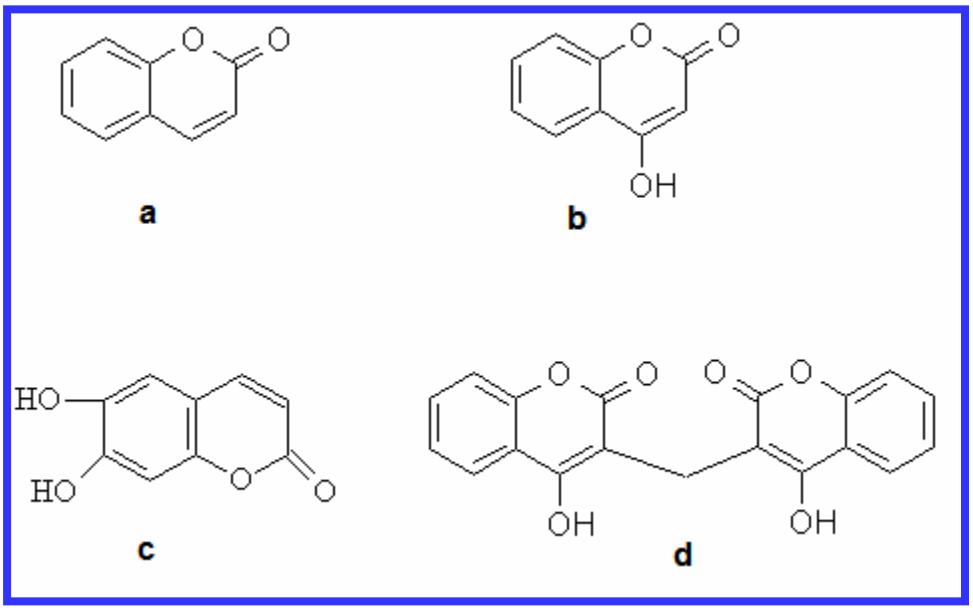

Figure 1.4: Chemical structures of some coumarins: a) coumarin; b) 4-hydroxycoumarin; c) 6,7-hydroxycoumarin and d) bishydroxycoumarin (http://www.people.vcu.edu/~urdesai/cou.htm).

Polyphenols (flavonoids and delphinidin) were found to be strong topoisomerase inhibitors, similar to some chemotherapeutic anticancer drugs including etoposide and doxorubicin. This property may be responsible for both an anticarcinogenic-proapoptotic effect and a carcinogenic, DNA damaging potential of the substances (Bandele *et al.,* 2008; Esselen *et al.,* 2009).

'Flavopiridol' (synthetic polyhydroxylated flavone) (cycline-dependent kinases (CDKs) inhibitor) synthesized by the Indian subsidiary of Hoechst (Aventis) following the isolation and synthesis of the plant-derived natural product, rohitukine, are currently in Phase III clinical trials both as a single agent and in combination with other agents, particularly with paclitaxel and cis-platinum (Cragg and Newman, 2005). Quercetin (3,3',4',5,7-pentahydroxyflavone) passed Phase I clinical trial with antiproliferative activity *in vitro* and is known to inhibit signal transduction targets including tyrosine kinases, protein kinase C, and phosphatidyl inositol-3 kinase (Ferry *et al.,* 1996). 'Genistein' is a well-known isoflavone and is a tyrosine kinase inhibitor. Studies have

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indicated that genistein elicits inhibitory effects on cell growth of various carcinoma cell-lines and may be a potential candidate for cancer therapy (Aoife and O'Kennedy, 2004).

1.2.3.4. Saponins and anticancer properties

Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution. The presence of saponins has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anticancer properties. There are more than 11 distinguished classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids (Man *et al.,* 2010). Saponins are amphipathic glycosides grouped by the soap-like foaming they produce when shaken in aqueous solutions, and by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. The aglycone (glycoside-free portion) of the saponins are termed ‘sapogenins’. The subset of saponins that are steroidal have been termed ‘saraponins’ (Hostettmann and Marston, 1995).

The pharmaceutical applications of saponins are varied as their origins and chemical structures. Just to mention a few: a number of saponins or saponin-rich mixtures have found to use as anti-inflammatory, antidiuretic, antipyretic, analgesic agent, central nervous system depressants and as treatment for ulcers (Cheeke, 1989). Due to the great variability of their structures, saponins always display anti-tumorigenic effects through varieties of antitumor pathways (Man *et al.,* 2010). The proposed mechanisms of anticarcinogenic properties of saponins include direct cytotoxicity, immune-modulatory effects, bile acid binding, and normalization of carcinogen-induced cell proliferation (Rao and Sung, 1995).

Extracts from *Maytenus diversifolia* have been found to inhibit growth of leukemic lymphocytes *in vivo* (Cheeke, 1989). Rao and Sung, (1995) showed that soybean saponins at the concentration of 150-600 ppm had a dose-dependent growth inhibitory effect on human carcinoma cells (HCT-15). Viability was also significantly reduced. Shibata, (2001) reported that tetracyclic triterpenoid saponins isolated from ginseng, the

root and rhizome of *Panax ginseng* C.A. Meyer (Araliaceae), showed the anti-carcinogenic activity in two-stage anti-cancer-promotion experiments *in vitro* and *in vivo*.

1.2.3.5. Terpenes and anticancer properties

Triterpenoids demonstrated a range of unique and potentially usable biological effects. The history of the use of plants with high saponin/triterpenoid content can be found in the first written herbariums (Dzubak *et al.,* 2006). Terpenes are naturally occurring substances produced by a wide variety of plants and animals. Terpenes are biosynthetically derived from isoprene units with the molecular formula C_5H_8. The basic formula of all terpenes is $(C_5H_8)_n$, where 'n' is the number of linked isoprene units (Gao and Singh, 1998). The most common forms of terpenes are the monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) also exist. A terpene containing oxygen is called a terpenoid (Bakkali *et al.,* 2008). The classification and source of terpenoids are presented in Table 1.1 and their chemical structures in Figure 1.5. Terpenes in high concentrations can be toxic and considered as key weapons against herbivores and pathogens. A broad range of the biological properties of terpenoids include; anticancer, cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory and antiparasitic activities (Dzubak *et al.,* 2006; Paduch *et al.,* 2007). Bardon *et al.,* (2002) claimed that various terpenes can act as inhibitors in a dose-dependent manner, to prevent the development of mammary, liver, skin, lung, colon, fore-stomach, prostate and pancreatic carcinomas (Fig 1.6).

Epidemiological studies suggest that dietary monoterpenes may be helpful in the prevention and therapy of cancers, of which among them, D-limonene and perillyl alcohol have been shown to possess chemopreventive and therapeutic properties against many human cancers (Paduch *et al.,* 2007). Chemotherapeutic activities towards human pancreatic cancers have also been shown for other terpenes, such as farnesol and geraniol (Burke *et al.,* 2002). Concerning more than 20000 triterpenoids, known to occur in nature; oleanolic acid (OA), ursolic acid (UA), synthetic oleanane-triterpenoids (SO) and rexinoids are highly effective for the prevention and treatment of cancer in many animal models, but as yet are not definitive agents in clinical practice.

To improve potency, many modifications of the basic triterpenoid structure have been made (Liby *et al.,* 2007).

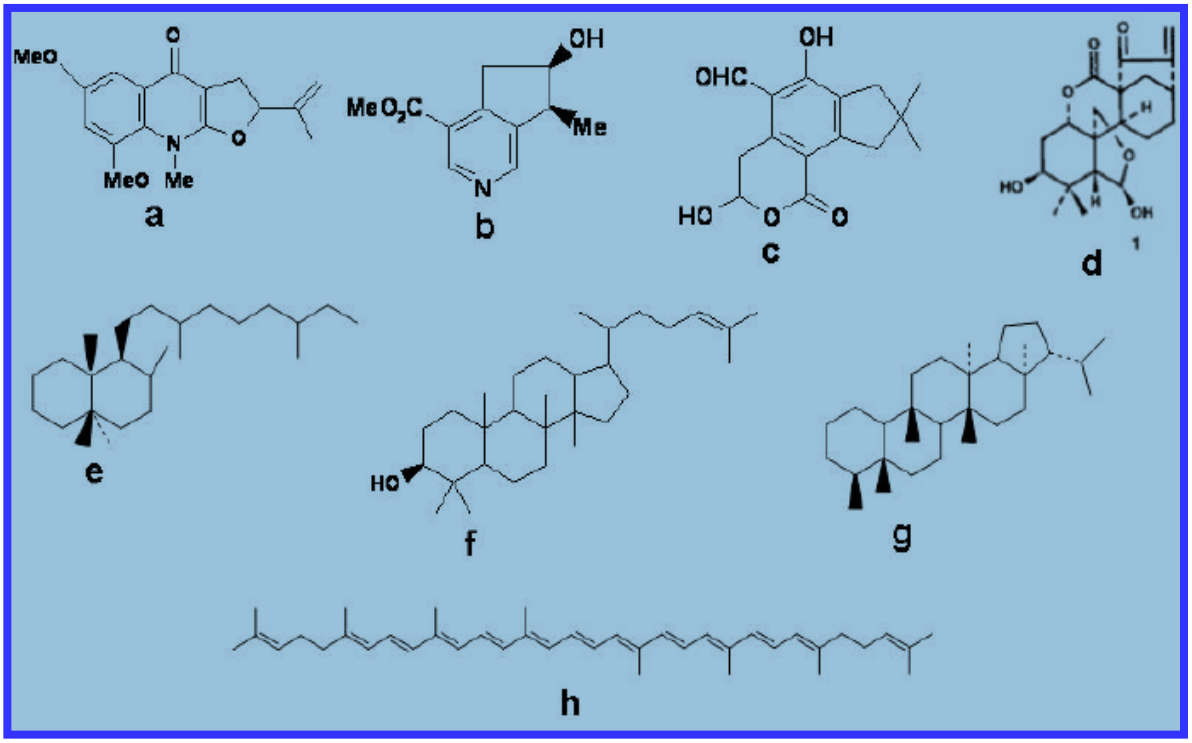

Figure 1.5: Classification of terpenoids: (a) hemiterpenoids (b) monoterpenoids (c) sesquiterpenoids (Manske, 1971); (d) diterpenoids (e) sesterpenoids (f) triterpenoids (g) tetraterpenoids and (h) polyterpenoids (Rahman, 1995).

1.2.3.5.1. Mechanisms of action of terpenes'cytotoxicity

Many attempts have been made to prove the influence of terpenoids on inducing apoptosis as a desired strategy of controlling cancers (Liby *et al.,* 2007; Paduch *et al.,* 2007; Yogeeswari and Sriram, 2005; Zhang *et al.,* 2004). Apoptosis is a process that develops in several phases: 1) an initiation phase, in which the biochemical pathways participating in the process depend on the apoptosis-inducing agent; 2) a decision phase, during which the cell "decides" to commit suicide; and 3) a common degradation phase, which is characterized by the activation of catabolic hydrolases (caspases and nucleases). Although the activation of caspases and nucleases is necessary for the acquisition of the full apoptotic morphology, it appears that the inhibition of such

enzymes does not inhibit cell death induced by a number of different triggers: Bax, Bak, c-Myc, PML), Fas-associated death domain (FADD), glucocorticoid receptor occupancy, tumor necrosis factor, growth factor withdrawal, CXCR4 cross-linking, and chemotherapeutic agents (Costantini *et al.,* 2000).

Table 1.1: Classification of terpenes (Daniel, 2006).

Class	Number of carbon atoms	Number of isoprenes	Sources
Hemiterpenoids	5	1	Volatile oils, esters
Monoterpenoids	10	2	Volatile oils, glycosides, mixed terpenoids
Sesquiterpenoids	15	3	Volatile oils, bitter principles
Diterpenoids	20	4	Resins, chlorophyll
Sesterpenoids	25	5	Rare (mostly in animals)
Triterpenoids	30	6	Resins, waxes, steroids, saponins, cardiac glycosides
Tetraterpenoids	40	8	Carotenoids
Polyterpenoids	α	n	Rubber and gutta

The activation of apoptosis by terpenes occurs via intrinsic cell death pathway, also known as the mitochondrial apoptotic pathway. Yang and Ping Dou, (2010) explained the activation of apoptosis triggered by inhibition of the fas-associated death domain and NF-αB pathways. It has been well documented that suppression of the ubiquitin-proteasome and NF-αB pathways are essential for induction of tumor cell apoptosis.

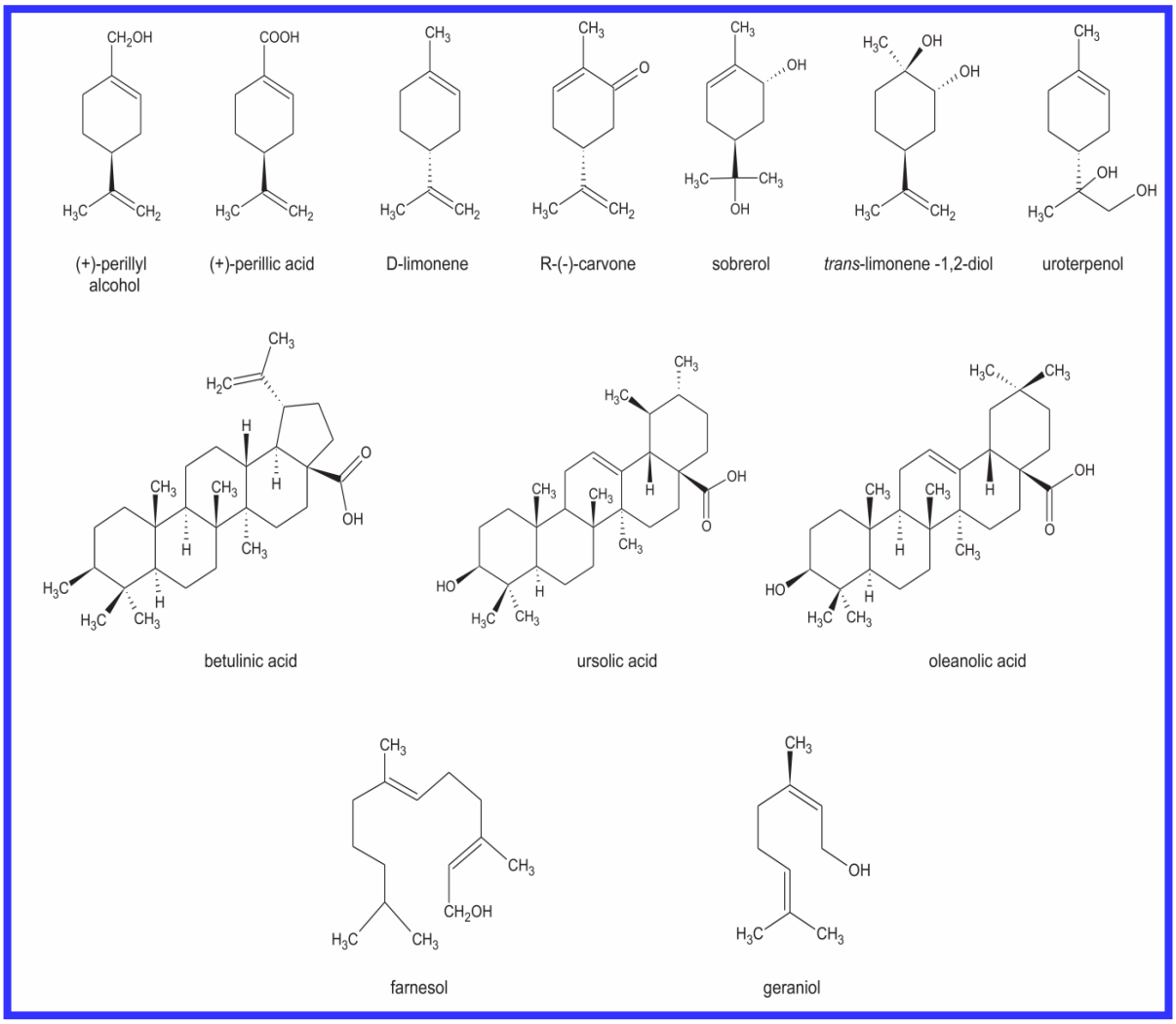

Figure 1.6: Various terpenes with anticancer properties (Paduch *et al.,* 2007).

A proposed mechanism expressed that monoterpenes may activate transforming growth factor (TGF)-β signaling which is produced in a latent form. This activation increases mRNA synthesis encoding (TGF)-β receptors and is closely associated with elevated synthesis of pro-apoptotic proteins (Bax, Bak, and Bad) without influencing p53 or Bcl-2 expression. Moreover, through TGF-β, the cell cycle is down-regulated, influencing the production of cyclin and cyclin-dependent kinases or their reciprocal interactions. In consequence, it leads to G1-phase arrest and cell apoptosis (Ahn *et al.,* 2003). Additional studies revealed that some monoterpenes can influence tumor cells by inhibiting the synthesis of coenzyme Q (CoQ) an important element of mitochondrial

respiratory metabolism. The reduction of CoQ in cell membranes may, therefore, limit cellular signal transduction and metabolism and induce apoptosis of tumor cells (Ahn *et al.,* 2003; Gould, 1995; Paduch *et al.,* 2007).

In another study Ulrich, (2007) showed treatment with ursolic acid (pentacyclic triterpenoid) leads to a significant time- and dose-dependent cell growth inhibition of colorectal cancer cells, coincident with the up regulation of the cell cycle1 regulators cyclin E, p21WAF1/Cip1 and p27Kip1. In addition, ursolic acid significantly induces apoptosis, which is mediated by an increase of BAX/Bcl-2-protein-ratio as well as an up regulation of TRAIL protein which meets in an induction of caspase-3 activity.

1.2.3.5.2. Chemoprevention of terpenes

Cancer chemoprevention, as first defined in 1976 by Sporn, is the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression (Sporn, 1976). According to the National Cancer Institute (NCI), five classes of mainly chemically synthesized chemopreventive agents are characterized of high priority: selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAIDs), calcium compounds, glucocorticoids and retinoids.

In parallel, the NCI identified about 40 edible plants possessing potential chemopreventive compounds, globally known as phytochemicals (National Center for Health Statistics, 2005). For instance, among dietary monoterpenes, D-limonene and perillyl alcohol have been shown to possess chemopreventive and therapeutic properties against many human cancers. There are multiple mechanisms of monoterpene chemopreventive actions. They may act during the initiation phase of carcinogenesis, preventing interaction of carcinogens with DNA, or during the promotion phase, inhibiting cancer cell development and migration. The chemopreventive and therapeutic activities of monoterpenes in later stages of carcinogenesis include induction of cancer cell apoptosis, re-differentiation of tumor cells, and influence on molecular mechanisms regulating their functions. The most important mechanism that monoterpenes influence is post-translational isoprenylation of proteins regulating the growth of cells. Figure 1.7 illustrates further chemoprevention strategies.

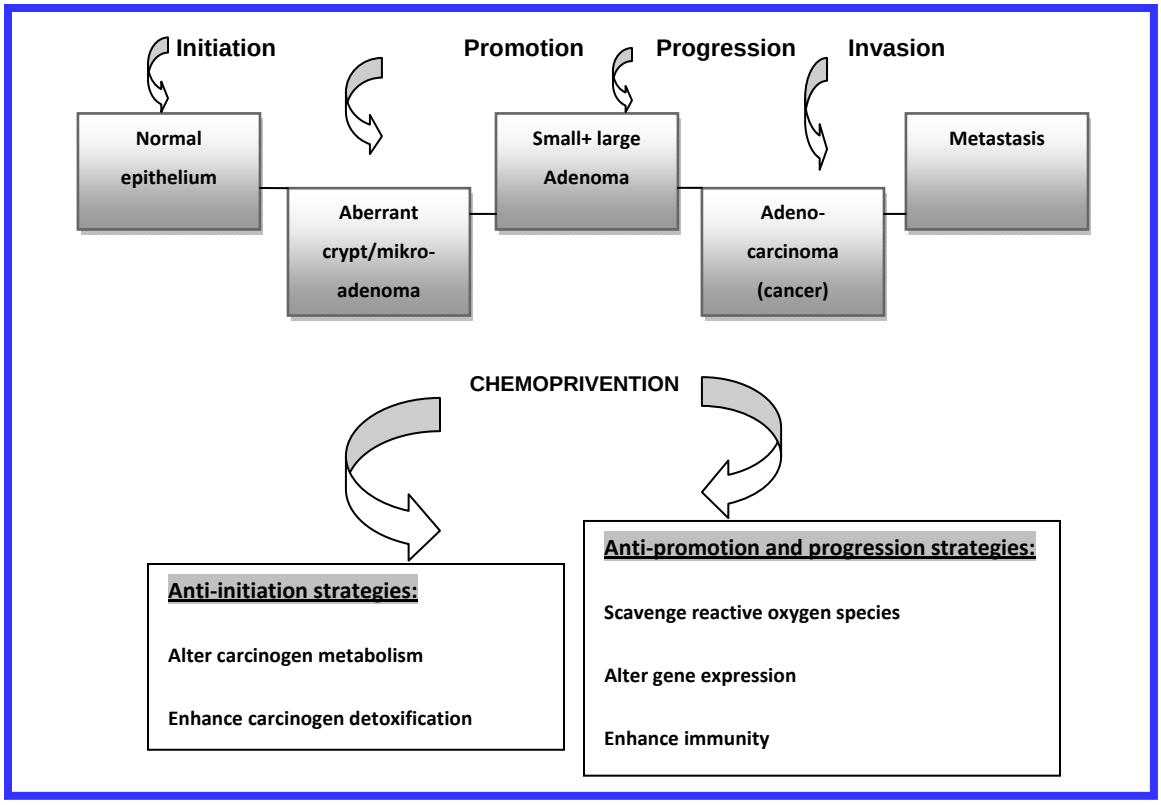

Figure 1.7: Chemoprevention strategies (Ulrich, 2008).

1.3. THE GENUS *Hyaenanche*

The genus *Hyaenanche* belongs to Euphorbiaceae family, which is one of the largest families of plants with about 300 genera and 7500 species, mostly monoecious herbs, shrubs and trees, sometimes succulent and cactus-like, that are further frequently characterized by a milky sap or latex material. Members of Euphorbeaceae family have been investigated as providing potential treatments for cancer, tumors and warts (Donato *et al.,* 2005). The chemistry of the Euphorbiaceae is among the most diverse and interesting of flowering plant families and is comparable to the biological diversity of the family. Because of the presence of unusual secondary metabolites, many species of euphorbiaceous plants are poisonous and have been involved in human and livestock poisoning. Plants of this family have been used in folk medicine, as pesticides or as arrow poisons. Several euphorbias are important food plants, which a number of them are important economically as ornamental plants and as sources of rubber, chemical precursors, lubricants and medicinal compounds (Seigler, 1994). Of all chemical

classes, the most useful for chemotaxonomic study of the Euphorbiaceae appear to be alkaloids, cyanogenic glycosides, diterpenes, glucosinolates, lipids, tannins and triterpenes (Hegnauer, 1963).

1.3.1. Compounds isolated and identified from the genus Hyaenanche

The genus *Hyaenanche* only contains a single species, which is known as *Hyaenanche globosa*. The seeds (possibly also other parts of plant) contain a deadly toxin with a strychnine-like action, which is used as an arrow poison to kill hyenas and other predators (Van Wyk *et al.,* 1997). This plant contains several toxic sesquiterpene lactones, such as; tutin, mellitoxin (also called hyenanchin), urushiol III and isodihydrohyaenanchine. Its main toxin, tutin, is known to cause convulsions, delirium and coma in humans (Van Wyk *et al.,* 1997; Van Wyk *et al.,* 2002).

1.3.2. Hyaenanche globosa

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Malpighiales*
Family: *Picrodendraceae*
Tribe: *Caletieae*
Subtribe: *Hyaenanchinae*
Genus: *Hyaenanche* Lamb.
Species: *H. globosa* (Gaertn.) Lamb. & Vahl
Synonym: *Jatropha globosa* Gaertn.

Hyaenanche globosa Lamb. is an endemic plant and is restricted to a single flat-topped mountain near Van Rhynsdrop in southern Namaqualand (a province in South Africa). This plant is the single species of *Hyaenanche*. It is a small, rounded tree, with dark green, leathery leaves, characteristically arranged in four along the stems. Male and female flowers are both small and occur on separate trees. The fruits are large rounded capsules with several segments (Fig 1.8). *Hyaenanche* is a Greek word for hyena

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poison and was chosen because the fruits were formerly used to poison carcasses in order to destroy hyenas and other vermin (Van Wyk *et al.,* 1997).

Figure 1.8: Aerial parts of *Hyaenanche globosa.*

1.4. THE GENUS *Maytenus*

The genus *Maytenus* belongs to Celastraceae (or staff vine or bittersweet family; syn. Canotiaceae, Chingithamnaceae, Euonymaceae, Goupiaceae, Lophopyxidaceae, and Siphonodontaceae in Cronquist system), which is indigenous to tropical and subtropical regions of the world, including North Africa, South America, and East Asia. This family consists of about 90-100 genera and 1,300 species of vines, shrubs, small trees, bushes, or lianas, which have resinous stems and leaves (Spivery *et al.,* 2002). 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 subpolar (http://en.wikipedia.org/wiki/Maytenus).

The variety of bioactivities of the Celastraceae in traditional medicine and agriculture is astonishing, which includes stimulant, appetite suppressive, sedative, emetic, purgative, memory restorative, male contraceptive, antitumor, anti-leukemic, antibacterial,

insecticidal and insect repellent activities (Costa *et al.,* 2008; Souza-Formigonia *et al.,* 1991; Spivery *et al.,* 2002). Cytotoxicity and antitumor activities of *M. ilicifolia, M. ovatus, M. cuzcoina, M. serrate*, *M. diversifolia, M*. *Molina*, *M. rigidi* and *M. emarginata* have been reported in literatures (Cargg and Newman, 2005; Hong, 2000; Hui *et al.,* 2009; Lee *et al.,* 1982; Martucciello *et al.,* 2010; Spivery *et al.,* 2002). Anti-microorganism activities of various *Maytenus ssp*. have been stated before (Avilla *et al.,* 2000; Lindsey *et al.,* 2006).

1.4.1. Compounds isolated and identified from the genus Maytenus

Over three past decades, a large number of secondary metabolites displaying a wide range of bioactivity have been extracted from the Celastraceae. Plants of 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-nicotinated 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 also been isolated from different species of the Celastraceae. However, the bulk of the bioactive constituents of this family, are terpenoids. All types of terpenoids are found in the extracts of *Maytenus ssp.* (Gonzaleza *et al.,* 2001; Queiroga *et al.,* 2000; Spivery *et al.,* 2002). Two steroids namely; β-sitosterol and β-sitosterol-βD-glucoside were isolated from the leaves of *M. floribunda* (Reiss). In addition, dulcitol, β-sitosterol and β-sitosterol-βD-glucoside; the flavonoids; 4'-O-methyl-(-)-epigallocatechin and proanthocyanidin A; the pentacyclic triterpenes: friedelin, friedelinol and 28-hydroxy-3-oxo-friedelane were isolated from the bark wood of *M. floribunda* (Salazar *et al.,* 1997). Orabi *et al.,* (2001) isolated eight compounds from the ethanol (EtOH) extracts of *M. heterophylla:* (A) dihydroagarofuran alkaloid, (B) 1β-acetoxy-9α-benzoyloxy-2β, 6α-dinicotinoyloxy-β-dihydroagarofuran, β-

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amyrin, maytenfolic acid, 3α-hydroxy-2-oxofriedelane-20α-carboxylic acid, lup-20(29)-ene-1β, 3β-diol,(−)-4′-methylepigallocatechin, and (−)-epicatechin. Compounds A and B with moderate antimicrobial activity have been reported.

The aerial parts of *M. undata* yielded different triterpenes such as: 3-oxo-11R-hydroxyolean-12-ene-30-oic acid, 3-oxo-olean-9(11), 12-diene-30-oic acid, 3,11-dioxoolean-12-ene-30-oic acid (3-oxo-18*α*-glycyrrhetinic acid), 3,4-seco-olean-4(23),12-diene-3,29-dioic acid (20-*epi*koetjapic acid), koetjapic acid, and the 12-oleanene artifact 3-oxo-11R-ethoxyolean-12-ene-30-oic acid (Muhammad *et al.,* 2000) (Fig 1.9). 'Pristimerin' (20α-3-hydroxy-2-oxo-24-nor-friedela-1-10,3,5,7-tetraen-carboxylic acid-29-methylester) purified from the ethanol extract of the root bark of *M. ilicifolia*, demonstrated anti-proliferative activity (Costa *et al.,* 2008) (Fig 1.9). Till date, there has been no report on the isolation of compounds from *M. procumbens*.

1.4.2. Maytenus procumbens

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Celastrales*
Family: *Celastraceae*
Genus: *Maytenus*
Species: *M. procumbens* (L.f.) Loes.

Maytenus procumbens (L.f.) Loes. is an indigenous native South African species, also known as Dune Koko (*duinekokoboom* in Afrikaans) tree 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 meter. Its bark is pale yellowish brown, which sometimes become fissured on old trees. The clusters of white to greenish white flowers may appear in winter, but are sometimes seen up to the end of the following summer. The inconspicuous flowers are followed by spherical fruits that burst open to reveal the bright orange seeds that are loved by birds. *M. procumbens*

occurs along much of the South African south and east coast in dune scrub and wooded areas up to the altitude of about 150 meter (Coates Palgrave, 2002) (Fig 1.10).

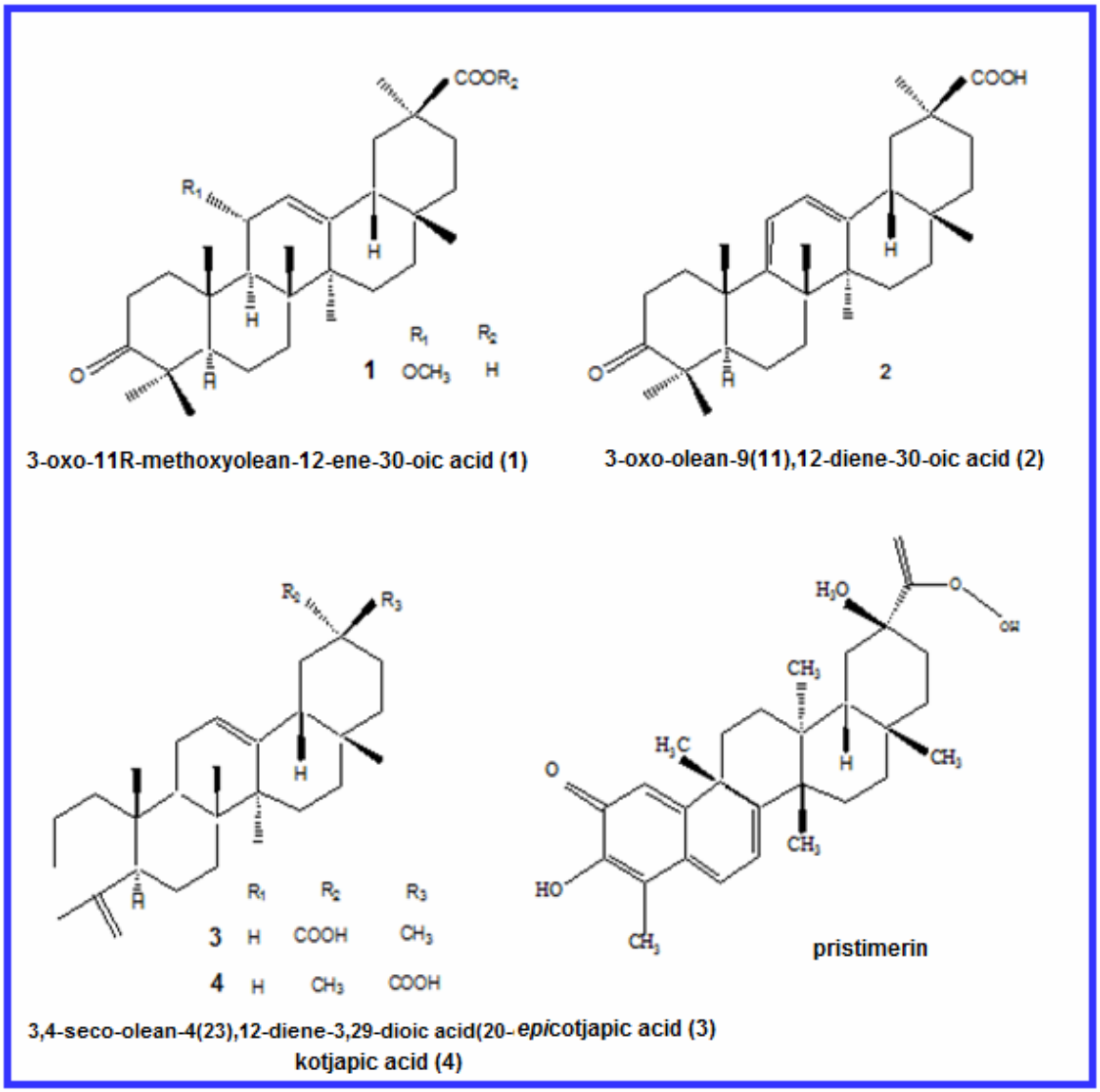

Figure 1.9: 'Pristimerin' isolated from *M. ilicifolia* (Costa *et al.,* 2008); '3-oxo-11R-hydroxyolean-12-ene-30-oic acid (1), 3-oxo-olean-9(11), 12-diene-30-oic acid (2), 3,4-seco-olean-4(23),12-diene-3,29-dioic acid (20-*epi*koetjapic acid) (3), koetjapic acid (4)' purified from *M. undata* (Muhammad *et al.,* 2000).

Figure 1.10: Aerial parts of *Maytenus procumbens.*

1.5. RATIONALE FOR THIS STUDY

Commonly scientific data representing plant-derived agents, display anticancer activity as individuals or in combination with other natural- or chemical-based components. The occurrence of non-selective *in vivo* toxicity and resistance of tumor cells to these drugs still remain problematic. It has been previously proven that different species vary in their biochemical contents resulting in diverse biological activities. In present study, two endemic South African species have been investigated (i) to establish whether these species induce selective cytotoxicity in cancerous cells, (ii) isolate novel compounds with possible cytotoxicity and, (iii) to identify mechanism involved in inducing cytotoxicity?

1.6. AIM AND HYPOTHYSIS

Interest in discovery of herbal-based anticancer agents has increased globally in past decades. Multiple natural agents have been recognized to block carcinogenesis in certain cancer cell lines or animal models. Although much progress has been made in reducing mortality rates, stabilizing incidence rates, and improving survival, cancer still accounts for more deaths all over the world after heart diseases. However, the appearance of drug-resistance tumor cells, non-selectivity of these cancer drugs, and high adverse effects still stay critical. Therefore, acceleration of further progression

regarding to discovery of novel anticancer agents seem to be crucial. Thus, the aim of our study was to characterize the effect of two plant species and their isolated compounds on some human cancer cell models.

1.7. OBJECTIVES

According to defined hypotheses, the objectives of this study were as follows:

a. To identify the most promising plant extracts by comparing the cytotoxicity of the experimental crude extracts on cancerous and non-tumour cell lines.

b. Isolation and identification of possible major compounds from the selected extracts.

c. To determine whether there is a correlation between the antioxidant properties and cytotoxicity of the test compounds.

d. To determine whether there is a connection concerning the anti-microorganisms activities and cytotoxicity of the selected compounds.

e. To determine the induced cell death pathway on a cancer cell line with a flow cytometric method.

f. To investigate whether the selected compounds have an effect on the mitochondrial membrane potentials of cancerous cells or not.

g. To measure if there is any chance of enhancing mutagenicity in selected cancer cells by test compounds.

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CHAPTER 2

Investigation of the possible biological activities of a poisonous South African plant; *Hyaenanche globosa* (Euphorbiaceae)*

2.1. ABSTRACT

The present study was undertaken to explore the possible biochemical activities of *Hyaenanche globosa* Lamb. and its compounds. Two different extracts (ethanol and dichloromethane) of four different parts (leaves, root, stem and fruits) of *H. globosa* were evaluated for their possible antibacterial, anti-tyrosinase and anticancer (cytotoxicity) properties. Two pure compounds were isolated using column chromatographic techniques. Active extracts and pure compounds were investigated for their antioxidant effect on cultured HeLa cells. Antioxidant/oxidative properties of the ethanolic extract of the fruits of *H. globosa* and purified compounds were investigated using reactive oxygen species (ROS), ferric-reducing antioxidant power (FRAP) and lipid peroxidation thiobarbituric acid reactive substance (TBARS) assays. The ethanolic extract of the leaves and fruits of *H. globosa* showed the best activity, exhibiting a minimum inhibitory concentration (MIC) of 3.1 mg/ml and a minimum bactericidal concentration (MBC) of 1.56 and 6.25 mg/ml, respectively, against *Mycobacterium smegmatis*. The ethanolic extract of the fruits of *H. globosa* (F.E) showed the highest percentage of inhibitory activity of monophenolase (90.4% at 200 µg/ml).

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Subsequently, F.E was fractionated using phase-partitioning with *n*-hexane, ethyl acetate, and *n*-butanol. The cytotoxicity of these fractions were determined *in vitro* using different cancer cell lines. The *n*-hexane fraction exhibited the highest activity of toxicity. Therefore, this fraction was subjected to further separation by chromatographic methods. Two pure compounds known as; 'tutin **1**' and 'hyenanchin **2**' were isolated and their structures were determined by NMR spectroscopic methods. Unpredictably, none of them showed significant ($P< 0.01$) inhibition on cell viability/proliferation at the concentrations that were used. F.E demonstrated potent inhibition of DPPH radical activity similar to vitamin C (positive control). 'Tutin **1**' and 'hyenanchin **2**' were found with marginal antioxidant activity of which 'compound **1**' showed more potent activity than 'compound **2**'. F.E showed significant anti-tyrosinase, antibacterial, and cytotoxicity effects, therefore it can be considered as an effective inhibitor alone or in combination with other plant extracts.

2.2. INTRODUCTION

There is great scope for new drug discoveries based on traditional medicinal plant use throughout the world (Hasani-Ranjbar *et al.,* 2009). Nowadays, at least 25% of the active compounds in the currently prescribed synthetic drugs were first identified in plant resources (Van Wyk *et al.,* 1997) and 20,000 plants have been used for medicinal proposes, of which, 4,000 have been used commonly and 10% of those are commercially available. The Euphorbiaceae family is one of the largest families of plants, with about 300 genera and 7,500 species of mostly monoecious herbs, shrubs, and trees that are further frequently characterized by a milky sap or latex material. Members of Euphorbeaceae family have been investigated for providing potential treatments for cancer, tumors, and warts (Lewis and Elvin-Lewis, 1995). The chemistry of Euphorbiaceae is one of the most diverse and interesting one of the flowering plant families and is comparable to the biological diversity of the family. Of all chemical classes, the most useful for a chemotaxonomic study of the Euphorbiaceae, above the level of genus, appear to be alkaloids, cyanogenic glycosides, diterpenes, glucosinolates, tannins and triterpenes. *Hyaenanche globosa* Lamb. (Euphorbeaceae) is

a narrow endemic plant and is restricted to a single flat-topped mountain near Van Rhynsdrop in southern Namaqualand. This plant is the single species of *Hyaenanche*. It is a small, rounded tree, with dark green, leathery leaves, characteristically arranged in four along the stems. Male and female flowers are both small and occur on separate trees. The fruits are large rounded capsules with several segments. *Hyaenanche* is a Greek word for hyena poison and was chosen because the fruits were formerly used to poison carcasses in order to destroy hyenas and other vermin. This plant contains several toxic sesquiterpene lactones, such as, tutin, mellitoxin, urushiol III and isodihydrohyaenanchine. Its main toxin, tutin, is known to cause convulsions, delirium, and coma in humans (Hasani-Ranjbar *et al.,* 2009; Van Wyk *et al.,* 1997).

Pigmentation has become an important phenotypical characteristic, in the pharmaceutical, medicinal as well as in the cosmetic field. Plants and their extracts are inexpensive and rich resources of active compounds that can be utilized to inhibit tyrosinase activity as well as melanin production. Natural and synthetic chemical agents can frequently modulate the metabolism of pigmentation produced. The methanolic extract of the aerial parts of *H. globosa* exhibited significant inhibitory effect on the monophenolase and diphenolase activated forms of tyrosinase *in vitro* (Momtaz *et al.,* 2008). Therefore, it was decided to prepare different extracts from this species to investigate the possible biological activities of the plant.

Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging and other degenerative diseases in humans (Halliwell, 1994; Poulson *et al.,* 1998). Ames *et al.,* (1995) expressed oxidative injury might induce gene mutation and promote carcinogenesis. In opposition, oxidative injury can lead to cell death (apoptosis). Oxidative stress can modulate the apoptotic programme (Bjelakovic *et al.,* 2004; Fruehauf and Meyskens, 2007). The role of plant extracts and natural purified compounds in alteration of pro-oxidant status in cancerous

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cell lines has described scantily in past. 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)

Based on the reported ethnobotanical information about the poisonous properties of the fruits of *H. globosa* (Van Wyk *et al.,* 1997), it was considered that the other parts also might have the same effects. To explore the possible bioactivities of this species, two different extracts (dichloromethane and ethanol) of fruits, leaves, roots and stem were prepared separately.

2.3. MATERIALS AND METHODS

2.3.1. Chemicals and reagents

Fetal bovine serum (FBS) and RPMI 1640 were purchased from Gibco (Paisley, UK). Penicillin/streptomycin was obtained from Roche (Mannheim, Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) powder, DCFH-DA (2,7-dichlorofluorescin diacetate), 1,2-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ) and all the other chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK). $FeCl_3.6H_2O$, sodium sulfate and $FeSO_4$, 2-thiobarbituric acid (TBA), Mueller Hinton agar (MHA) and Sabouraud dextrose agar (SDA) were obtained from Merck (Tehran, Iran). *L*-Tyrosine, *L*-DOPA, tyrosinase, arbutin and kojic acid were obtained from Sigma-Aldrich (Kempton Park, South Africa). All chemicals and solvents were of the highest commercial grade.

2.3.2. Preparation of plant extracts

The *H. globosa* (leaves, roots, stem and fruits) materials 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 (Voucher herbarium specimen number: S.M. 95499). Forty grams of each powdered part (shade dried) was soaked in 200 ml of ethanol and dichloromethane separately for four hours and after filtration the solvents were removed under vacuum (BUCHI, Rotavapor, R-200) to yield dry extracts (F.E: Fruits, ethanol extract; F.DC: Fruits, dichloromethane extract; L.E: Leaves, ethanol extract; L.DC: Leaves, dichloromethane extract; R.E: Root, ethanol extract; R.DC: Root, dichloromethane extract; S.E: Stem, ethanol extract; S.DC: Stem, dichloromethane extract).

2.3.3. Antibacterial bioassay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined against *Mycobacterium smegmatis* (MC^2 155, American Type, USA Culture Collection) as described previously (Mativandlela *et al.,* 2007; Mativandlela *et al.,* 2008). The sample extracts were dissolved in 10% dimethyl sulfoxide (DMSO) in a sterile Middlebrook 7H9 broth base, to obtain a stock concentration of 50.0 mg/ml. Serial two-fold dilutions of each sample to be evaluated were made with 7H11 broth, to yield volumes of 200 µl/wells, with final concentrations ranging from 12.5 mg/ml to 0.390 mg/ml. The highest percentage of DMSO (10%), which was not toxic to bacteria, was used in this assay. Ciprofloxacin at a final concentration of 0.156 mg/ml, served as a positive drug control.

The samples were also tested against *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 (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) (Appendices C.6.1-C.6.3).

2.3.4. Inhibition of tyrosinase activity and DOPA auto-oxidation

This assay was performed using methods as described earlier (Curto *et al.,* 1999; Nerya *et al.,* 2003). The extracts were dissolved in DMSO to a final concentration of 20 mg/ml. This extract stock solution was then diluted to 600 µg/ml in a 50 mM potassium phosphate buffer (pH 6.5). The extracts were tested only at two concentrations, 20 and 200 µg/ml, for their inhibitory effect on the monophenolase and diphenolase activated forms of tyrosinase *in vitro*. Arbutin and kojic acid (positive controls) were also tested at the above-mentioned concentrations. In a 96-well plate, 70 µl of each extract dilution was combined with 30 µl of tyrosinase (333 units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, 110 µl of substrate (2 mM *L*-tyrosine or 12 mM *L*-DOPA) was added to each well. Incubation commenced for 30 minutes at room temperature. The optical densities of the wells were then determined at 492 nm with the BIOTEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

2.3.5. Isolation of active constituents

The ethanolic extract of the fruits of *H. globosa* (F.E) exhibited the highest cytotoxicity effect of HeLa cells compared to the other extracts. The ethanolic extract was selected for the isolation and identification of active principle(s). One thousand two hundred (1,200) grams of air-dried fruits of the plant were milled into a fine powder using a commercial grinder. The powder was extracted thrice, each time with 3 L of ethanol at 50°C for 24 hours. The combined ethanol extract was filtered and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator.

The dried ethanolic extract of the fruits of *H. globosa* (70 g) was re-dissolved in 80% ethanol (ethanol/distilled water; 75:25) and partitioned with *n*-hexane and ethyl acetate. The organic layers were evaporated to dryness at 40°C to give 22 g, 28 g and 18 g of *n*-hexane, ethyl acetate and aqueous fractions, respectively (Appendices A.1). The bioassay of these fractions of *H. globosa* showed that the *n*-hexane fraction demonstrated the highest inhibition of cell growth/proliferation (82% at 100 µg/ml) in the HeLa cells. It was therefore subjected to fractionation on a Silica gel column LH-20

(7×50 cm) using a gradient of *n*-hexane: ethyl acetate of increasing polarity (0 to 100% ethyl acetate). Forty-two fractions were collected and those with similar thin-layer chromatography (TLC) profiles were combined. TLC plates were developed using (*n*-hexane: ethyl acetate; 9:1) as eluent. Acidic vanillin was used as a detecting agent. Fractions exhibiting similar TLC profiles were combined together to provide 14 major fractions (1B to 14B) (Appendices A.1).

The pure compound 'tutin **1**' was crystallized from 12B spontaneously (white hairy crystals, yield: 456 mg; 0.038%) (Fig 2.1) (The ^{1}H & ^{13}C NMR spectra are presented in Appendices A.2.1-A.2.2). Fractions 13B and 14B (2.645 g) were chromatographed separately using silica gel column LH-20 (Sigma-Aldrich, Jet Park, South Africa) using *n*-hexane: ethyl acetate of increasing polarity (0 to 90% ethyl acetate) as an eluent, to obtain pure 'hyenanchin **2**' from 13B (white rounded crystals, yield: 347 mg; 0.028%) (Fig 2.1) (The ^{1}H & ^{13}C NMR spectra are presented in Appendices A.3.1-A.3.2). The compounds were identified by mass spectrometric and NMR data, which were identical to those in the literature. The schematic presentation of the isolation steps are shown in Appendices A.4.

2.3.6. Cell culture

Six cancerous cell lines HT29/219 (Human, Colon, epithelial-like, Carcinoma), HeLa (Human, Cervix, epithelial-like, Carcinoma), Caco-2 (Human, Colon, Adenocarcinoma), NIH-3T3 (Swiss NIH mouse, embryo fibroblast), K562 (Human, Pleural effusion, Lymphoblast-like) and T47D (Human, Breast, ductal-carcinoma), and one normal cell line (HPLF) were purchased from the Pasteur Institute, Tehran, Iran. The cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 0.28 units/ml insulin, 100 µg/ml streptomycin, 100 units/ml penicillin and 0.3 mg/ml glutamine. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2, in air (Appendices C.1.1-C.1.2).

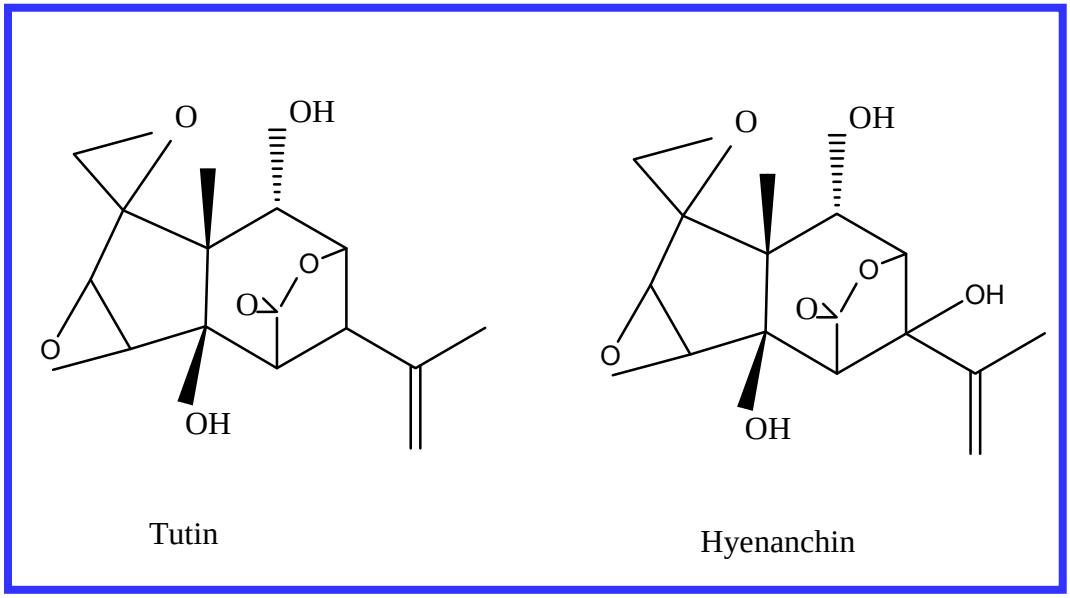

Figure 2.1: Chemical structures of the isolated compounds from the ethanolic extract of F.E (fruits, ethanol extract) of *H. globosa*.

2.3.7. Cytotoxicity

The cytotoxicity of the different extracts of *H. globosa* and the isolated compounds from the ethanolic extract of fruits ('tutin **1**' and 'hyenanchin **2**') was assayed using the MTT cytotoxicity assay with modifications (Mosmann, 1983; O'Brien *et al.,* 2000) (Appendices C.1.3). The cells (3×10^4) were plated in 500×1 of medium/well in 48-well plates (NUNC Cell Culture Flasks, Roskilde, Denmark). After an overnight incubation at 37°C, in 5% CO_2, and a humidified atmosphere, the extracted samples were added to the cells to a final concentration of 500 µg/ml.

'Metotherexate' (positive control) and pure compounds were examined at concentrations ranging from 5, 10, 20, 40, 80 and 100 µg/ml. The plates were incubated at 37°C, in 5% CO_2, humidified atmosphere, for 48 hours. After 48 hours, 50 µl of 5 mg/ml MTT (dissolved in PBS) was added per well. After three hours of incubation, the MTT solution was removed and the cells were washed with 100 µl of PBS, twice. One hundred and fifty microlitres of DMSO was added per well, to solubilize the formazan crystals. The optical densities of the wells were then measured at 570 nm (690 nm

reference wavelength). By referring to the control (medium with DMSO), the cell survival was assessed.

2.3.8. 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.3.1-C.3.2).

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

As mention earlier (section 2.3.7), HeLa cells ($1X10^6$) were seeded in 25-cm^2 cell culture flasks (Falcon) (NUNC Cell Culture Flasks, Roskilde, Denmark) in a minimum essential medium RPMI 1460 (Gibco, Paisley, UK), until nearly confluent. After an overnight incubation at 37°C, in 5% CO_2, and a humidified atmosphere, F.E was added to the cells to form final concentrations of 12.5-400 µg/ml. 'Tutin **1**' and 'hyenanchin **2**' (isolated pure compounds) were examined at concentrations ranging from 5, 10, 20, 40, 80 and 100 µg/ml. The plates were incubated at 37°C, in 5% CO_2, and a humidified atmosphere for 48 hours. Thereafter, the medium was removed and 2 ml of 'trypisin' was added to each flask to harvest the cells. The cells were centrifuged at 2000 rpm for five minutes and were resuspended in PBS, twice. The pellets were used for FRAP and TBARS.

2.3.10. Ferric-reducing antioxidant power assay (FRAP)

Following the procedures as described by Dehghan *et al.,* (2007) the total antioxidant capacities of (F.E), 'tutin **1**' and 'hyenanchin **2**' were determined by measuring the ability of the medium to reduce Fe^{3+} to Fe^{2+}. The complex between Fe^{2+} and TPTZ gave a blue color, with absorbance at 593 nm (Appendices C.2).

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. To precipitate the cell's proteins, 500 µl of trichloroacetic acid (TCA) 20% (m/V) was added into 100 µl of the sample, which was then centrifuged at 1500 rpm for 10 minutes. Then 500 µl of sulfuric acid (0.05 M) and 400 µl TBA (0.2%) were added to the sediment, shaken and incubated for 30 minutes in a boiling water bath. Subsequently, 800 µl of *n*-butanol was added and the solution was centrifuged, cooled, and the supernatant absorption was recorded at 532 nm, using a Synergy4 BIOTEK multi-well plate reader (BIOTECK, Vermont, USA). The calibration curve was obtained using different concentrations of 1,1,3,3-tetramethoxypropane as a standard to determine the concentration of thiobarbituric acid/malondialdehyde (TBA/MDA) adducts in samples (Sarkheil *et al.,* 2007; Satho, 1978). Data were normalized by dividing the TBA content on 'HeLa cells' survival in related concentrations of samples.

2.3.12. Measurement of intracellular reactive oxygen species

This assay was performed using methods as described by Yong Sun *et al.,* (1999); Wang and Joseph, (1999) with slight modifications. On day one, 1×10^4 number of HeLa cells were seeded in 96-well black fluorescent cell culture plates. The intracellular generation of ROS was measured using the oxidation- sensitive fluorescent dye 2,7-dichlorofluorescin diacetate (DCFH-DA). On the second day, the cells were incubated with 500 µl of different concentrations of samples (final concentrations of 12.5-400 µg/ml for the sample extract (F.E) and 3.1-100 µg/ml for pure compounds). After an hour, the medium was removed and the cells were washed with HBSS (Life Technologies, Inc.) twice. The cells were then incubated with 500 µl of HBSS containing 10 µg/ml of DCFH-DA 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 intensity was used to represent the generation of net intracellular ROS. Nontreated cells were used as negative control in contrast to H_2O_2 as positive control in concentrations of 125 to

2,000 mM.

2.4. RESULTS AND DISCUSSION

Nowadays, the discovery of novel phyto-pharmaceuticals from natural sources is extremely encouraging. Despite the variety and frequency of the Euphorbiaceae species, very little information on the medicinal values of *H. globosa* is available. Based on the ethnobotanical information about the toxicity effect of the fruits of this species (Van Wyk *et al.,* 1997), it was considered that the other parts also might have the same effects. To explore the possible bioactivities of *H. globosa*, two different extracts (dichloromethane and ethanol) of fruits, leaves, roots and stem were prepared separately. The antibacterial, anticancer and anti-tyrosinase activities of these extracts were examined.

In antimicrobial assay, L.E, R.DC, L.DC, F.DC and F.E exhibited the MIC values of 1 mg/ml against *S. aureus*. The growth inhibitory rate of *B. subtilis* was 1 mg/ml while R.DC, L.E and F.E were used. R.DC showed the MIC of 3 mg/ml against *A. niger* while L.DC exhibited the MIC of 6 mg/ml. *H. globosa* showed more effective on gram positive bacteria than those of negative. Amongst pure compounds, only 'tutin **1**' showed inhibitory activity exhibiting MICs of 400 and 800 µg/ml for *S. aureus* and *P. aeruginosa,* respectively. None of pure compounds inhibited the growth of fungi tested (Table 2.1).

Of the eight different extracts of *H. globosa*, against *M. smegmatis*, R.DC and L.DC were found to be the most effective. They exhibited MIC values of 0.39 mg/ml against *M. smegmatis*. The L.E and F.E were the next best extracts, which inhibited growth at 3.13 mg/ml. The F.DC, R.E, S.DC and S.E had the same MIC of 6.25 mg/ml. Ciprofloxacin (positive drug control for *M. smegmatis*) inhibited the growth of bacteria at a concentration of 0.15 mg/ml (Table 2.2). Mativandlela *et al.,* (2008) reported that the ethanolic extracts of *Artemisia afra*, *Drosera capensis* and *Galenia africana* exhibited MIC values of 1.56, 3.1 and 0.78 mg/ml against *M. smegmatis*. Comparison of the data obtained in this study with the previously published results shows

that the different extracts of *H. globosa* represent promising growth inhibitory activity of *M. smegmatis*, even better than some pure compounds. Epigallocatechin, catechin and umckalin, isolated from the butanolic extract of the root of *Pelargonium sidoides* showed a minimum inhibitory concentration (MIC) of 7.8, 31.25 and 62.5 mg/ml, respectively against *M. smegmatis*.

The ethanolic extracts from the fruits, leaves, and roots of *H. globosa* showed 90.4%, 87% and 86.8% inhibition of tyrosinase activity at 200 µg/ml ($P< 0.01$), respectively. They also demonstrated 31%, 8.4% and 13.7% inhibition of DOPA auto-oxidation, respectively, at 200 µg/ml ($P< 0.01$). Other extracts showed a marginal inhibition of tyrosinase and DOPA auto-oxidation activity. None of the isolated pure compounds represented significant inhibitory anti-tyrosinase activity. Kojic acid significantly showed 100% inhibition of monophenolase activity at 200 µg/ml ($P< 0.01$), while arbutin exhibited 32.4% anti-tyrosinase activity ($P< 0.01$). The inhibition of *L*-DOPA auto-oxidation was determined as 83.3% and 0% by Kojic acid and arbutin, respectively (Table 2.3). In our previous study, the methanolic extract of the leaves of *H. globosa* showed 92% and 42% inhibition of monophenolase and diphenolase activities at 500 µg/ml, respectively (Momtaz *et al.,* 2008). Another publication reviewed, *Glycyrrhiza glabra*, *Morus alba* and *Gastrodia ellata* (80% ethanol extract), which showed 65%, 68% and 85% tyrosinase inhibition at the concentration of 333 µg/ml, respectively (Lee *et al.,* 1997).

Table 2.1: Antibacterial activities of different extracts of *H. globosa.*

	MIC (mg/ml) [a]					
Bacteria	***B. subtilis***	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***	***A. niger***
Samples						
F. E	1	1	8	2	-	-
F. DC	1	1	-	-	-	-
L. E	8	1	-	6	-	8
L. DC	-	1	-	8	-	6
R. E	8	-	-	-	-	-
R. DC	1	1	8	-	-	3
S. E	-	6	-	8	-	8
S. DC	-	6	-	-	-	8
b	0.01	0.01	0.01	0.05	N	N
c	N	N	N	N	0.001	0.0002

F.E: fruits, ethanol extract; F.DC: fruits, dichloromethane extract; L.E: leaves, ethanol extract; L.DC: leaves, dichloromethane extract; R.E: root, ethanol extract; R.DC: root, dichloromethane extract; S.E: stem, ethanol extract; S.DC: stem, dichloromethane extract.
[a] Minimum inhibitory concentration.
[b] Streptomycin sulfate.
[c] Amphotricin B.
[N] Not tested.
[-] MICs were more than the highest concentration tested.

In recent years, the anticancer property of various sesquiterpene lactones has attracted a great deal of interest and extensive research has been carried out to characterize the anticancer activity, the molecular mechanisms, and the potential chemotherapeutic application of them (Zhang *et al.,* 2005). Among the eight different extracts of *H. globosa*, R.E and F.E demonstrated IC_{50} values of 46.5 µg/ml and 37.7 µg/ml of the HeLa cells, respectively ($P< 0.01$). The other extracts did not show significant inhibition of the cell growth or proliferation (Table 2.4). Accordingly, the F.E phase partitioned into three fractions (section 2.3.5), of which the *n*-hexane fraction

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demonstrated the highest inhibition of HeLa cells growth/proliferation (82% at 100 µg/ml). Subsequently, two known pure compounds; 'tutin **1**' and 'hyenanchin **2**' were isolated. Following this, their cytotoxicity and antioxidant activities were examined using conventional methods. F.E exhibited IC_{50} values of 25.1, 25.9, 82.1, >120, >120 and 49.2 µg/ml when Caco-2, HeLa, HT29, NIH3T3, K562 and T47D were used, respectively (Appendices A.5).

Fuentealba *et al.,* (2000) reported the concentration-dependent inhibitory effect of tutin, obtained from *Coriaria ruscifolia* subspecies *ruscifolia*, on spinal glycine receptors. In another study, tutin isolated from the essential oils of the *Pimpinella* species, was not cytotoxic to the mammalian cells that were explored (SK-MEL, SK-OV3, BT-549, and KB) (Tabanca *et al.,* 2007). The literature review showed an epileptogenic action by tutin, derived from *Coriaria Lactone* (a mixture that has been used to establish animal models of epilepsy) in rats, demonstrating that tutin is a potent convulsant (Zhou *et al.,* 2006).

Hall, (1978) found that tutin and hyenanchin were present in common foods, such as potatoes, rice, carrots and honey. Their safety depended on the amount of 'tutin **1**' and 'hyenanchin **2**' present in the food. The bioactivities that are reported in this study are novel, and to the best of our knowledge there are no other multi-sides about *H. globosa* that have been studied to date.

'Tutin **1**' and 'hyenanchin **2**' did not show any significant reduction on cell viability/proliferation on the tested cell lines (Appendices A.6-A.7). The IC_{50} value of 60 µg/ml was observed for all samples in the 'HPLF' normal control cells (Table 2.5). The effect of methotrexate (anticancer drug) on the viability of different cancer cell lines is shown in Table 2.6.

Table 2.2: Antibacterial activities of different extracts of *H. globosa* against *M. smegmatis*.

Samples	MIC [a] (mg/ml)	MBC[b] (mg/ml)
F. E	3.1±0.4	6.2±1.4
F. DC	6.2±0.9	3.1±0.6
L. E	3.1±0.6	1.5±0.6
L. DC	0.39±0.4	25±3.3
R. E	6.2±1.1	1.5±2.7
R. DC	0.39±0.7	25±3.4
S. E	6.2±1.3	6.2±0.4
S. DC	6.2±4.1	NA[c]
CIP	0.15±0.1	3.12±1.8

F.E: fruits, ethanol extract; F.DC: fruits, dichloromethane extract; L.E: leaves, ethanol extract; L.DC: leaves, dichloromethane extract; R.E: root, ethanol extract; R.DC: root, dichloromethane extract; S.E: stem, ethanol extract; S.DC: stem, dichloromethane extract; CIP: Ciprofloxacin.
[a] Minimum inhibitory concentration.
[b] minimum bactericidal concentration
[c] NA, no activity at highest concentration tested.
Data are mean ±SD of three separate experiments.

Only few investigations have been performed that led to the isolation of a few active principles of this plant. As mentioned before, *H. globosa* contains several toxic sesquiterpenes, such as, tutin, mellitoxin, urushiol III and isodihydrohyaenanchine (Corbella *et al.,* 1969; Hasani-Ranjbar *et al.,* 2009; Van Wyk *et al.,* 1997). Several studies have reported that tutin is the major neurotoxin in the New Zealand shrubs of the genus *Coriaria*. Kinoshita *et al.,* (2005) succeeded in isolating 'tutin' from the acetone extracts of achenes separated from the *Coriaria japonica* berries. The hydroxy derivative 'hyenanchin' (also called mellitoxin) is a major active component in toxic honey (Perry *et al.,* 2001; Porter, 1969; Sutherland, 1992).

Table 2.3: Inhibitory activities of mushroom tyrosinase and DOPA auto-oxidation by different extracts of *H. globosa.*

Sample	% Inhibition of DOPA auto-oxidation (%) at 20 µg/ml	Inhibition of DOPA auto-oxidation (%) at 200 µg/ml	Inhibition of tyrosinase (%) at 20 µg/ml	Inhibition of tyrosinase (%) at 200 µg/ml
F. E	15.7±0.03	31.7±0.05	13±0.01	90.4±0.03
F. DC	15.5±0.02	19.1±0.09	0	1.8±0.02
L. E	0	8.4±0.06	4.8±0.04	87±0.02
L. DC	13.3±0.03	13.6±0.02	0	0
R. E	9±0.03	13.7±0.02	53.8±0.03	86.8±0.06
R. DC	14.8±0.06	18.3±0.02	0	0
S. E	0.9±0.03	0	0	40.2±0.01
S. DC	7.4±0.04	10.1±3	0	0
Kojic acid	42.2±0.2	83.3±0.2	99±0.1	100±0.5
Arbutin	0	0	8.7±0.8	32.6±0.1

F.E: fruits, ethanol extract; F.DC: fruits, dichloromethane extract; L.E: leaves, ethanol extract; L.DC: leaves, dichloromethane extract; R.E: root, ethanol extract; R.DC: root, dichloromethane extract; S.E: stem, ethanol extract; S.DC: stem, dichloromethane extract.
Data are mean ±SD of three separate experiments.

Various compounds derived from the plant's secondary metabolites are commonly used in cancer chemotherapy, but only a few are potent and effective. The MTT analysis showed that pristimerin (triterpenoid), isolated from *Maytenus ilicifolia* Martius (ethanolic extract of root bark) exhibited IC_{50} values of 0.55 to 3.2 µg/ml, against MDA/ MB435 and K562 (Costa *et al.,* 2008).

Sayyah *et al.,* (2002) described that the essential oil of the leaves of *Croton flavens* exhibited IC_{50} values of 27.4 µg/ml for A-549 (human lung carcinoma) and 28.3 µg/ml

for DLD-1 (human colon adenocarcinoma). In another study, two fractions of *Myrica gale* (60-minute and 30-minute fractions) were assessed against A-549 and DLD-1. The 60-minute fraction showed higher anticancer activity against both tumor cell lines with an IC_{50} value of 88.1 µg/ml. The 30-minute fraction had an IC_{50} value of 184.4 µg/ml for A-549 and 160.3 µg/ml for DLD-1. The higher cell growth inhibition induced by the 60-minute fraction, as compared to the 30-minute fraction, could be due to sesquiterpene enrichment (Sylvestre *et al.,* 2006).

Table 2.4: Effect of eight different extracts of *H. globosa* on the viability of HeLa cells using MTT assay.

Samples	IC_{50} (µg/ml)
F. E	37.7±3.2
F. DC	> 120
L. E	> 120
L. DC	> 120
R. E	46.5±4.6
R. DC	> 120
S. E	> 120
S. DC	> 120

F.E: fruits, ethanol extract; F.DC: fruits, dichloromethane extract; L.E: leaves, ethanol extract; L.DC: leaves, dichloromethane extract; R.E: root, ethanol extract; R.DC: root, dichloromethane extract; S.E: stem, ethanol extract; S.DC: stem, dichloromethane extract.
Data are mean ±SD of three separate experiments.

Table 2.5: Effect of *H. globosa* (F.E) and its isolated compounds on the viability of different cancer cell lines by using MTT assay.

Cell lines	F. E IC_{50} (µg/ml)	Tutin IC_{50} (µg/ml)	Hyenanchin IC_{50} (µg/ml)
HeLa	25.9±0.8	> 120	> 120
NIH3T3	> 120	> 120	> 120
T47D	61.5±0.1	> 120	> 120
Caco-2	25.1±0.02	> 120	> 120
HT29	82.1±0.3	> 120	> 120
K562	> 120	> 120	> 120
HPLF	>60	>60	>60

F.E; fruits, ethanol extract.
Data are mean ±SD of three separate experiments.

Table 2.6: Effect of methotrexate on the viability of different cancer cell lines using MTT assay.

Cell Lines	Methotrexate IC_{50} (µg/ml)	Methotrexate IC_{50} (nmol)
L929	0.46±0.03	101.2
NIH3T3	0.24±0.013	50
T47D	0.16±0.09	31
Caco-2	0.23±0.04	70.4
HT29	0.23±0.02	50

Methotrexate as an anticancer drug was used as a positive control. Data are mean ±SD of three separate experiments.

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 the concentrations tested ($P< 0.05$). Radical scavenging assay revealed F.E played almost a similar inhibition of DPPH activity as vitamin C after

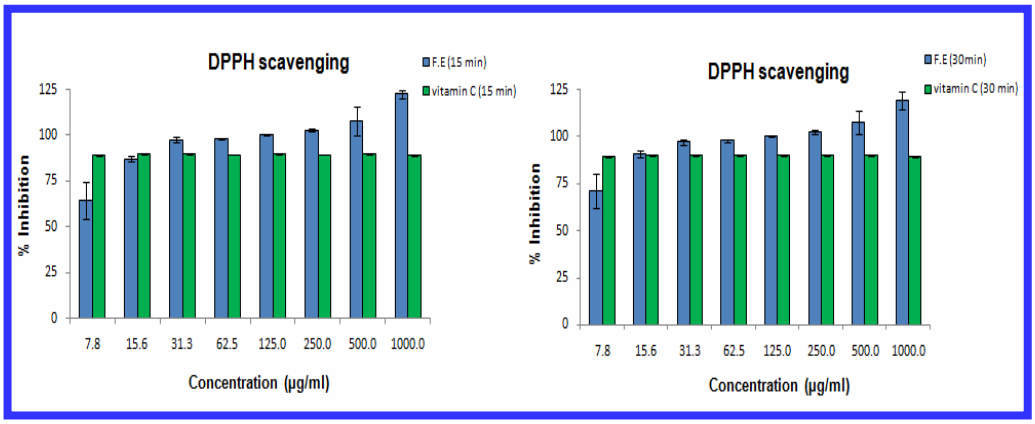
30 minutes. F.E showed DPPH discoloration between almost 70% and 120% at concentrations 7.8-1000 µg/ml after 30 minutes (Fig 2.2.a). 'Tutin **1**' showed the highest antioxidant activity (50% inhibition of DPPH at the highest concentration tested after 30 minutes) which was followed by 'hyenanchin **2**' (less than 35% antioxidant activity at all the concentrations tested). The amount of DPPH discoloration increased along time with both compounds (Fig 2.2.b).

The mean FRAP in the control cells were 399.6 µmol/L, reaching 170.7 µmol/L, in treated HeLa cells by F.E ($P< 0.01$) (Fig 2.3). Treatment of cells with pure compounds could not decrease the cell TBARS, significantly ($P< 0.01$) (Fig 2.4). As a standard for an ROS assay (to compare the production of ROS), we first tested H_2O_2 to explore the concentration-response relationship of the exposed cells. Figure 2.5 shows that the levels of ROS detected with the fluorescent dye DCFH-DA in the HeLa cells demonstrated an enhancement with time in all the samples. Among pure compounds, the ROS level did not seem to jump up very much further than the control, but F.E exhibited a very good level of ROS production at 400 µg/ml.

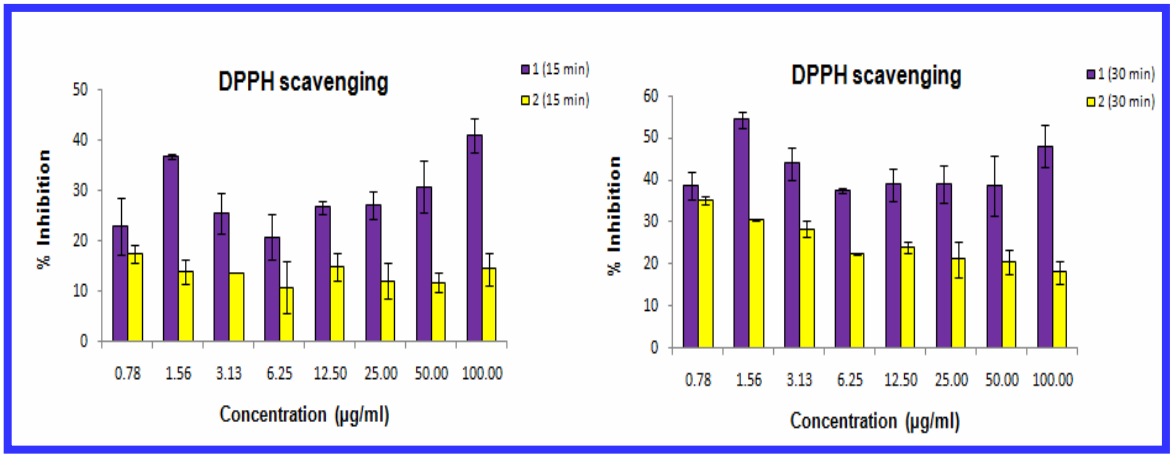
2.5. CONCLUSION

In summary, in spite of our great expectation about the toxicity of pure compounds isolated from the ethanolic extract of the fruits of *H. globosa* ('tutin **1**' and 'hyenanchin **2**'), they did not show any significant cytotoxic effects on the examined cancer cell lines, while the crude extract was well known for its poisonous effects. The poisonous effect of this plant could be due to the activity of the compounds that were not isolated yet. It could be concluded that the ethanolic extract of the fruits of *H. globosa* showed significant anti-tyrosinase, antibacterial and cytotoxic effects, therefore, it could be considered as an effective inhibitor alone or in combination with the other plant extracts. Although the data are still inconclusive and further scientific attempts are needed to confirm the traditional information or to investigate the novel medicinal aspects of this plant. A further study aims to determine the anticancer properties of other major constituents of *H. globosa*, as well as identify the unknown compounds required to fully

understand its bioactivity.



(a)



(b)

Figure 2.2: The percentage inhibition of 1,2-diphenyl-2-picrylhydrazyl (DPPH) activity after 15 and 30 minutes by; the ethanolic extract of the fruits of H. globosa (F.E), vitamin C (standard control) (a); 'tutin **1**' and 'hyenanchin **2**' (b). Each data point represents the mean of data from three wells (n= 3).

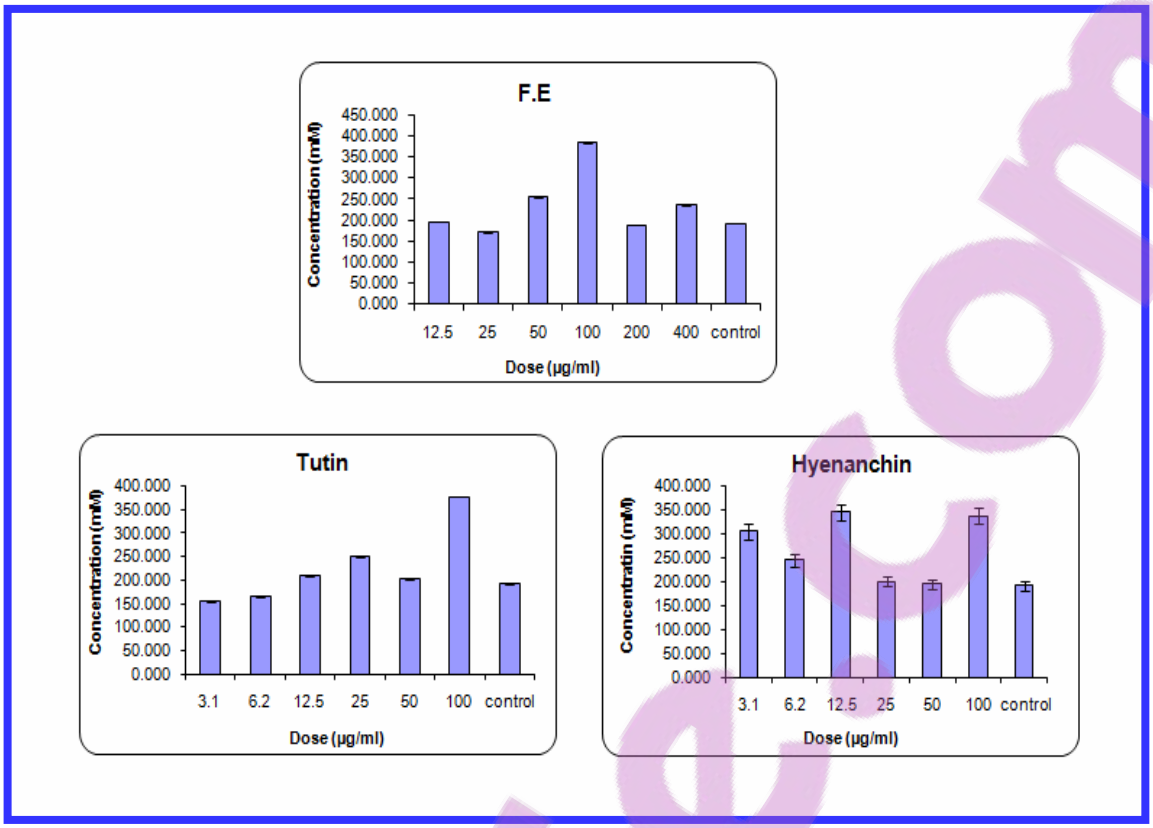


Figure 2.3: Ferric-reducing antioxidant power (FRAP) potential of the F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**' in cultured HeLa cells.

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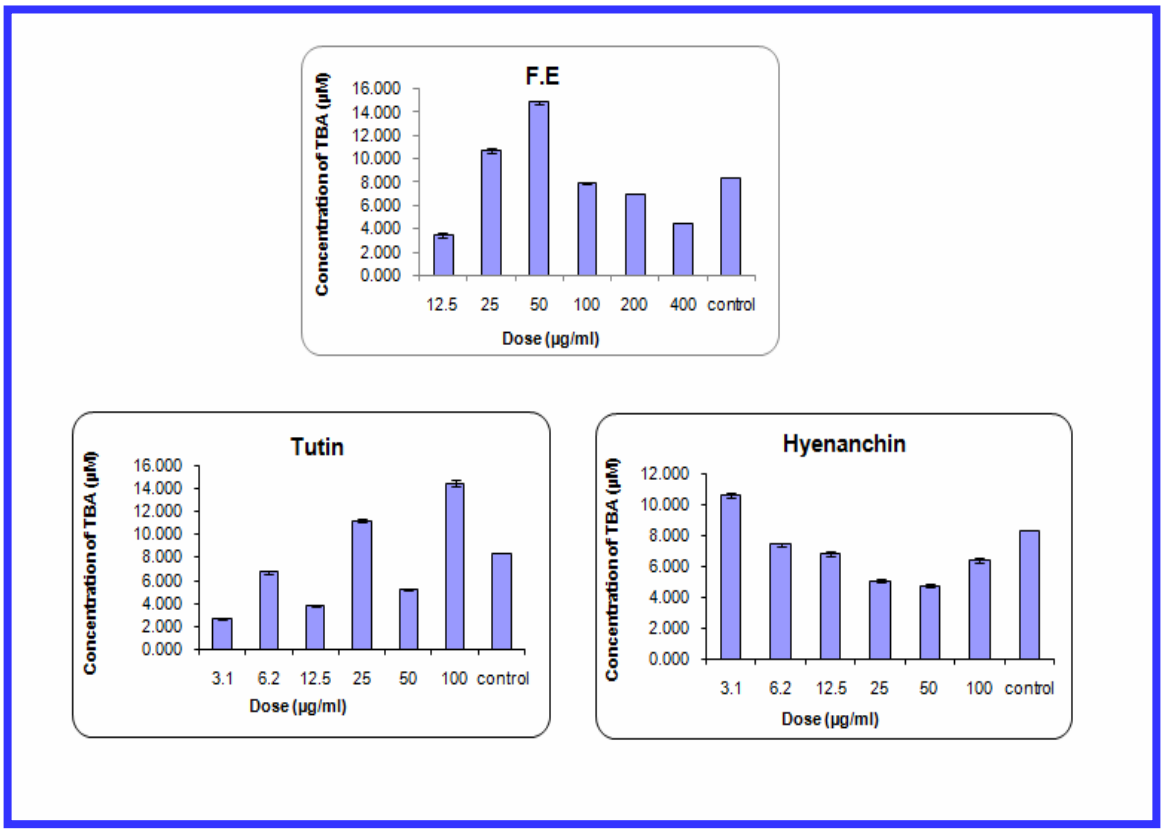

Figure 2.4: Lipid peroxidation thiobarbituric acid reactive substance (TBARS) potential of the F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**' TBARS in cultured HeLa cells.

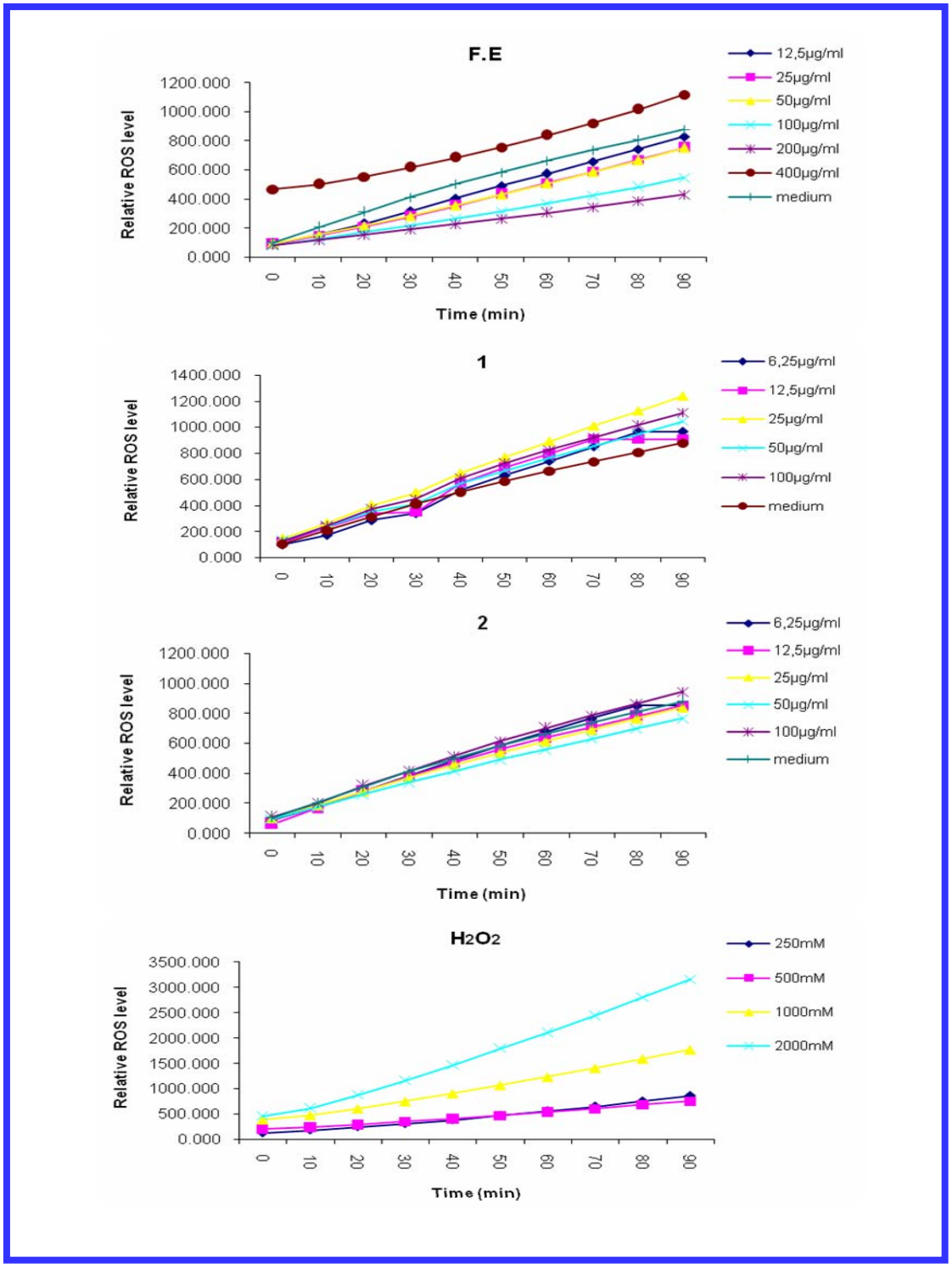

Figure 2.5: Time-response curve for DCF fluorescence in HeLa cells after 90 min exposure to various concentrations of F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**'. Each data point represents the mean of data from three wells (n= 3).

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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).

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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

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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.

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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. $[\alpha]_D$), 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; 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^oC 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, $J_{12,11\beta}$ = 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.

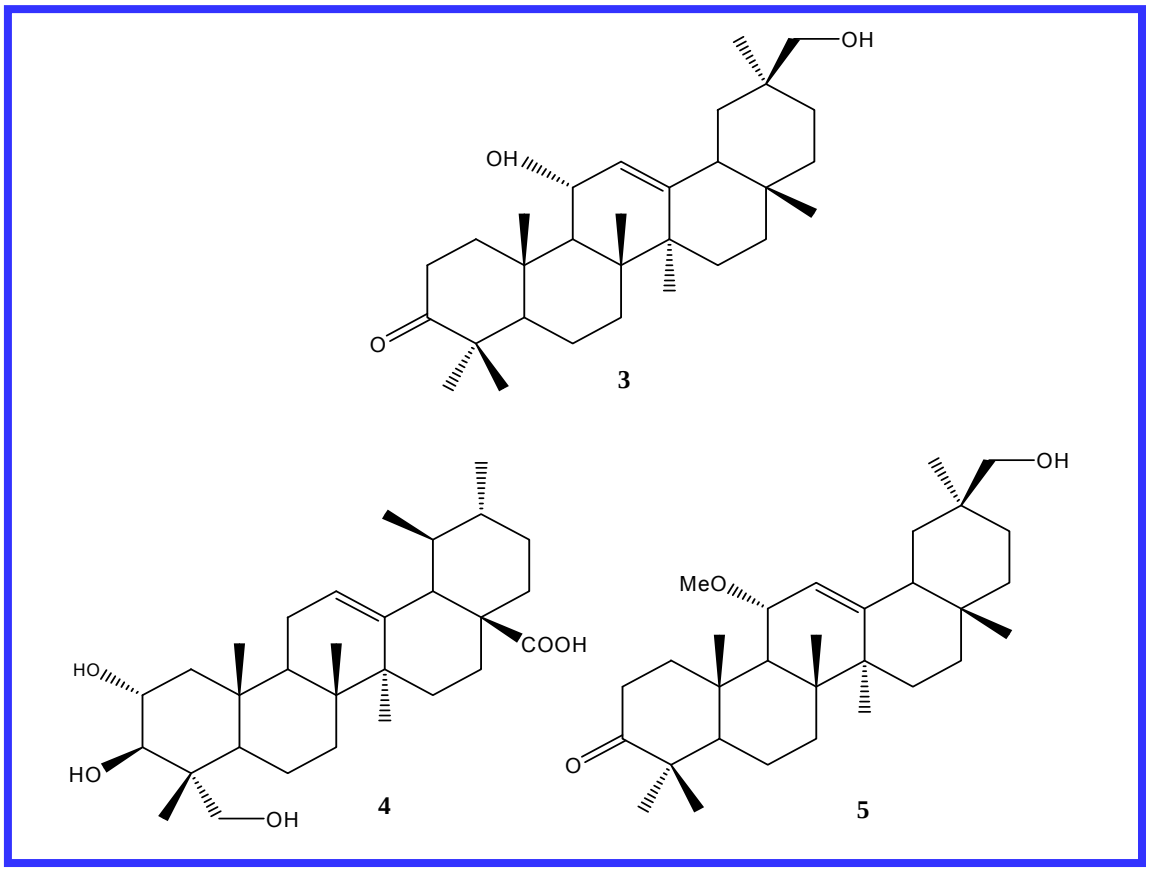

Figure 3.1: Chemical structures of the isolated compounds from the acetonic/ethanolic extract of the leaves of *M. procumbens* (L.M.P): '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**', 'asiatic acid **4**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

'Compound **5**' was isolated as amorphous colorless powder, IR (KBr disc): 3420, 2950, 2925, 2870, 1727, 1702, 1464, and 1385 cm^{-1}; $[\alpha]_{589.3}^{25}$ +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. ^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, OCH_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

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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).

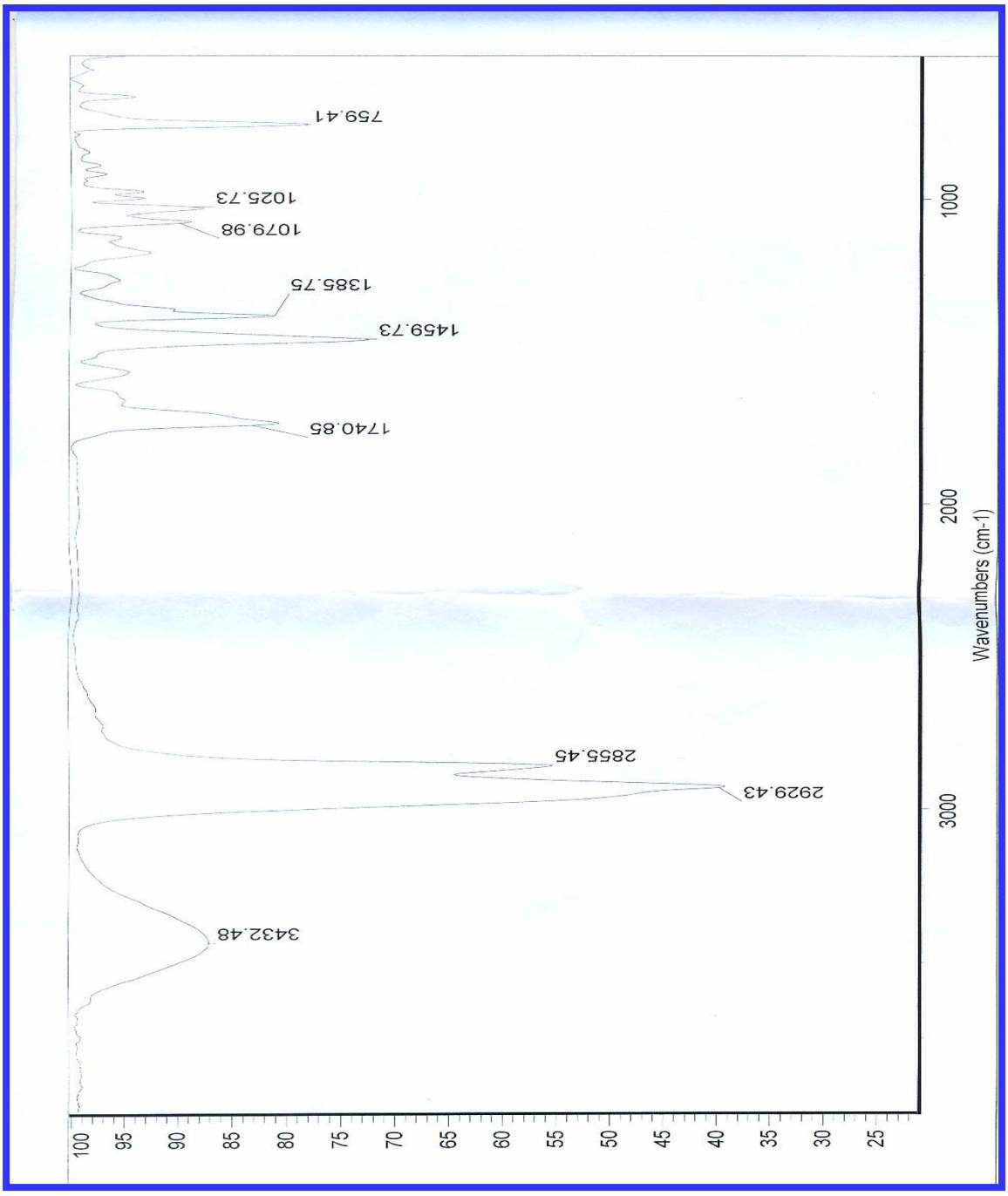

Figure 3.2: The Infra red (IR) spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

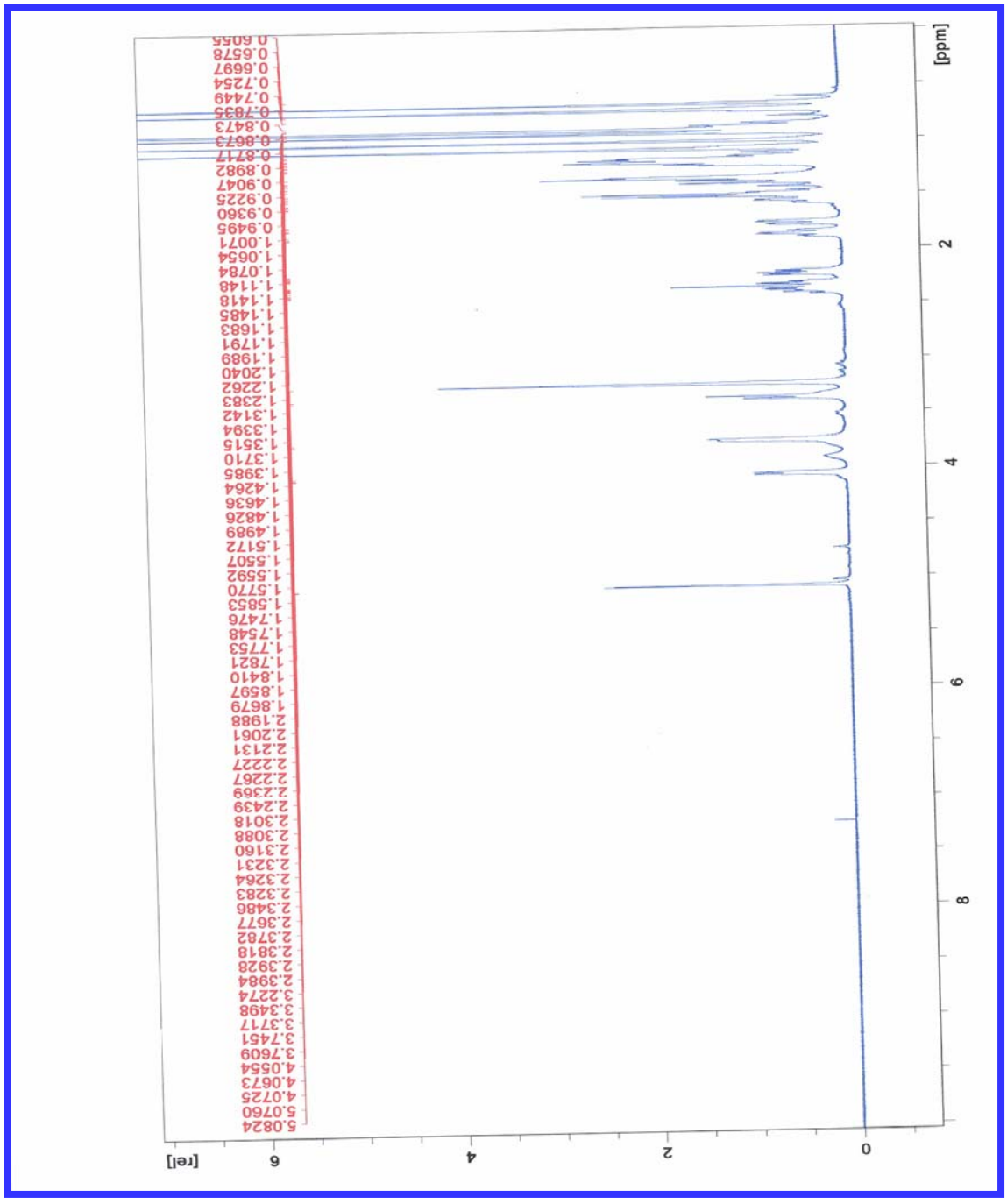

Figure 3.3: The ^{1}H NMR spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one 3'.

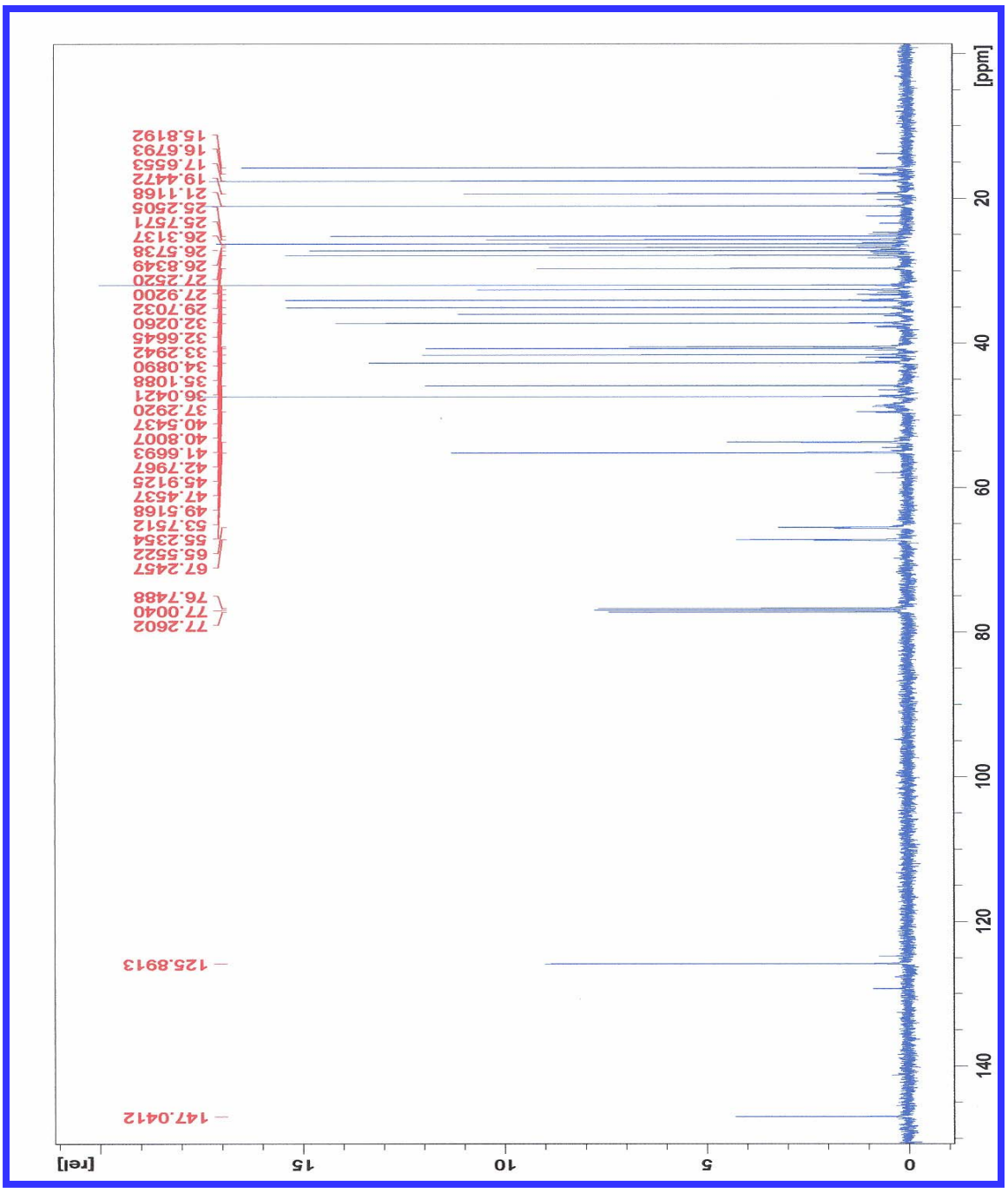

Figure 3.4: The ^{13}C NMR spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

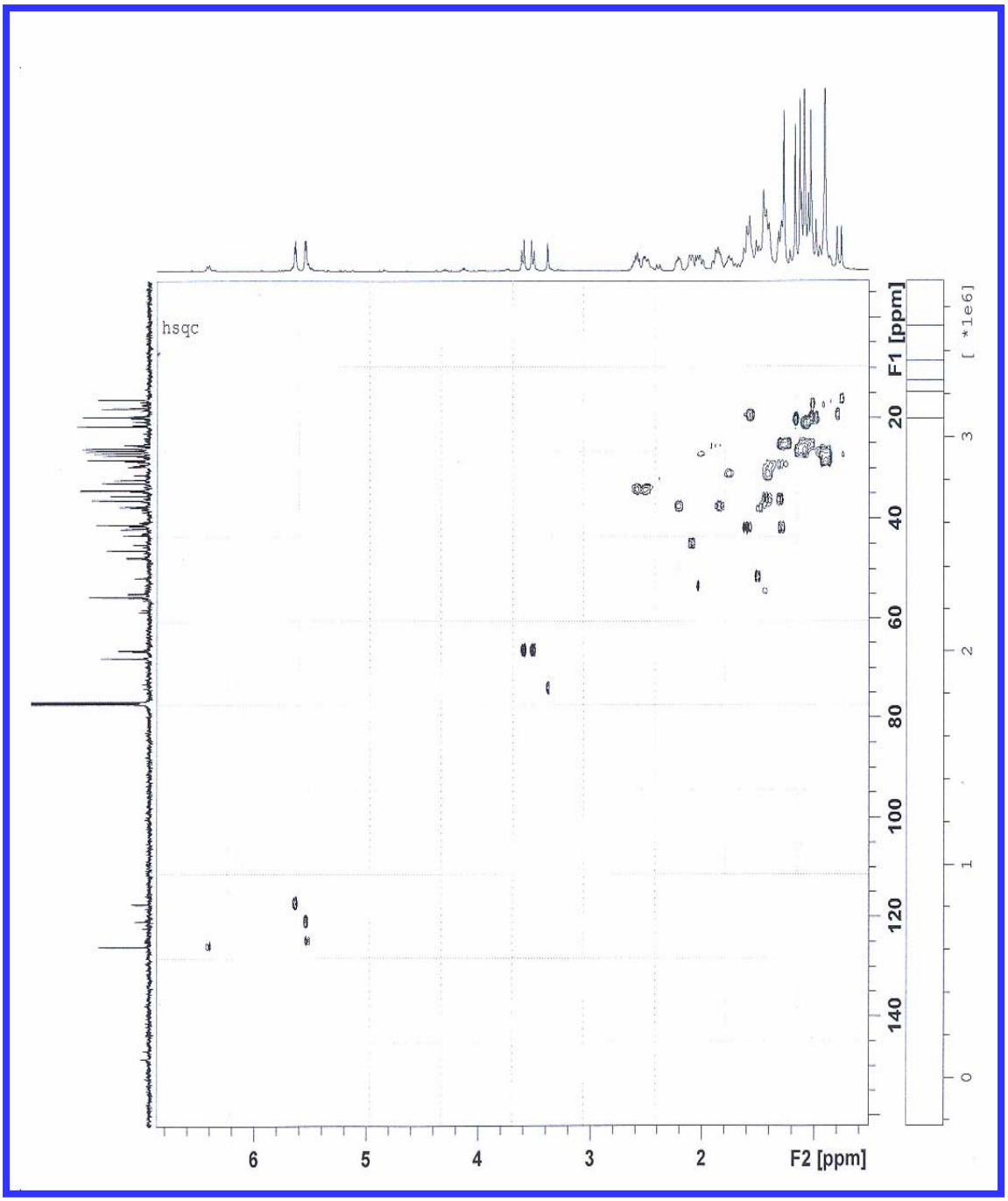

Figure 3.5: The HSQC spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

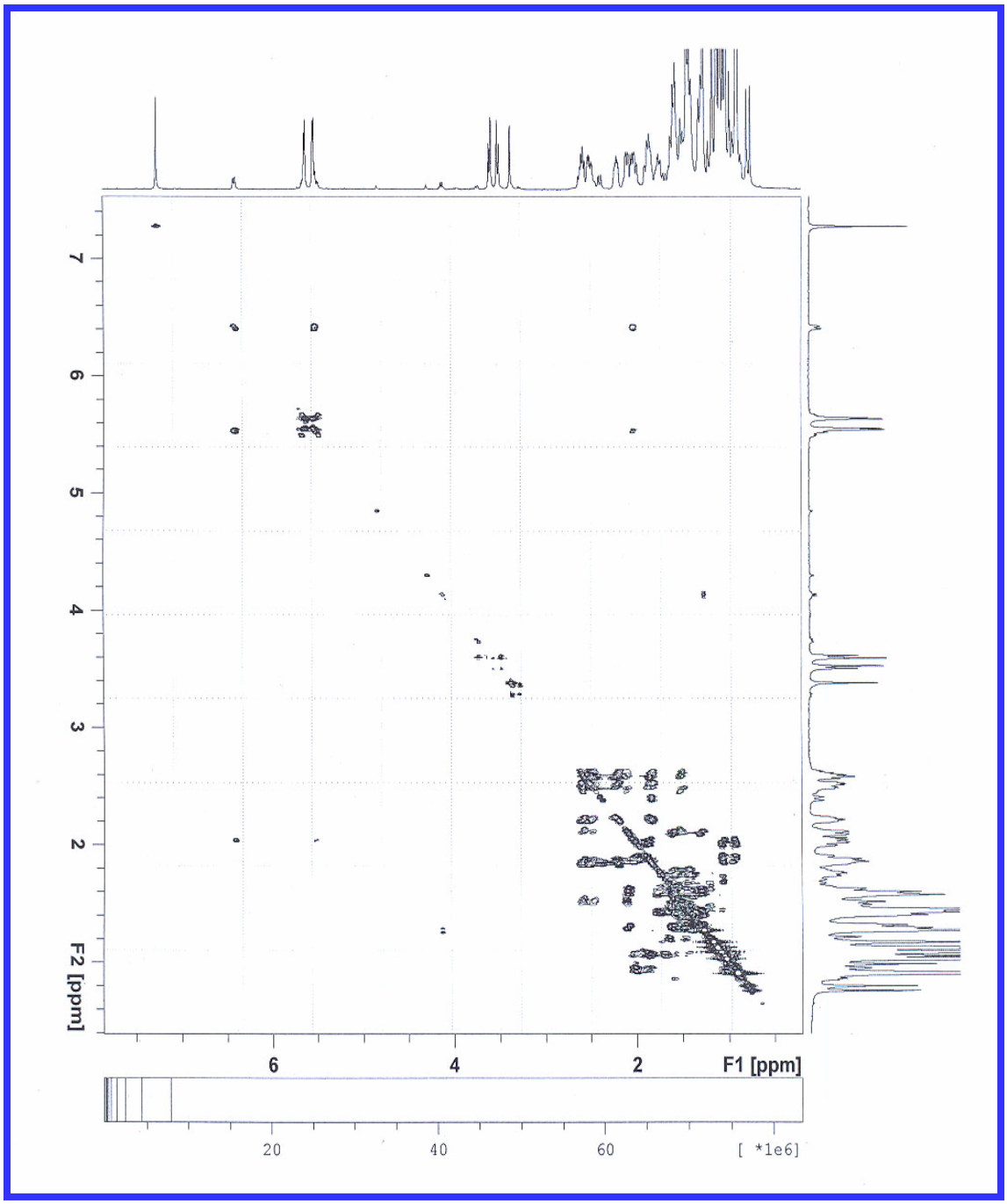

Figure 3.6: The COSY spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

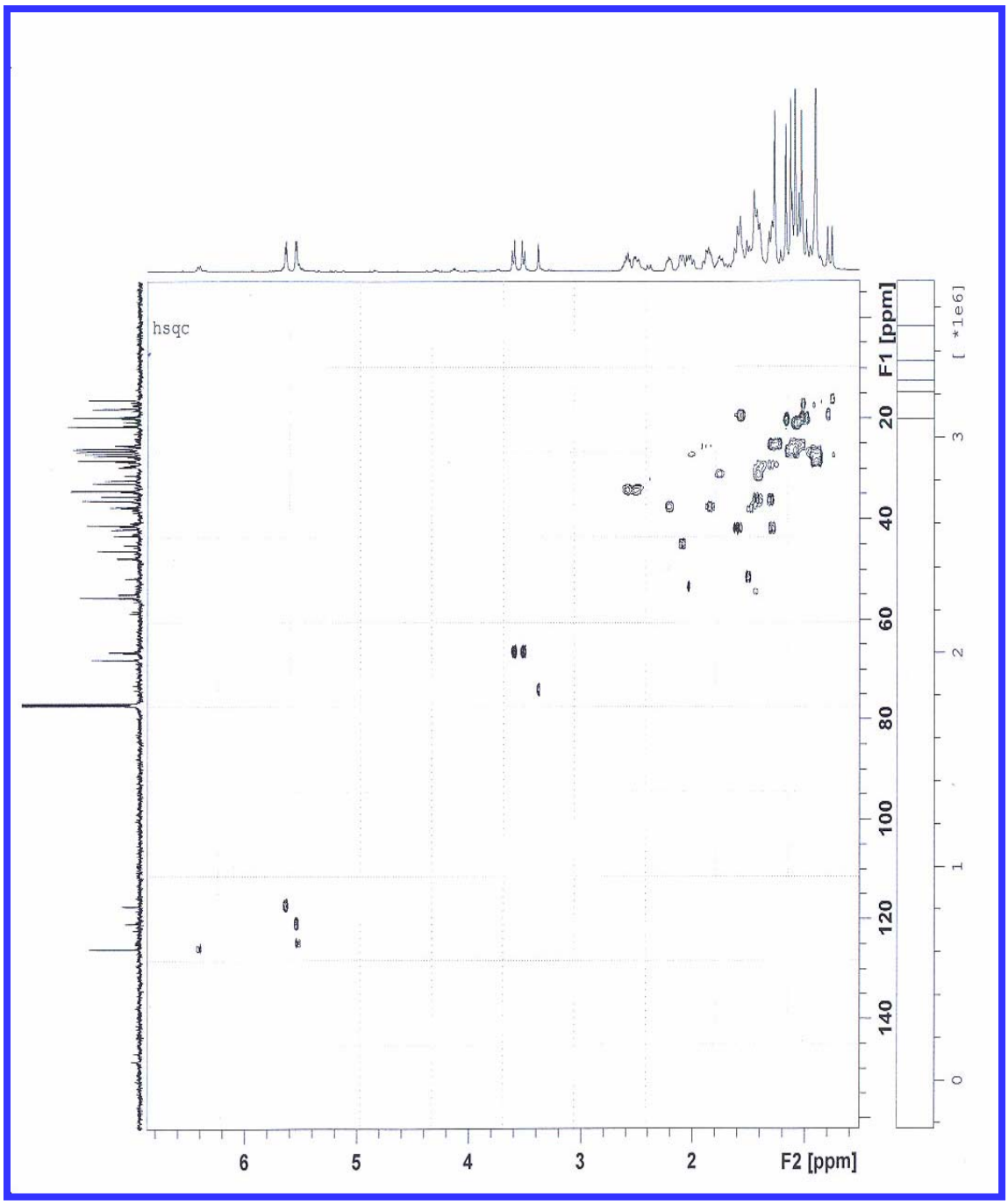

Figure 3.7: The HMBC spectra of '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**'.

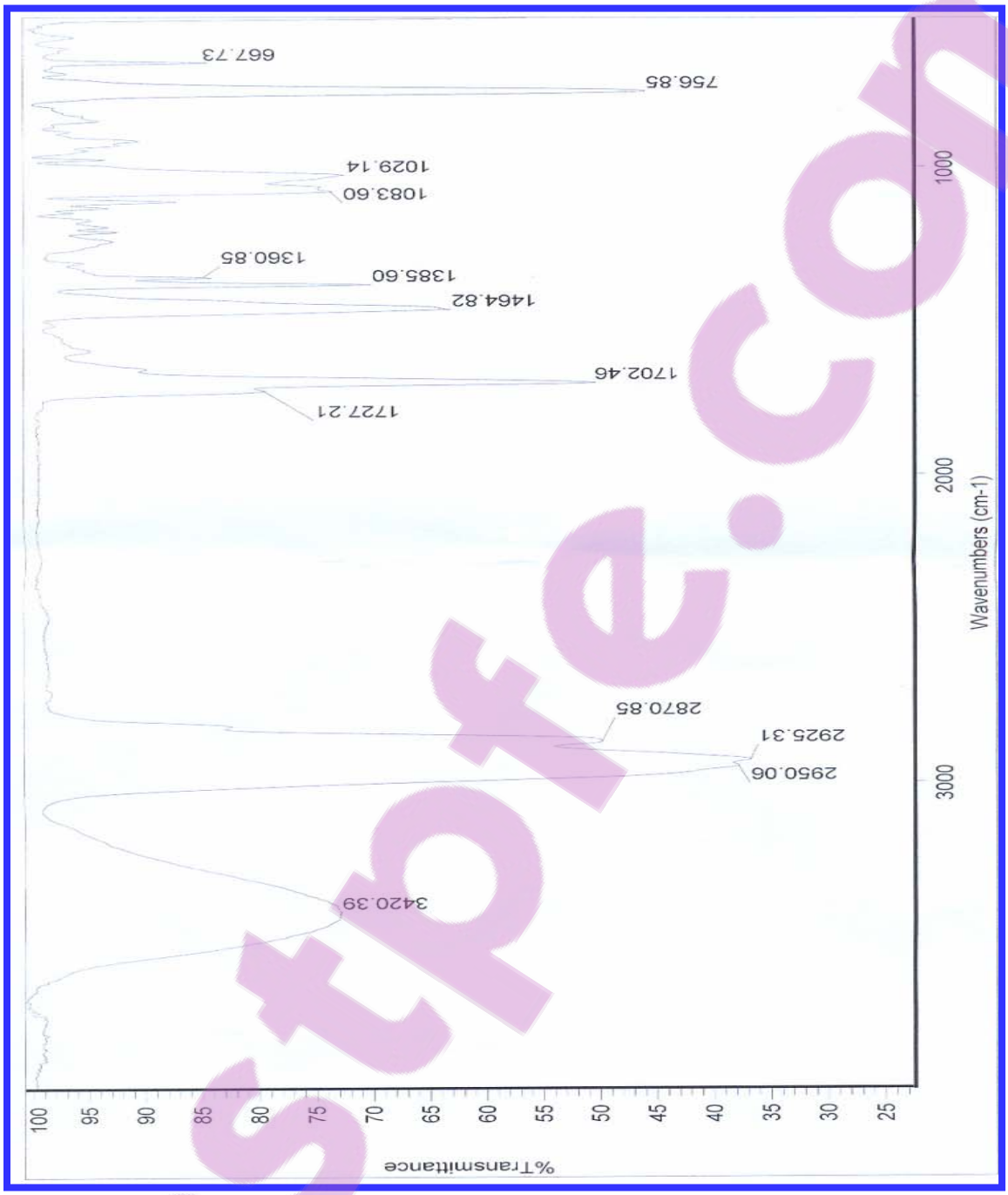

Figure 3.8: The Infra red (IR) spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

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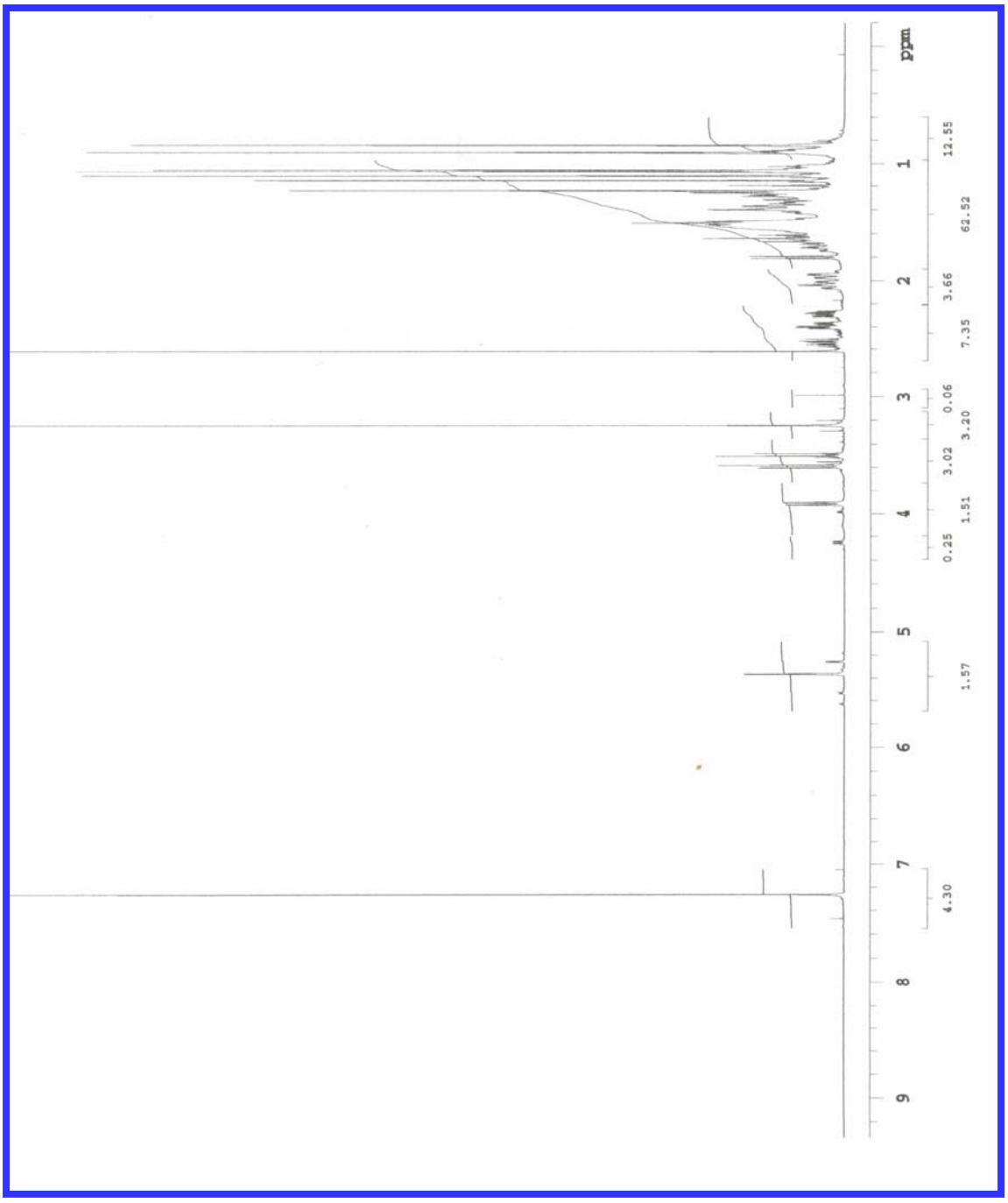

Figure 3.9: The ^{1}H NMR spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

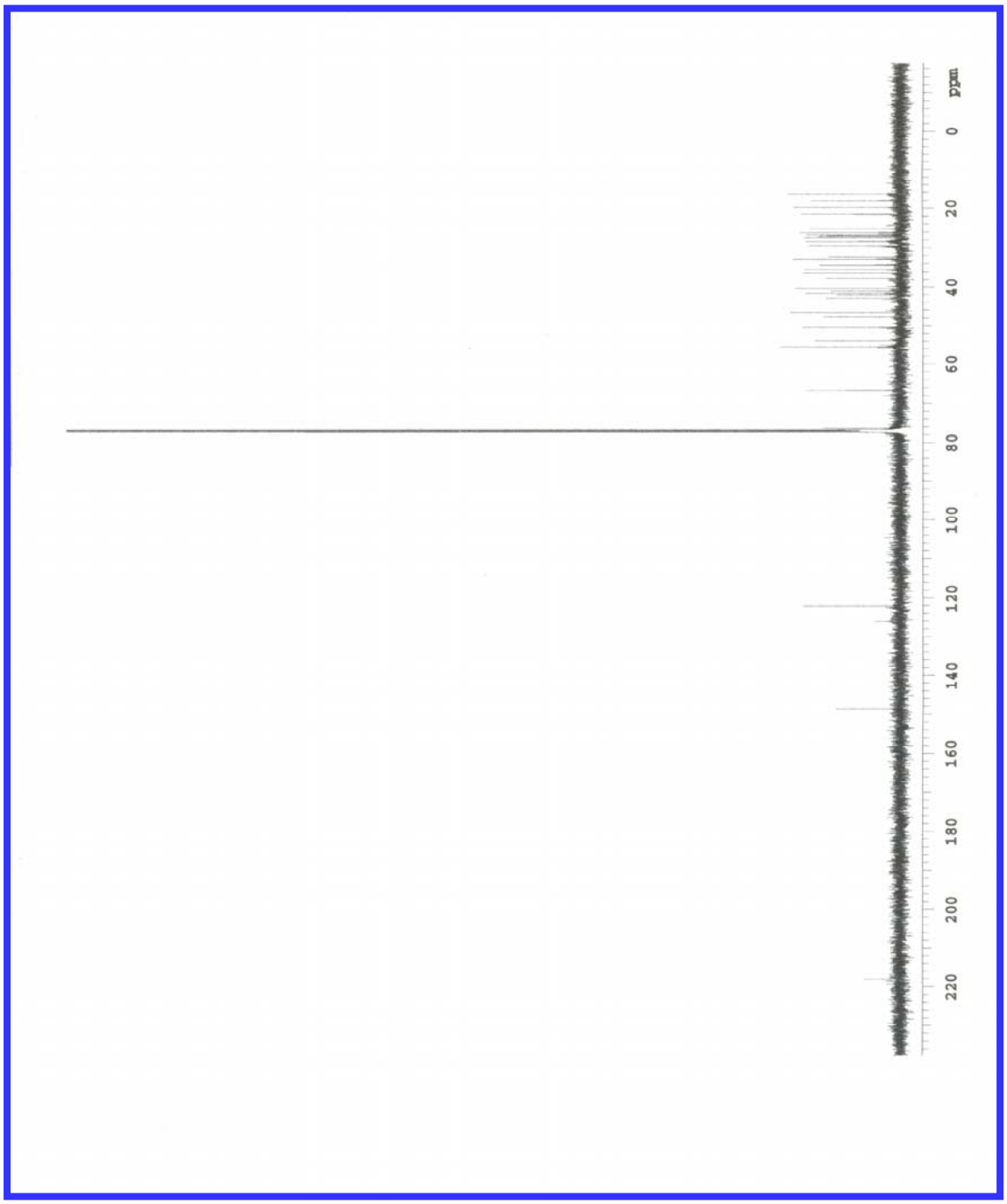

Figure 3.10: The ^{13}C NMR spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

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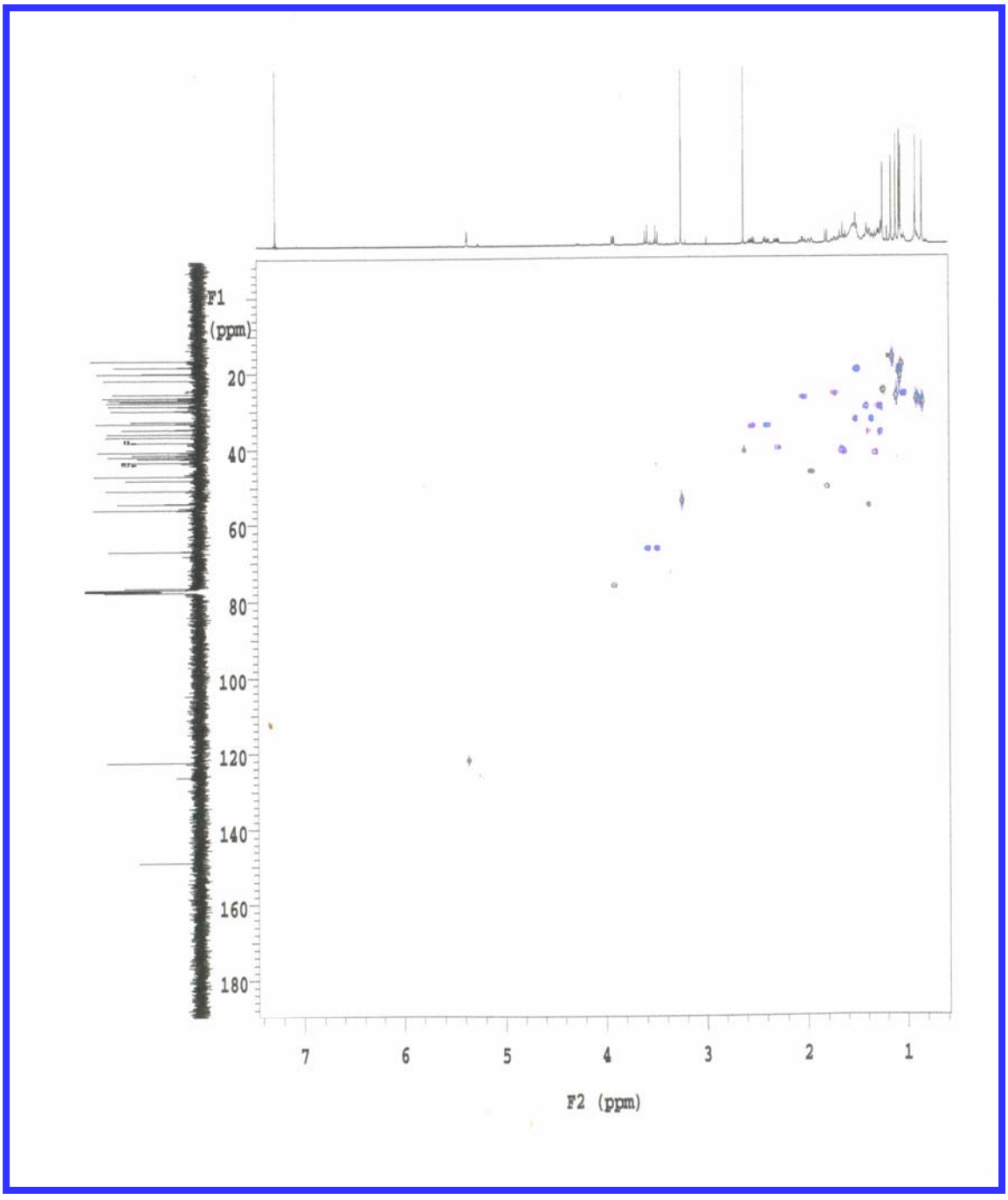


Figure 3.11: The HSQC spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

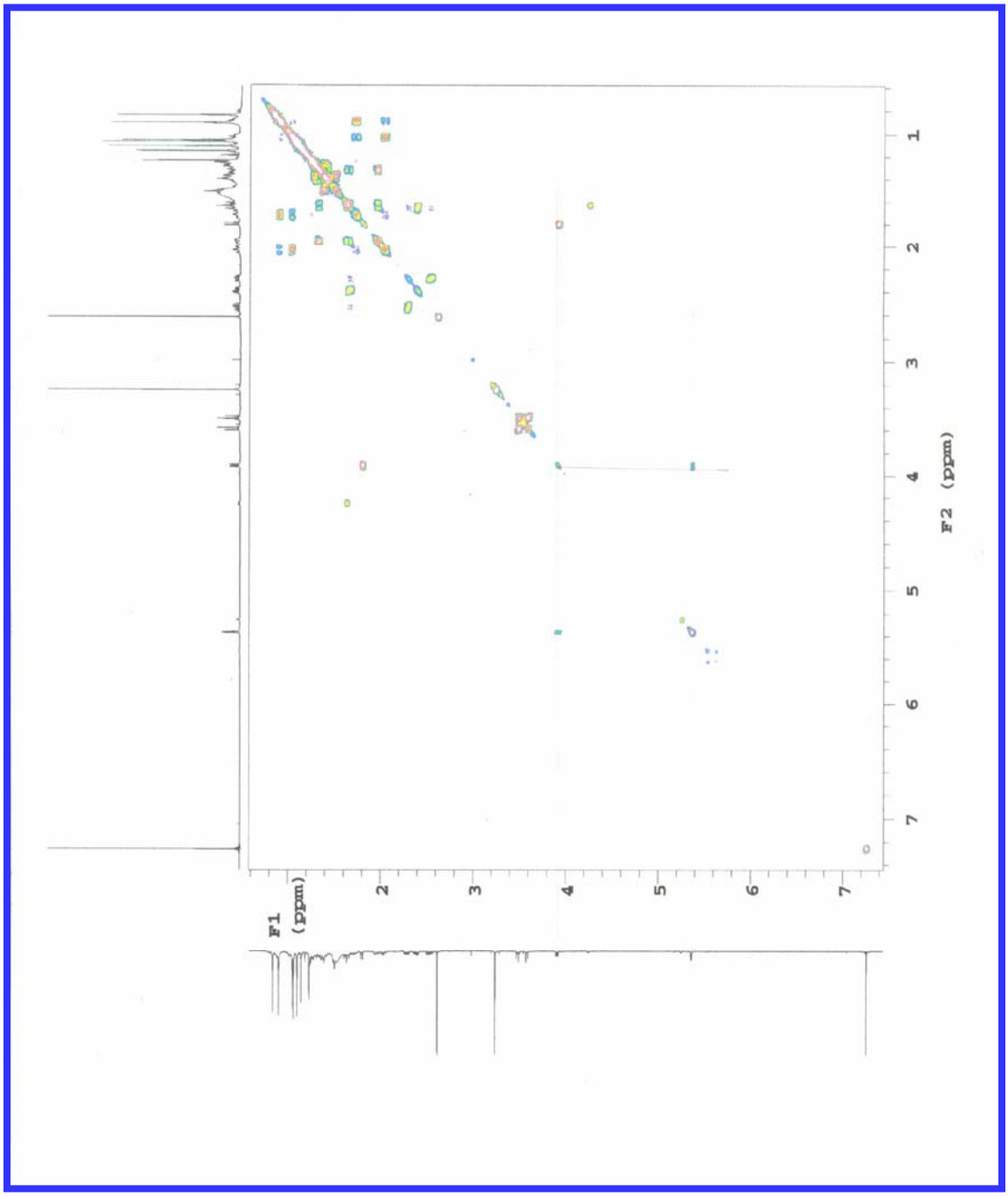


Figure 3.12: The COSY spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

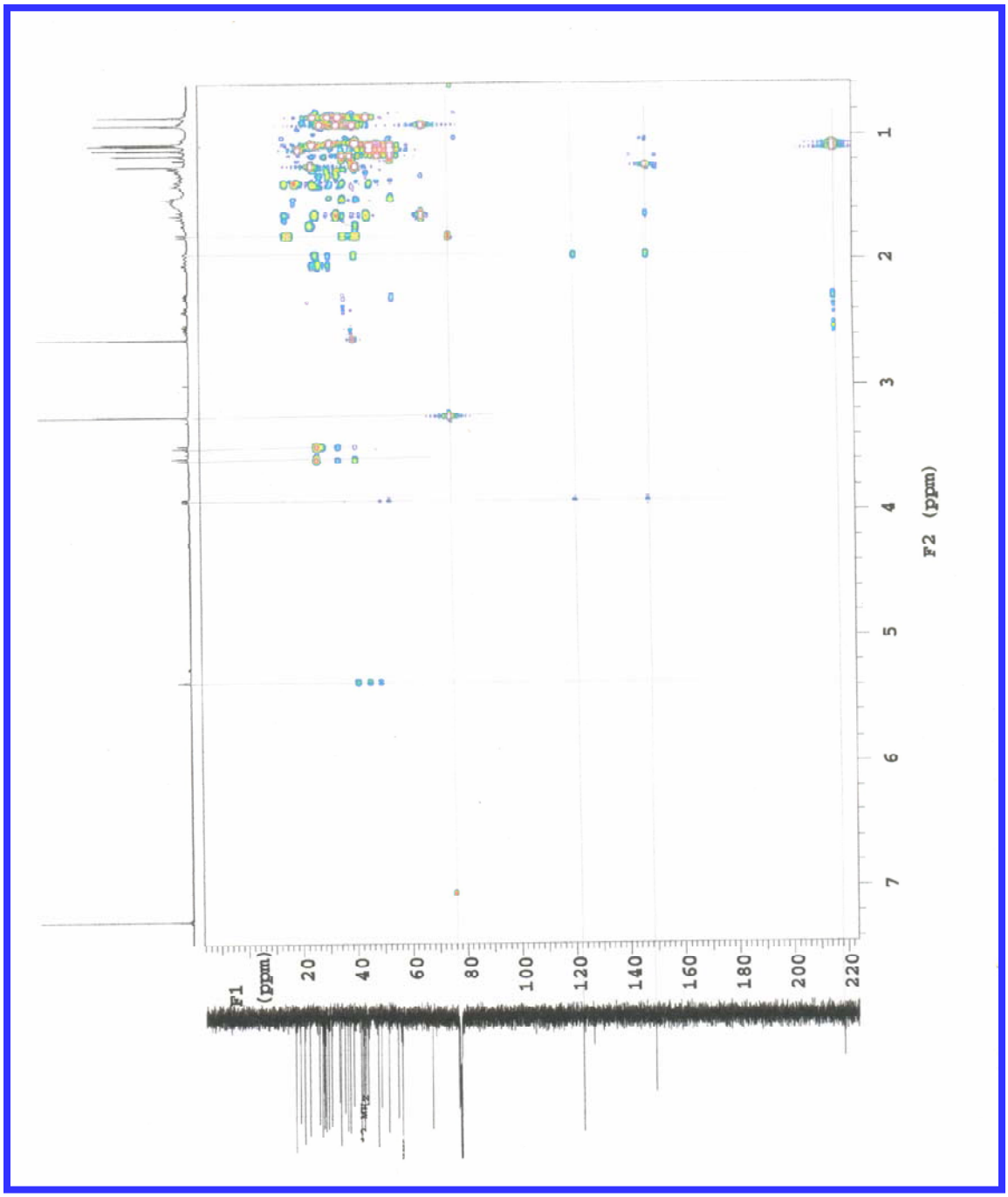


Figure 3.13: The HMBC spectra of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

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.

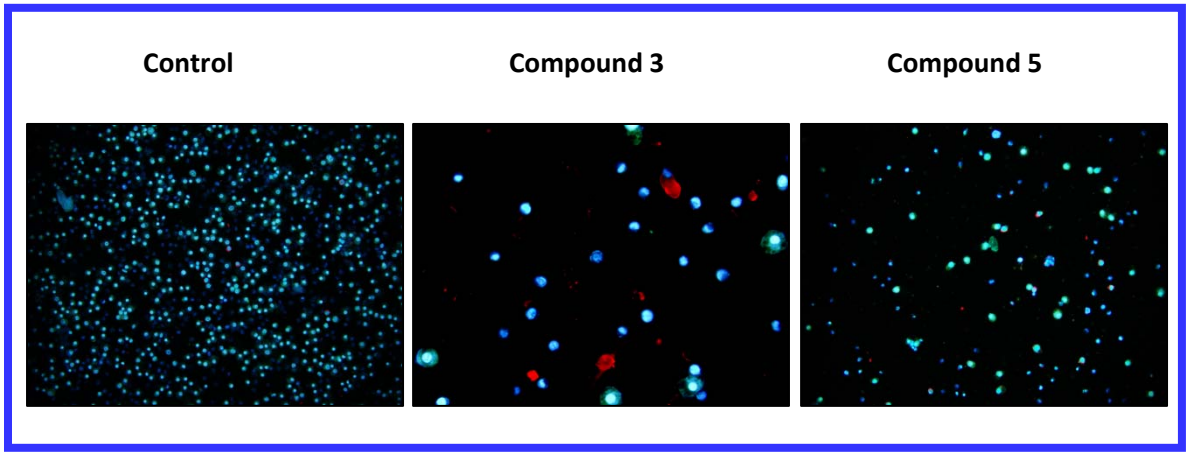
Table 3.2: Percentage of alive, apoptotic, and necrotic HeLa cells treated with IC_{50} concentration of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' using flow cytometry method by Annexin V-fluorescein isothiocyanate and propidium iodide (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**.

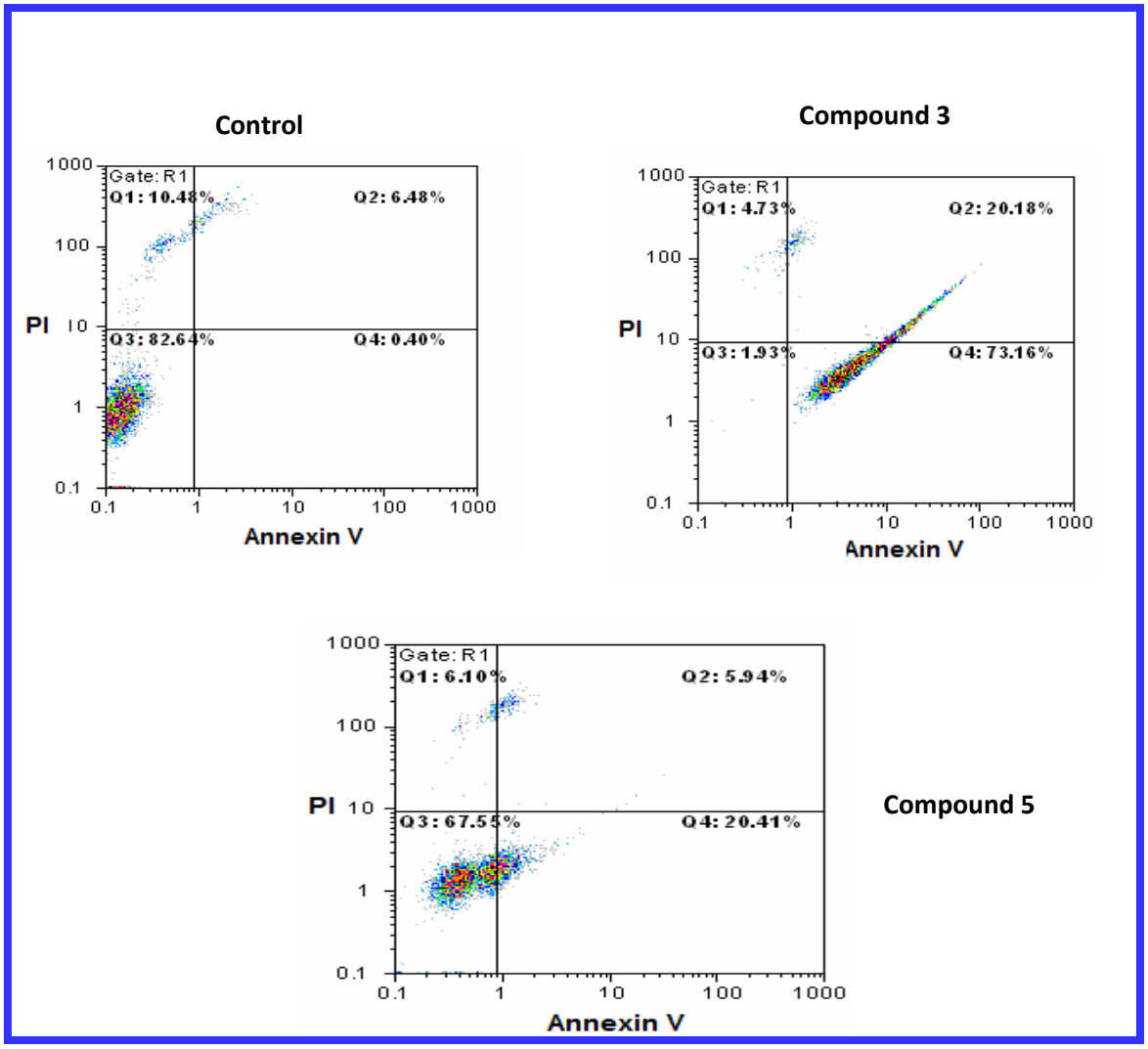
3.4.4. Comet assay

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 $^{-}$).

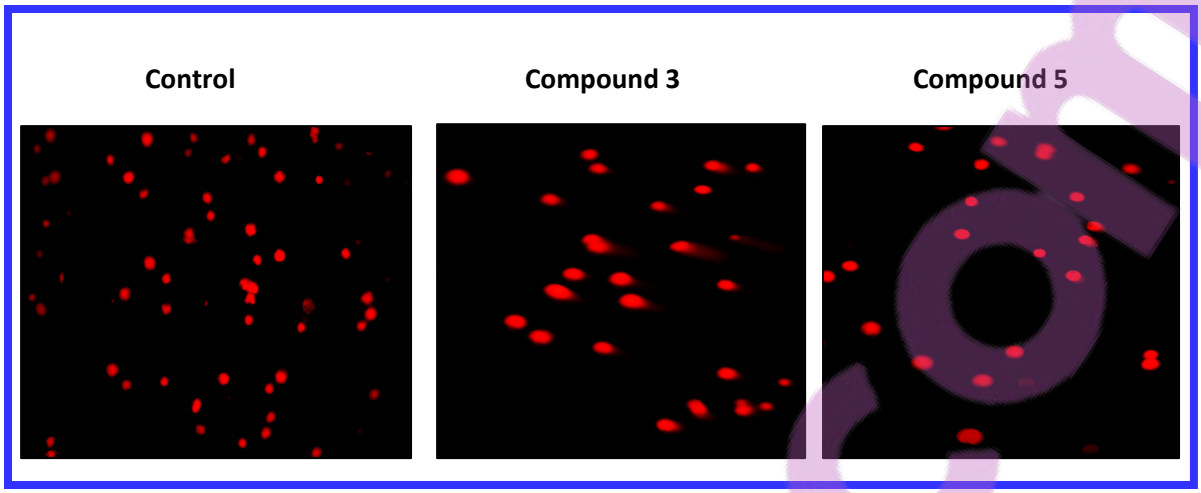


Figure 3.15: Microscopic analysis of HeLa cells 72 h after treatment with '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' with alkaline comet assay. The microscopic pictures were taken by fluorescence microscope, enlargement 200X.

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$^{\bullet}$) 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.

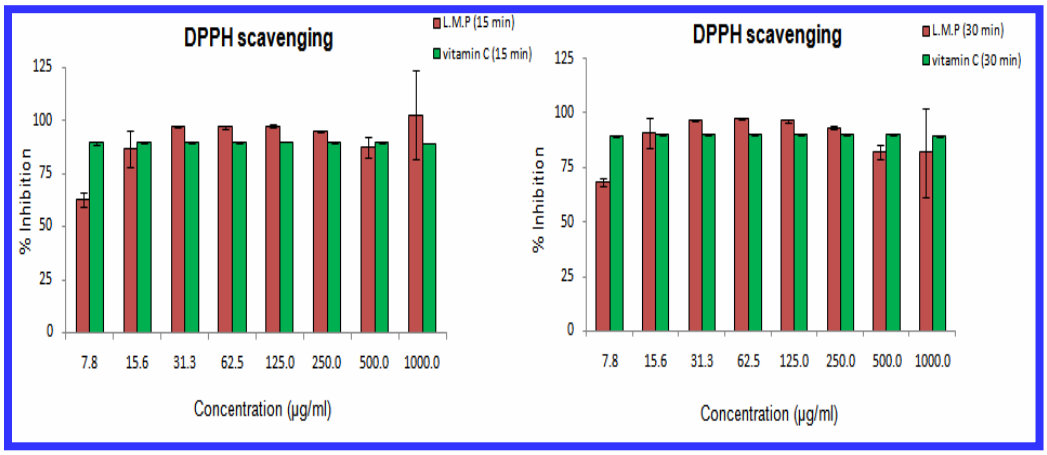
Table 3.3: Average median values (±SD) of the amount of DNA damage induced by '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

	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

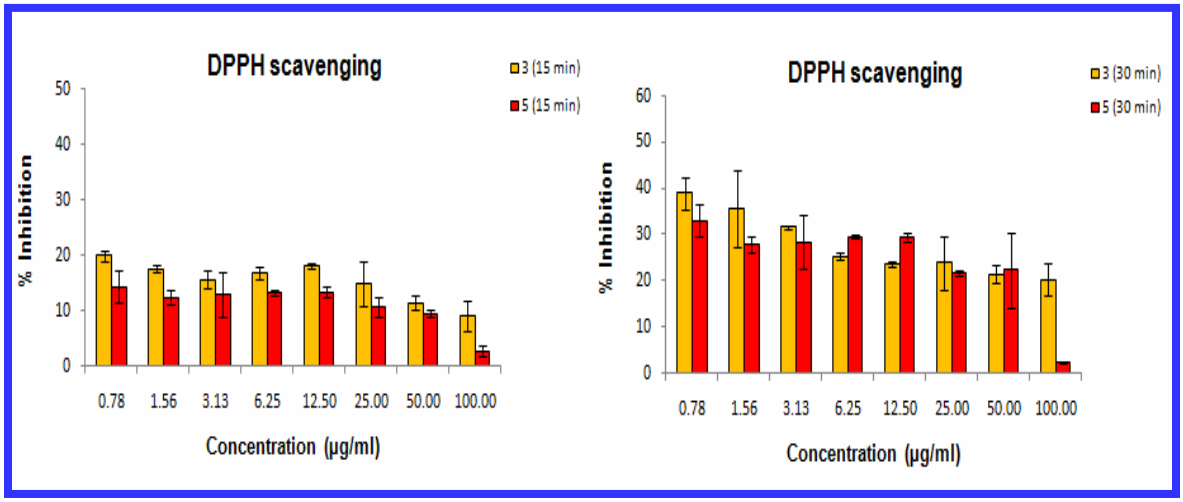
[a] L Tail = the length of tail.
[b] L comet = the length of entire comet (from head to the end of tail).
[c] TM (tail moment) = [tail length] × [percentage of DNA in tail].
[d] OTM (Olive tail moment) = [The horizontal distance between the center of DNA in tail to the center of DNA in head] × [percentage 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).

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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).

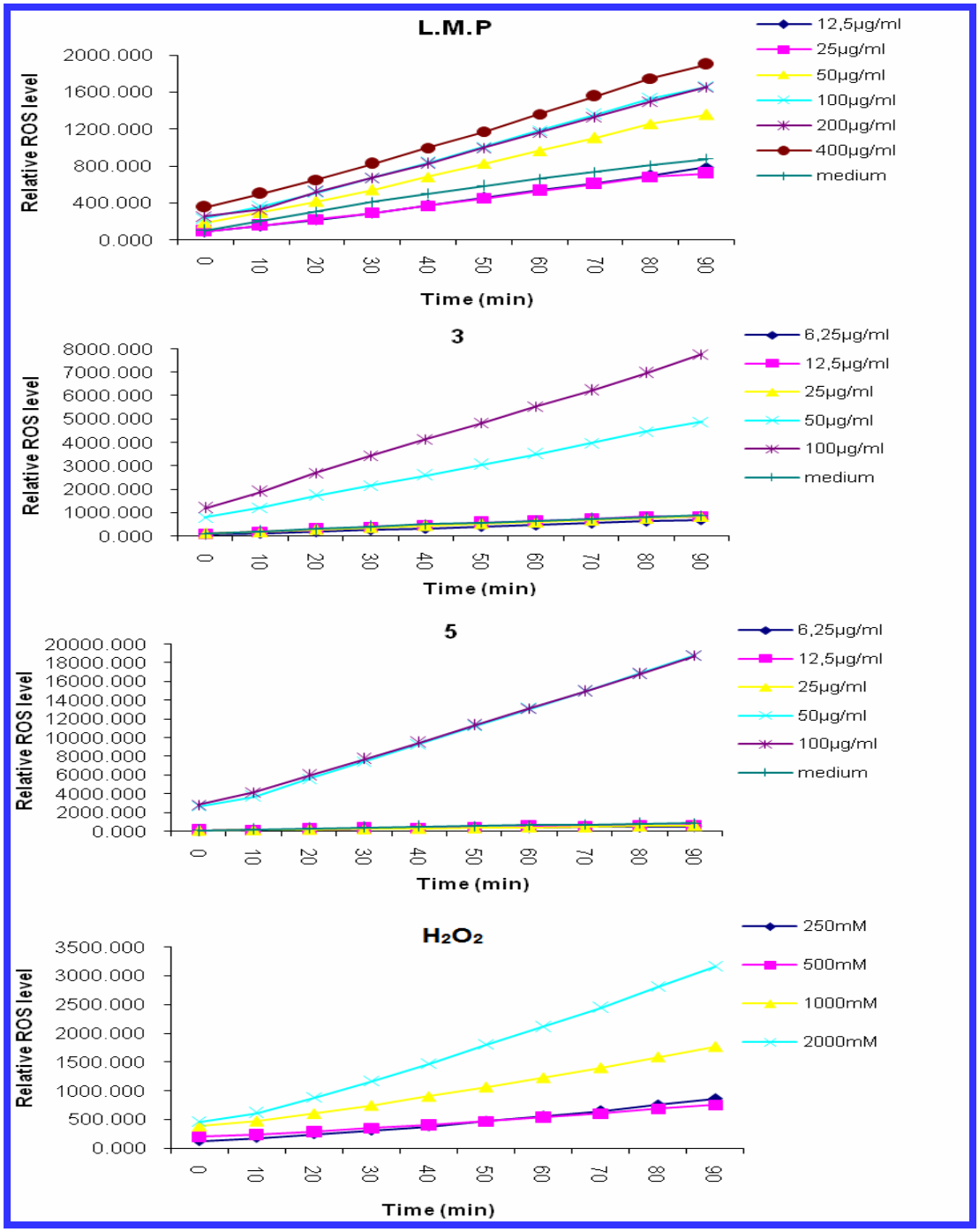

Figure 3.17: Time-response curve of increase of relative ROS level (DCF fluorescence) in HeLa cells after 90 minutes exposure to various concentrations of the ethanolic/acetonic extract of the leaves of *M. procumbens* (L.M.P), '30-hydroxy-11α-hydroxy-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one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

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CHAPTER 4

4.1. Motivations for this study

Cancer is the second leading cause of death in many nations after cardiovascular diseases in the world. Chemoprevention or chemotherapy via natural based agents could be one approach for decreasing the incidence of different type of cancers. Despite the discovery of numerous natural and synthetic anticancer agents, attempts for sighting of an effective anticancer agent with low toxicity effects on healthy cells, high efficacy against multiple cancers, and low cost have been absorbed many attentions.

For centuries people have used plants as medicine or food additives with varying success to cure and prevent diseases. Written records about medicinal plants date back at least 5,000 years to the Sumerians. According to World Health Organization (WHO) around 80% of the population in developing countries is dependent on herbal medicine for basic healthcare needs. Even at the dawn of the twenty-first century, 11% of the 252 drugs considered as basic and essential by WHO were exclusively of flowering plant origin.

The selected plants for present investigation were indigenous to South Africa. Only a few studies were found on the biological activities of *Hyaenanche globosa*. Genus *Maytenus* has been studied intensively in different countries while astonishingly; literature review demonstrated a gap of biological index about *M. procumbens* in between. Thus, we were encouraged to evaluate the new possible biological activities from these species with a particular emphasis on their antiproliferative properties.

4.2. BIOLOGICAL ACTIVITIES OF *HYAENANCHE GLOBOSA*

Ethnobotany and traditional usage of *Hyaenache globosa* Lamb. (Euphorbeaceae) led to the selection of this plant to explore its possible cytotoxicity effects. This genus contains several toxic sesquiterpene lactones, such as, tutin, mellitoxin, urushiol III, and isodihydrohyaenanchine (Van Wyk *et al.,* 1997).

Phytochemical studies of the ethanol extract of the fruits of *H. globosa* (F.E) resulted in isolation of two known pure sesquiterpene lactones; 'tutin **1**' and 'hyenanchin **2**'. The crude extract (F.E) and its isolated constituents were tested on four cancerous and a normal cell lines. F.E exhibited the highest antiproliferative activity on HeLa cells which followed by Caco-2 cells. None of the isolated compounds (**1** and **2**) were found to be toxic to the cells tested in this experiment.

Antioxidant/pro-oxidant activities of F.E, 'tutin **1**' and 'hyenanchin **2**' were determined extracellular (DPPH radical scavenging method) and intracellulary (in cultured HeLa cells) via three different methods; FRAP, TBARS and ROS assays. F.E demonstrated potent inhibition of DPPH radical activity similar to vitamin C (positive control). 'Tutin **1**' and 'hyenanchin **2**' were found with marginal antioxidant activity of which 'compound **1**' presented more potent than 'compound **2**'. As data characterized, F.E, 'tutin **1**' and 'hyenanchin **2**' enhanced FRAP values in HeLa cells at higher concentrations tested compared to non-treated cells. None of samples were capable to enhance lipid peroxidation in HeLa cells, significantly. Nevertheless, the amounts of ROS radicals formed by pure compounds (**1** and **2**) were not significantly higher than those of controls. However, F.E elevated the fluorescence intensity of DCF at the highest concentration tested (400 µg/ml).

The crude extract of *H. globosa* showed the highest antibacterial activity of 1 mg/ml against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and the lowest of 8 mg/ml against Gram-negative bacteria (*P. aeruginosa*). Only 'tutin **1**' showed inhibitory activity exhibiting MICs of 400 and 800 µg/ml for *S. aureus* and *P. aeruginosa,* respectively.

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Compound **2** did not show any significant growth inhibition of microorganisms tested. None of pure compounds inhibited the growth of fungi tested in this study.

In summary, in spite of our great expectation about the toxicity of pure compounds isolated from the ethanolic extract of the fruits of *H*. *globosa* ('tutin **1**' and 'hyenanchin **2**'), they did not show any significant cytotoxic effects on the examined cancer cell lines, while the crude extract was well known for its poisonous effects. The poisonous effect of this plant could be due to the activity of the compounds that were not isolated yet. Moreover, it has been proven the toxicity effects of sesquiterpene lactones are dose dependent. Although the data are still inconclusive and further scientific attempts are needed to confirm the traditional information or to investigate the novel medicinal aspects of this plant. A further study aims to determine the anticancer properties of other major constituents of *H*. *globosa*, as well as identify the unknown compounds is required to fully understand its bioactivity.

4.3. BIOLOGICAL ACTIVITIES OF *MAYTENUS PROCUMBENS*

Biochemical studies of the acetonic/ethanolic extract of the leaves of *M. procumbens* (L.M.P) resulted in isolation of two new triterpenes namely; '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'. In addition, a known terpene was isolated which identified as 'asiatic acid **4**'. Due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested.

M. procumbens (L.M.P) exhibited the highest inhibition of cells growth on HeLa cells among tested cell lines. The reduction of proliferation was followed in Caco-2, T47D and HT29, though L.M.P showed cytotoxicity against normal NIH3T3 cells. Both pure compounds (**3** and **5)** showed cytotoxicity of all experimental cancer cell lines. They also appeared toxic to the normal NIH3T3 cells. The cytotoxicity of triterpenes have been proven frequently thus confirm our findings well.

Following the MTT assays, the induction of apoptosis by pure compounds (at the concentration of their IC_{50}) were investigated in HeLa cells. The affinity of the isolated compounds for Annexin V and PI were determined through microscopic and flow cytometric analysis. These compounds induced apoptosis in HeLa cells at their IC_{50} concentrations. Significant elevation of DNA damage in concept of tail moment was detected in cultured human HeLa cells by both compounds. 'Compound **3**' presented more apoptotic and genotoxic than 'compound **5**' *in vitro*.

To see whether induction of apoptosis by compounds **3** and **5** in HeLa cells depends on their prooxidant/antioxidant properties; RSC, FRAP, TBARS and ROS assays were utilized. L.M.P and its isolated constituents exhibited marginal antioxidant properties as compared to vitamin C. There were significant elevations of ferrous content by L.M.P and pure compounds in HeLa cells. The experimental samples were unable to increase HeLa cells TBARS significantly at any concentration tested as compared to those of controls. The ROS intensity of HeLa cells was elevated significantly by L.M.P and the isolated compounds compared to control cells ($P< 0.05$).

The moderate to weak antioxidant potential of L.M.P and the isolated compounds might be a logical explanation for enhancement of ROS levels *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, compounds **3** and **5** induced ROS generation dose and time-dependently in the HeLa cellular environment. Thus, 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. Such a dual-property role for antioxidants has also been reported previously (Turley *et al.,* 1997; Yang *et al.,* 2006; Zou *et al.,* 2001).
This study proved compounds **3** and **5** were capable to induce apoptosis at their IC_{50} concentrations 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

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compounds **3** and **5**, other pathways may also be involved. The elucidation of these mechanisms by which these samples induce 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*. This project resulted in isolation of two new triterpenes from L.M.P for the first time. There is no report until date on phytochemical index, anticancer, antioxidant and antibacterial properties of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens* (L.M.P), '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

4.4. FUTURE PERSPECTIVES

Regarding the novelty of the isolated compounds, there are limited information about their medicinal properties except from those reported in this study. Wide spectrum of biological activities of triterpenes have been recognized such as bactericidal, fungicidal, antiviral, cytotoxic, analgetic, anticancer, spermicidal, cardiovascular, antiallergic, and so on. In the next step, the other biological aspects of this species should be explored.

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 the clinical application of them 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. The combination of two or more agents acting on different mechanisms to produce a synergistic anticancer effect should be considered.

4.5. REFERENCES

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CHAPTER 5

5.1. ACKNOWLEDGEMENTS

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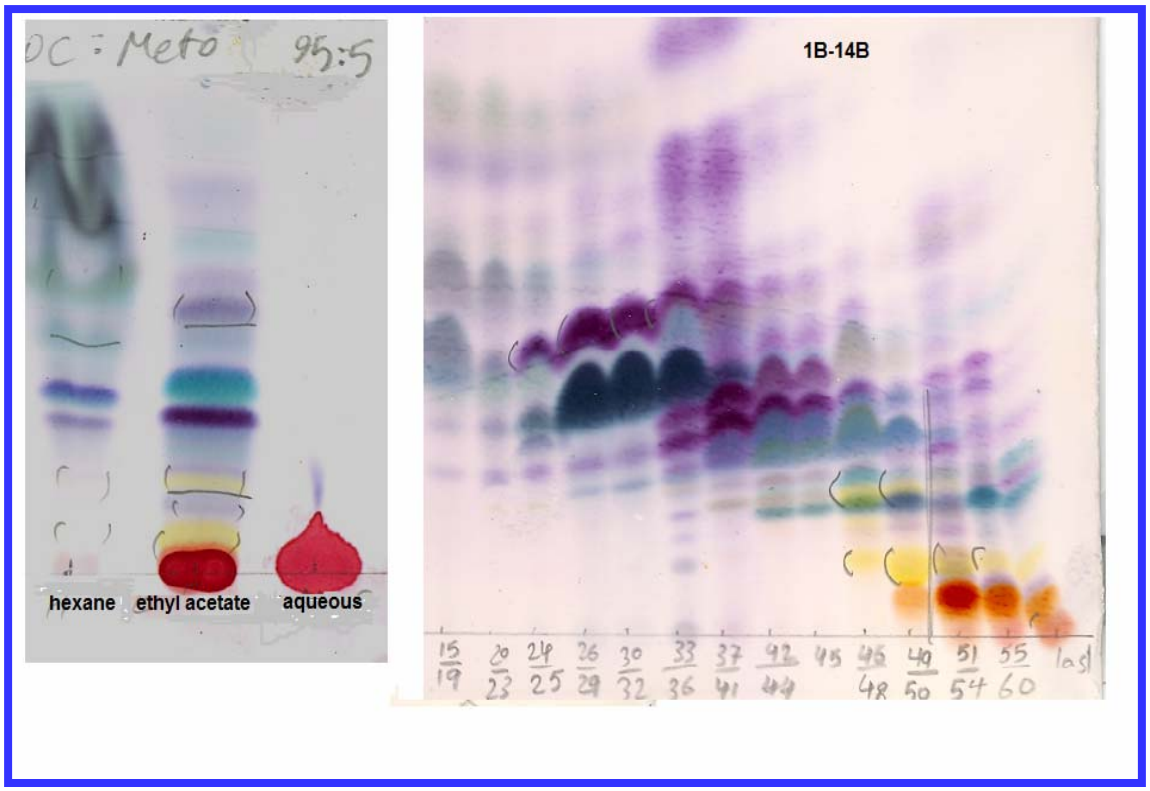
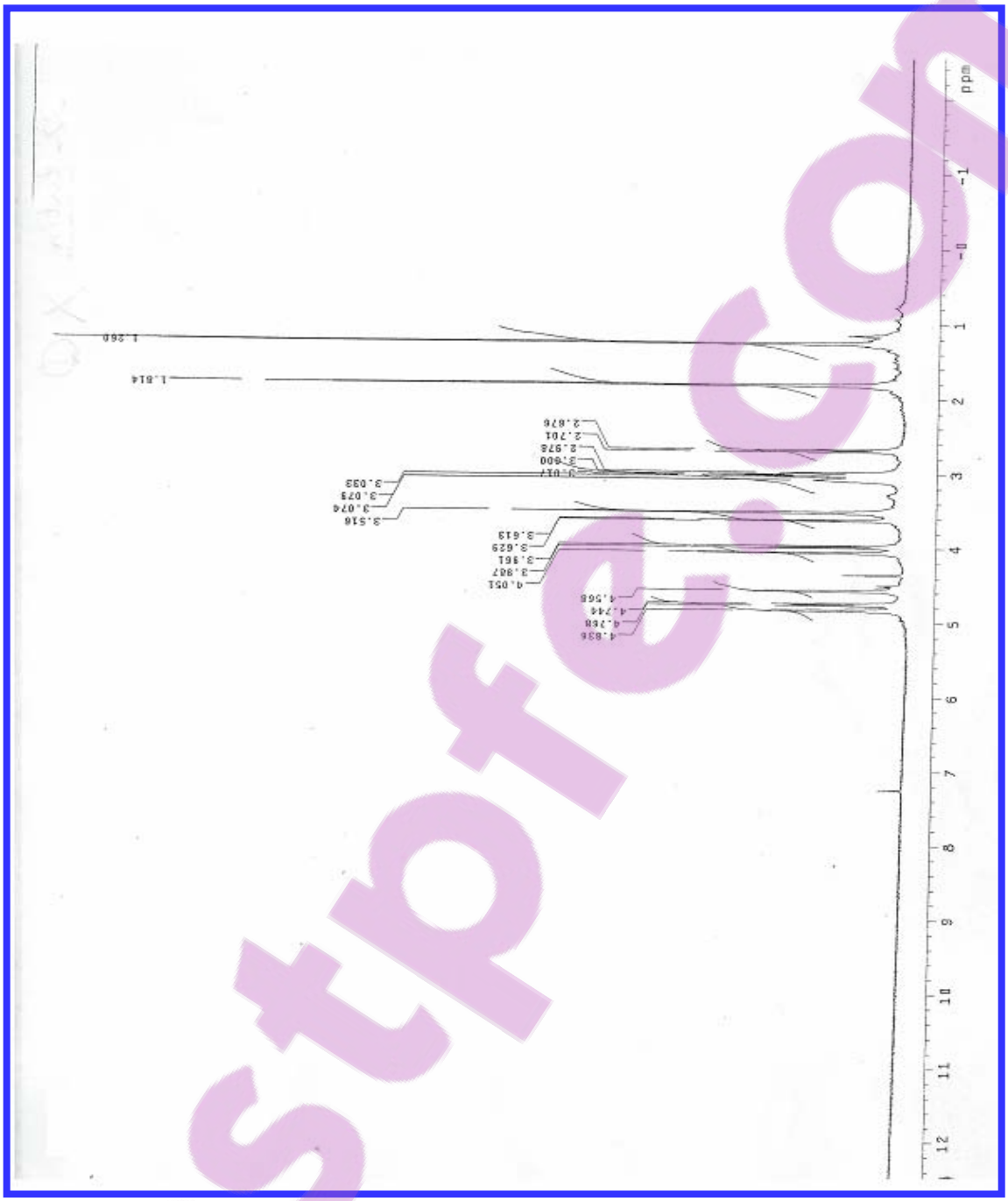
- To my colleagues in Faculty of Pharmacy, and Pharmaceutical Sciences Research Center for their friendships and supports in University of Medical Sciences, Tehran, Iran.

CHAPTER 6

APPENDIX A: *HYAENANCHE GLOBOSA*



Appendix A.1: Hexane, ethyl acetate and aqueous fractions of the fruits of *H. globosa* (F. E) and 14 main fractions (1B-14B) of the hexane fraction.



Appendix A.2.1: The ^{1}H NMR spectra of 'tutin **1**'.

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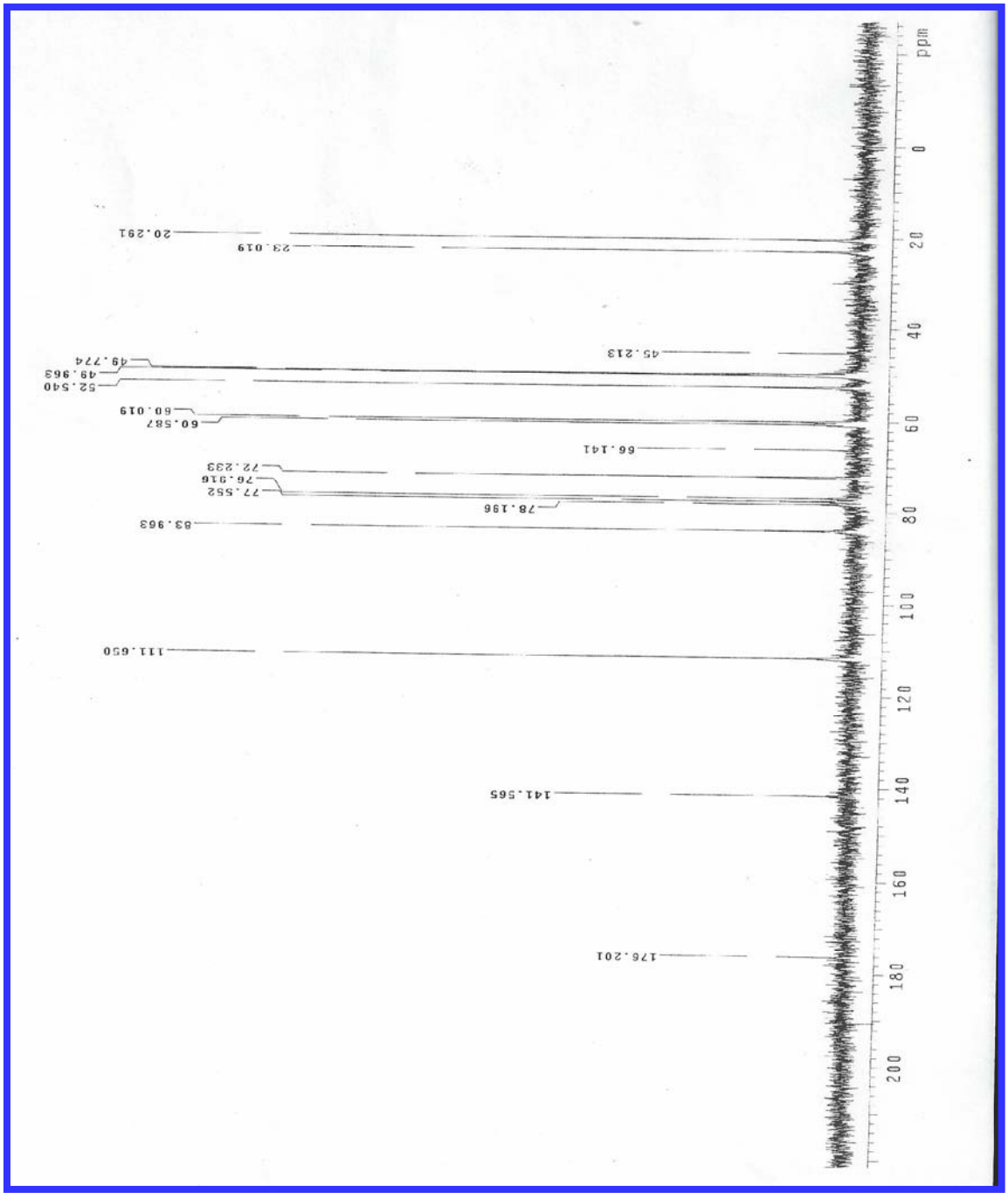
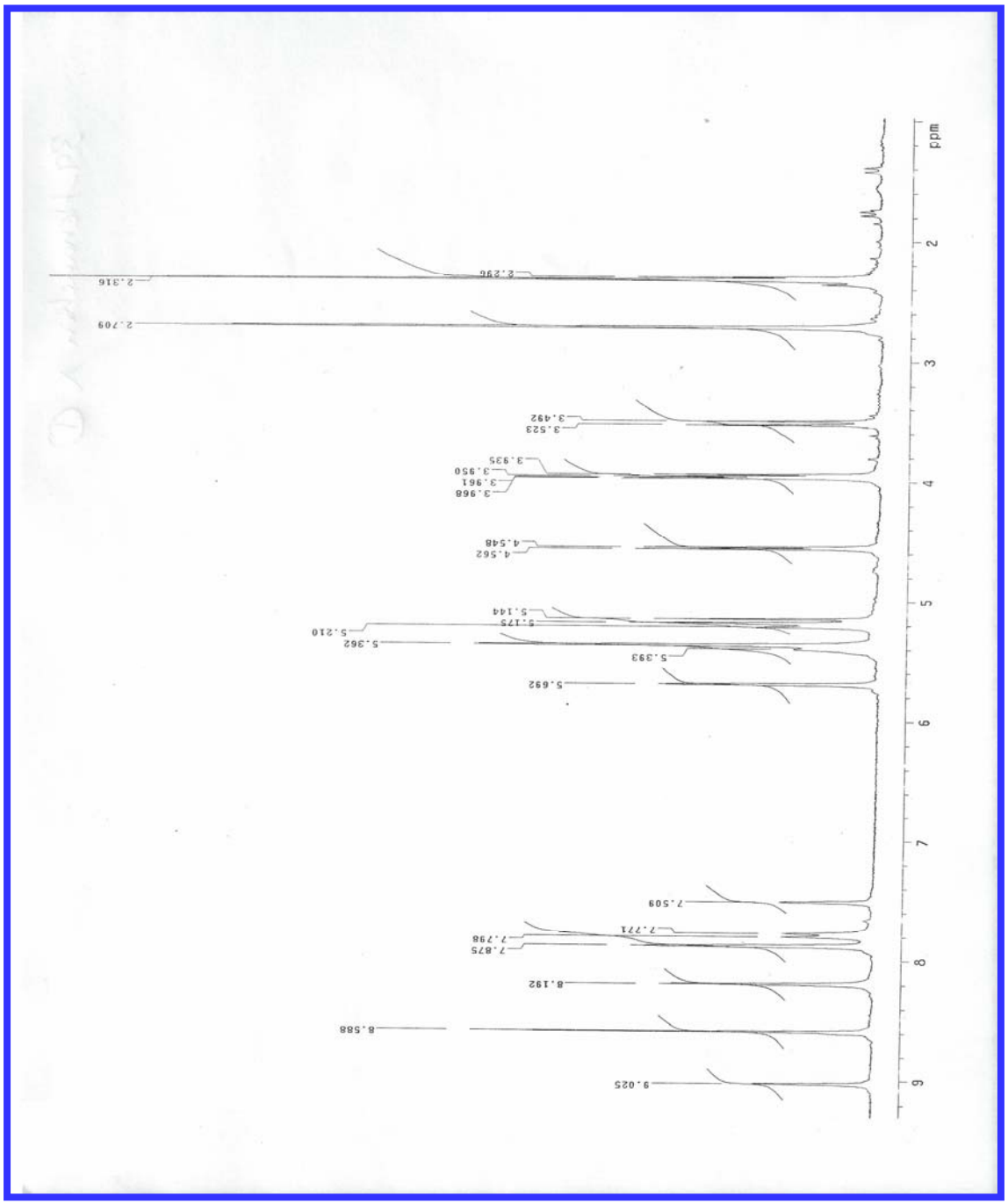


Appendix A.2.2: The ^{13}C NMR spectra of 'tutin **1**'.



Appendix A.3.1: The ^{1}H NMR spectra of 'hyenanchin 2'.

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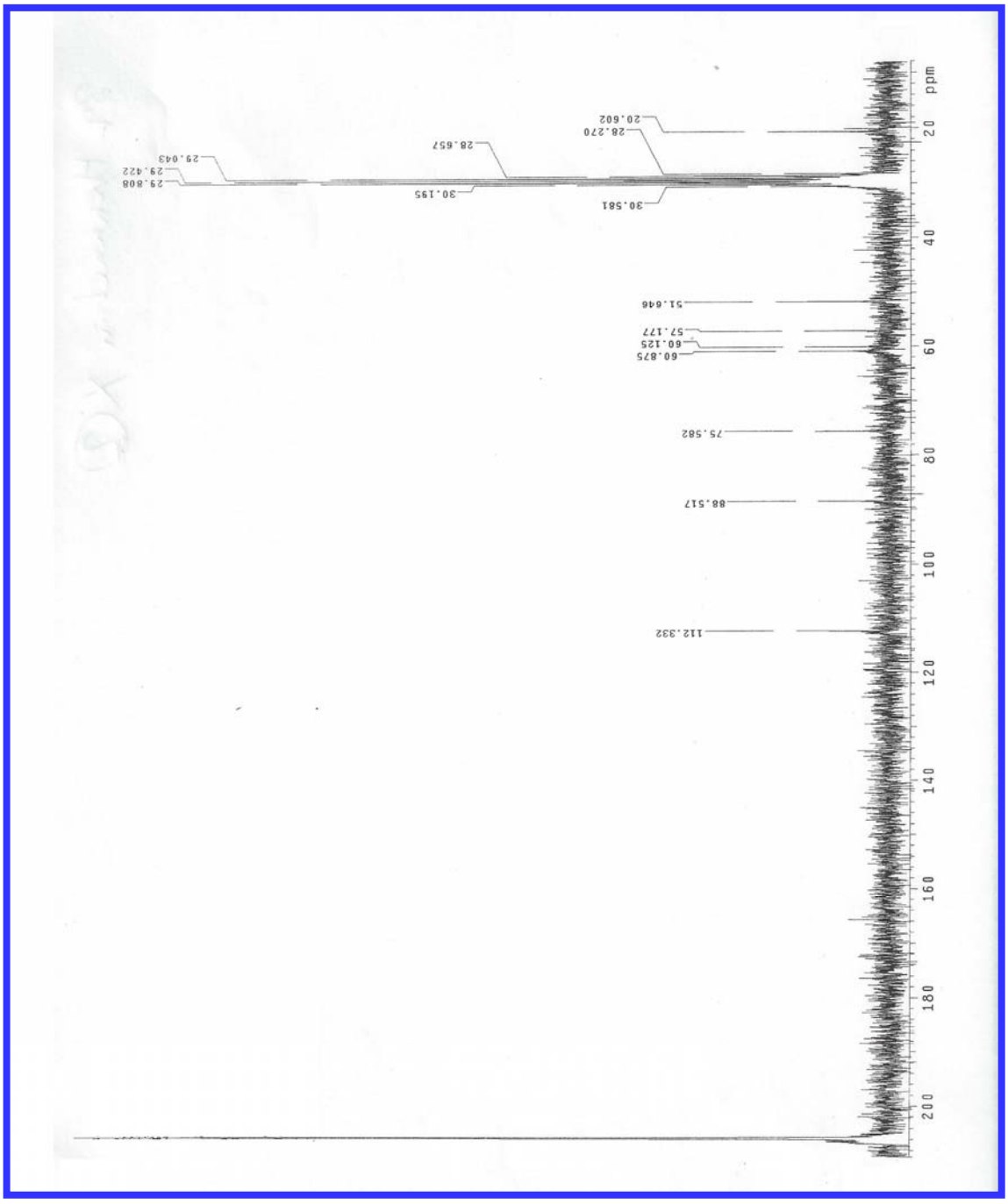
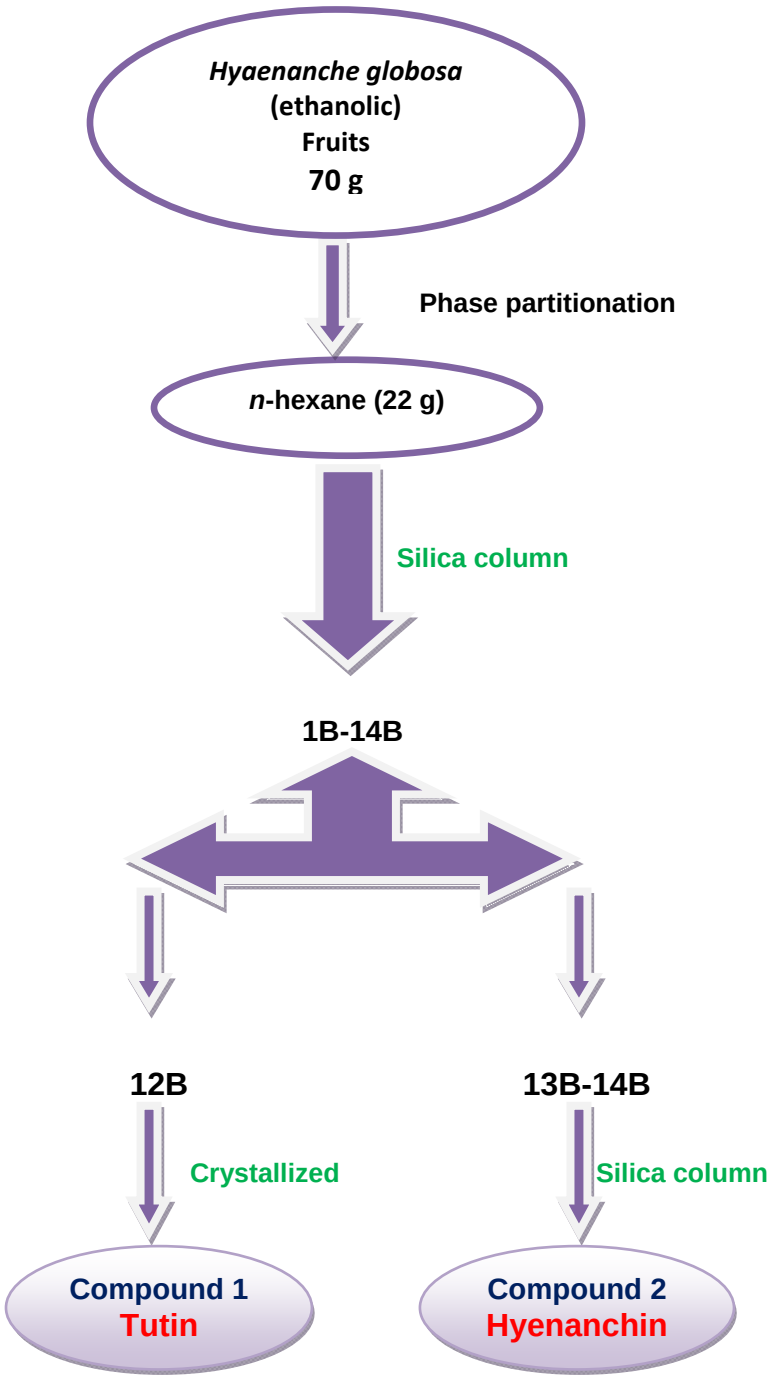
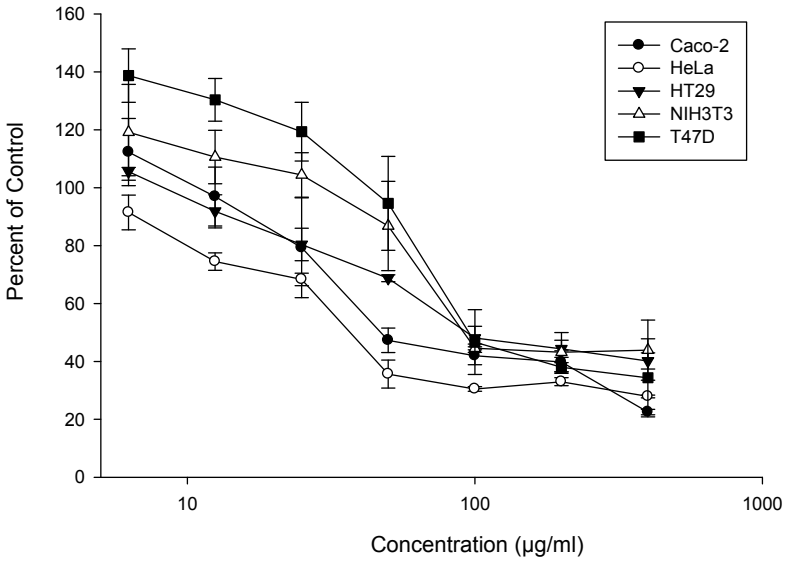


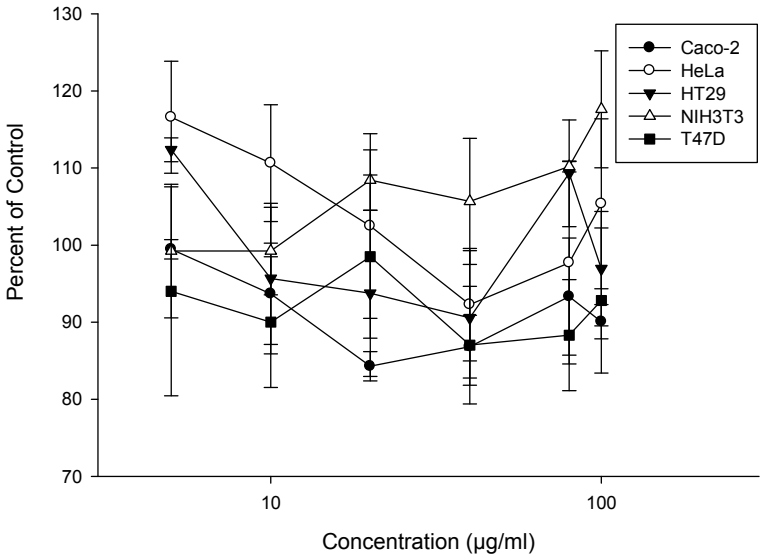
Appendix A.3.2: The ^{13}C NMR spectra of 'hyenanchin 2'.



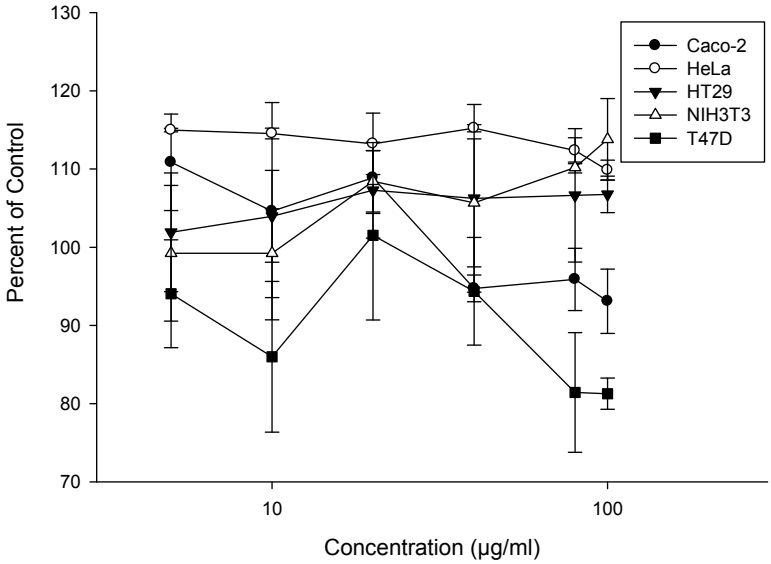
Appendix A.4: The schematic presentation of the isolation steps of *H. globosa* ethanolic extract of the fruits (F.E).



Appendix A.5: The cytotoxicity effects (MTT assay) of the ethanol extract of the fruits of *H. globosa* (F.E) on different cell lines, (mean ± SD, n=3).



Appendix A.6: The cytotoxicity effects (MTT assay) of 'tutin **1**' on different cell lines, (mean ± SD, n=3).



Appendix A.7: The cytotoxicity effects (MTT assay) of 'hyenanchin **2**' on different cell lines, (mean ± SD, n=3).

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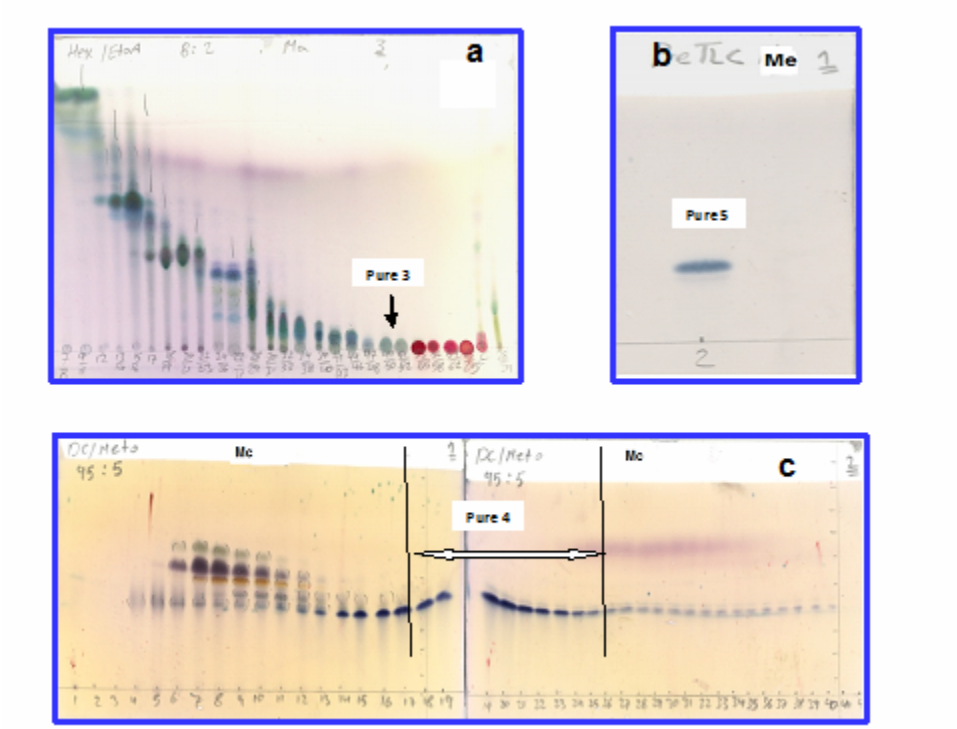
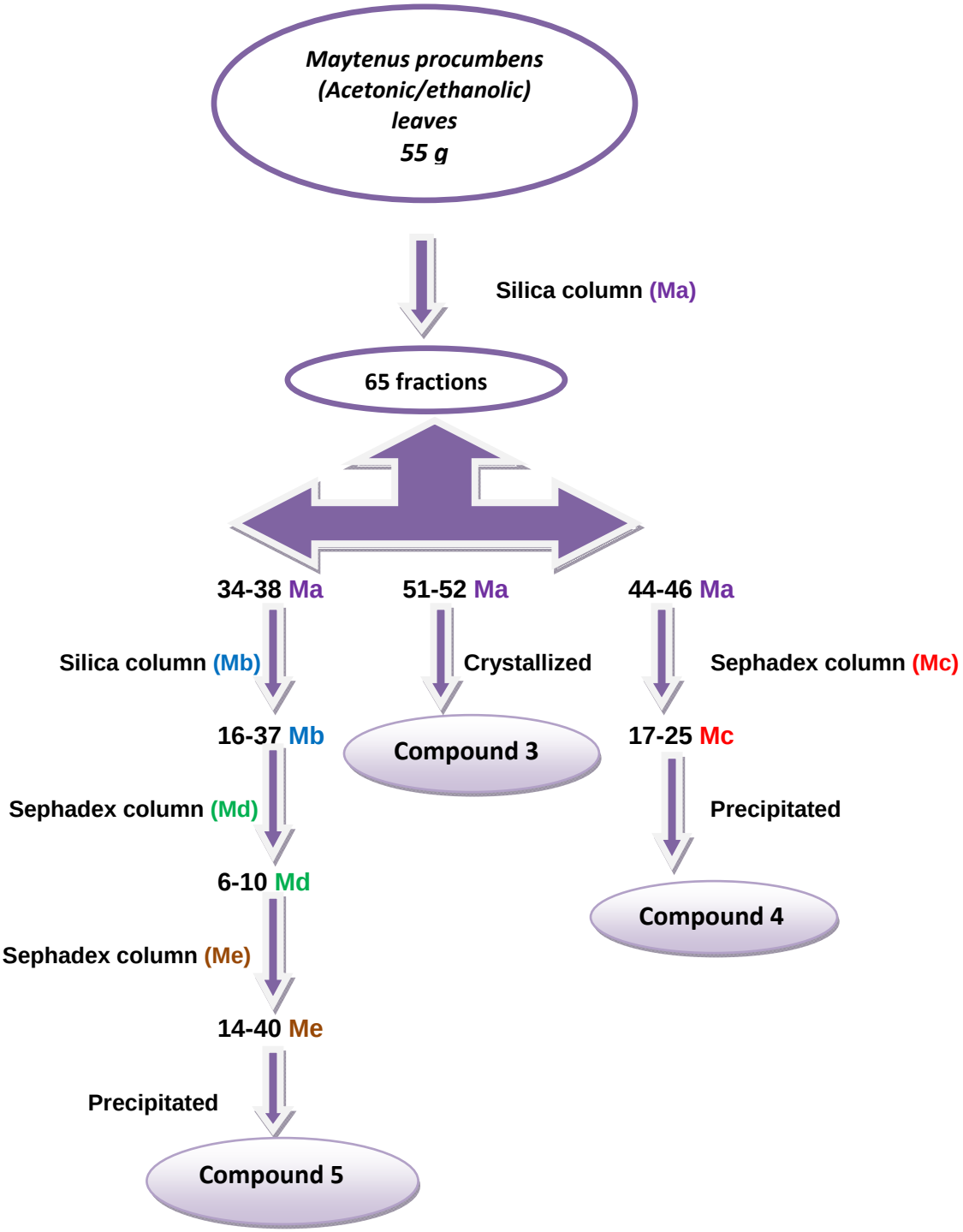
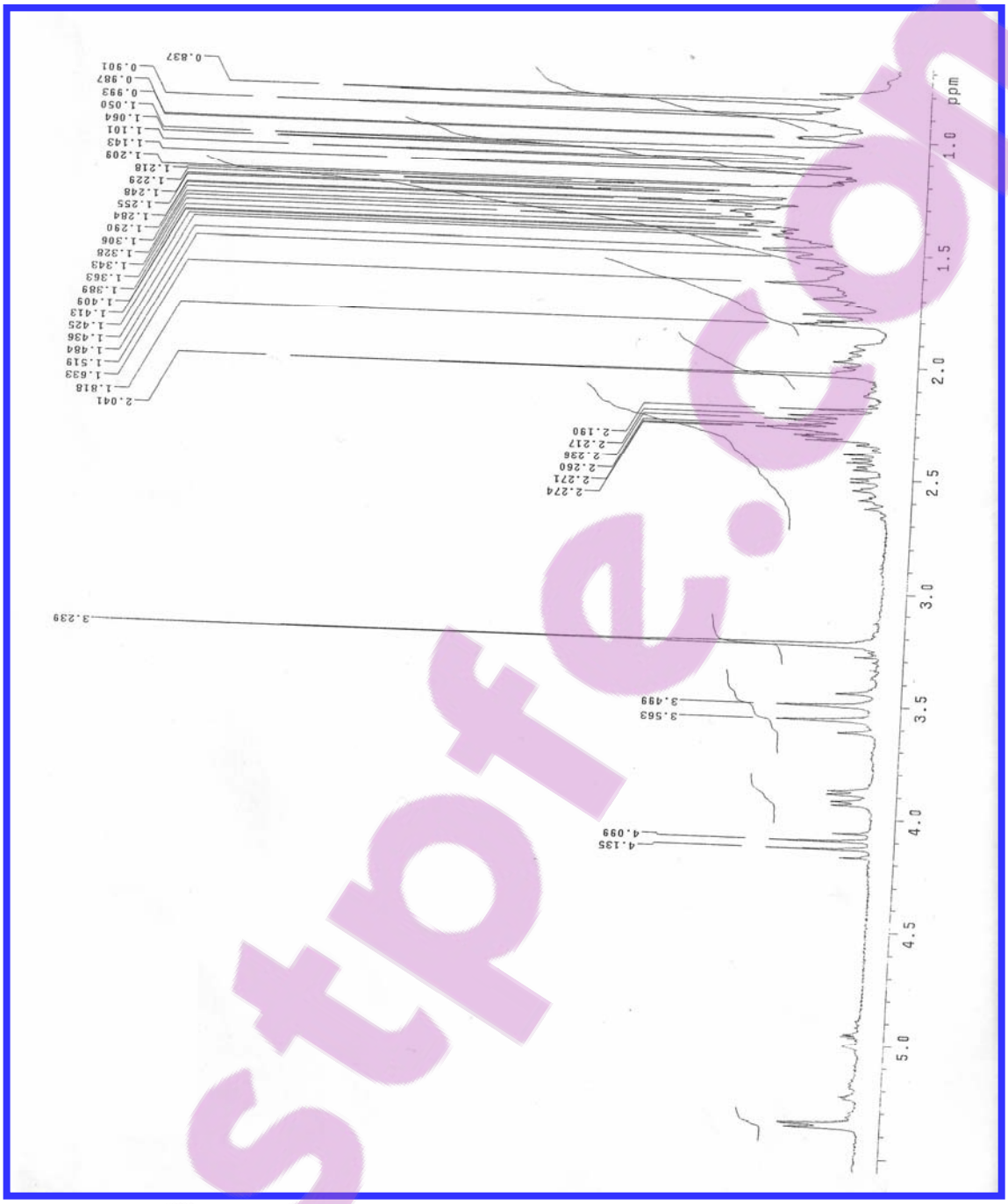
APPENDIX B: *MAYTENUS PROCUMBENS*



Appendix B.1: Twenty six main-fractions collected from **Ma** of which a pure powder was crystallized from 51-52**Ma** (compound **3**) (a), a pure compound was precipitated from fractions 16-37**Mb** (compound **5)** (b), and collected fractions 17-25**Mc** were combined, which resulted in a pure powder (compound **4**) (c).



Appendix B.2: The schematic presentation of the isolation steps of *M. procumbens* total extract (L.M.P).



Appendix B.3: The ^{1}H NMR spectra of ‘asiatic acid 4’.

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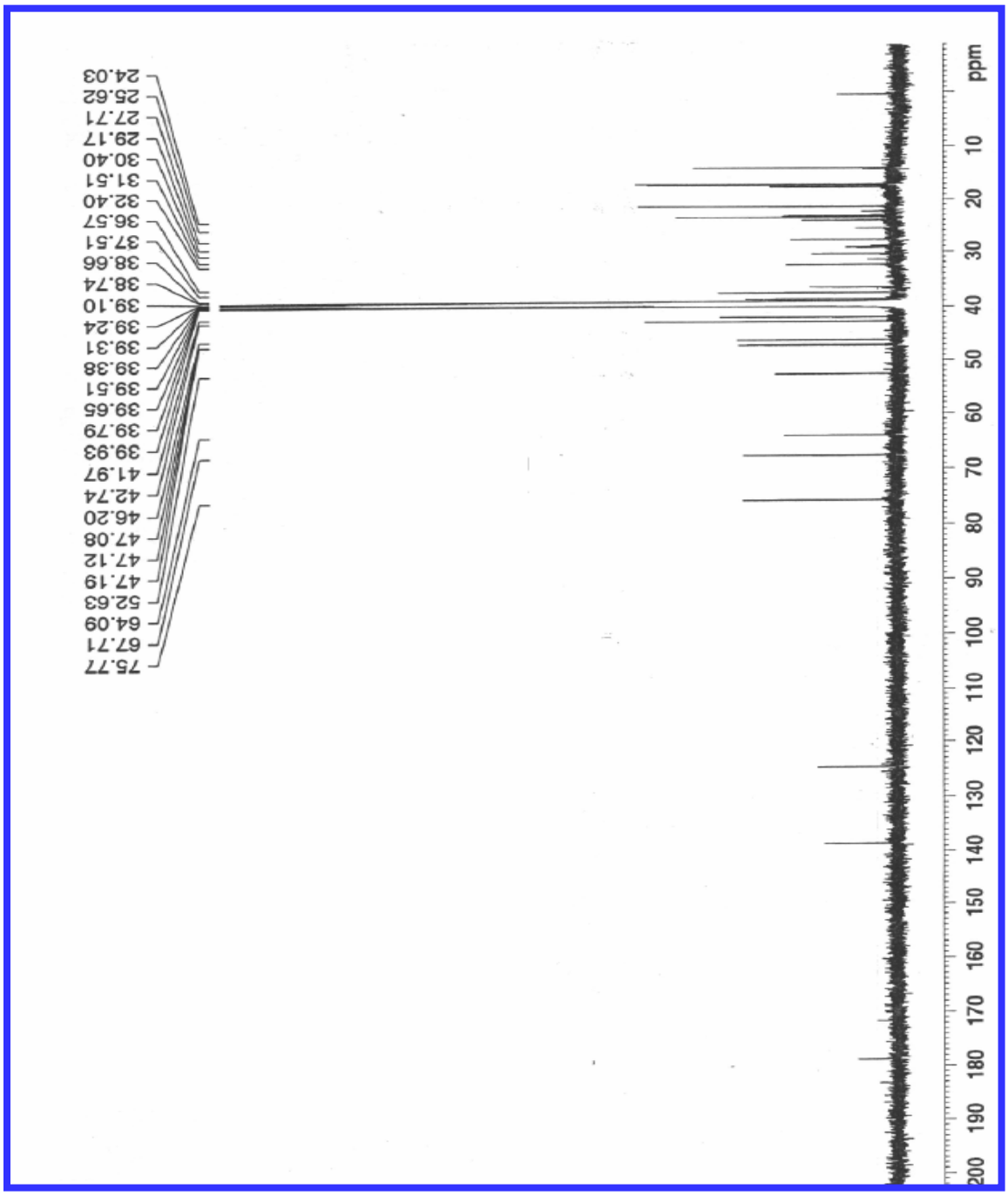
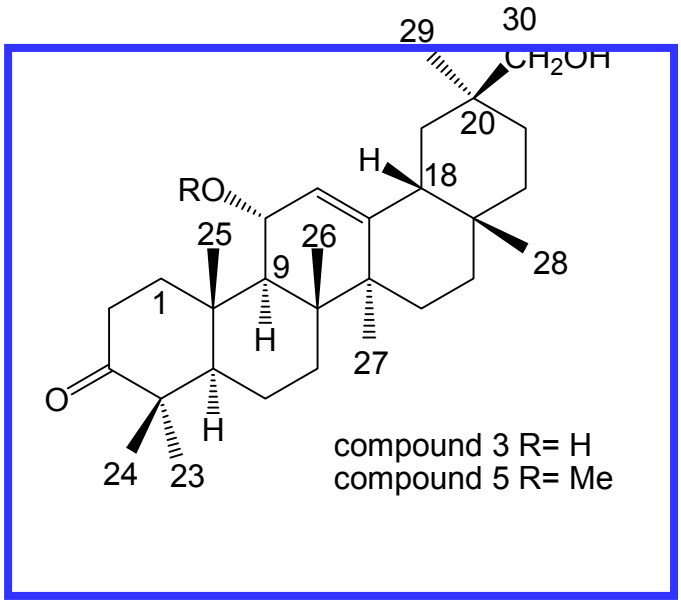


Appendix B.4: The ^{13}C NMR spectra of 'asiatic acid 4'.



Appendix B.5: A methoxy group at position C-12 of 'compound **5**'.

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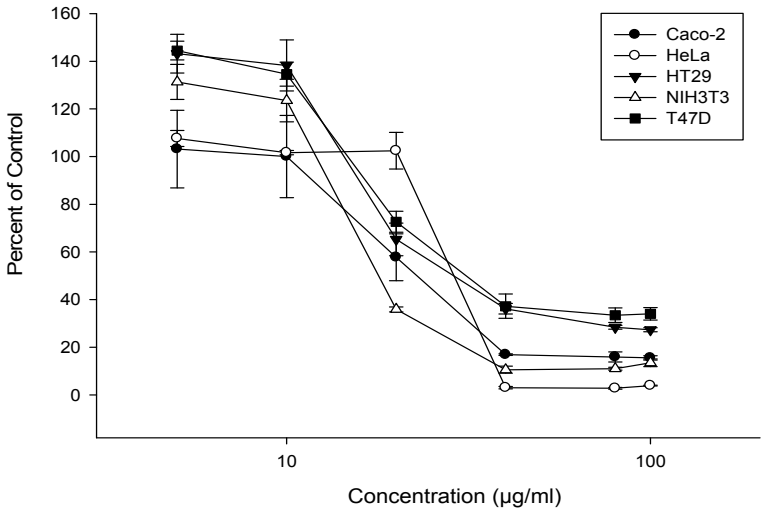
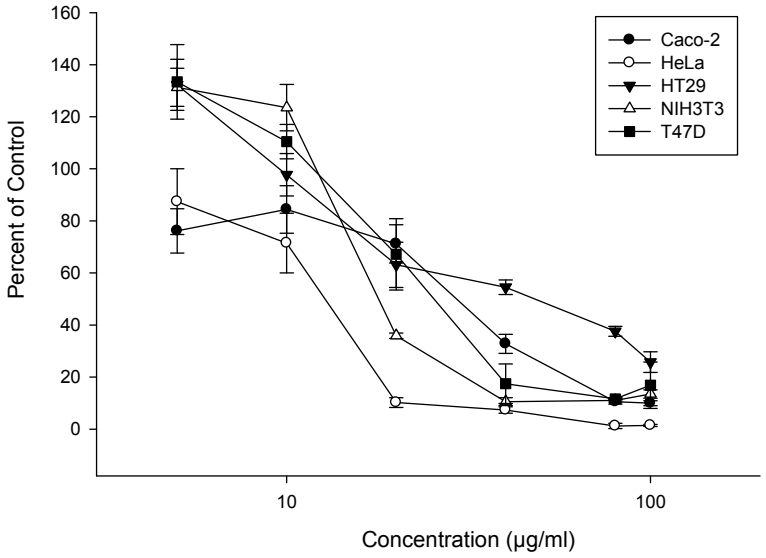
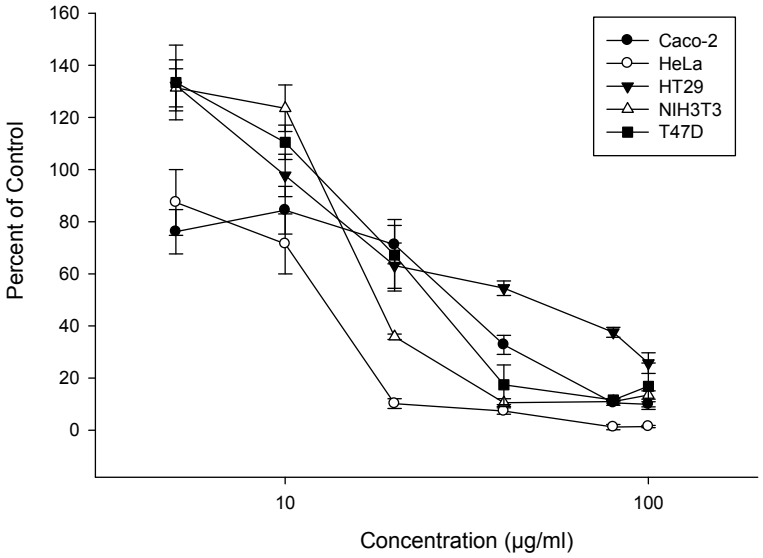


Appendix B.6: The cytotoxicity effects (MTT assay) of the acetone/ethanol extract of the leaves of *M. procumbens* (L.M.P) on different cell lines, (mean±SD, n=3).



Appendix B.7: The cytotoxicity effects (MTT assay) of '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' on different cell lines, (mean±SD, n=3).



Appendix B.8: The cytotoxicity effects (MTT assay) of '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**' on different cell lines, (mean±SD, n=3).

CHAPTER 6

APPENDIX C: *METHODS*

Appendix C.1: Cell culture

C.1.1. Transferring cells to 96-well plates

- The culture medium was discarded from the tissue culture flask (250 ml, 75 cm^2, (Nunc, Denmark) with the confluent cells.
- The confluent culture was washed with 5 ml of PBS (Phosphate Buffer Saline, without Ca^{2+} or Mg^{2+}).
- The PBS was later removed from the culture flask and 3 ml trypsin/versene (10%) was added to the cells.
- The flask was incubated at 37°C with 5% CO_2 for 10-20 minutes where the cells are detached.
- Once the cells were detached, 5 ml of complete medium supplemented with FBS was added to the flask in order to neutralise the action of trypsin/versene.
- The latter suspension was then aspirated to a 15 ml conical tube and centrifuged at 1,000 rpm for 5 minutes.
- The cells were pelleted and supernatant was discarded after which the cells were re-suspended in 2 ml complete medium and aspirated to form a suspension.

C.1.2. Cell counting

A 1:1 dilution of the cell suspension in trypan blue solution (e.g. 20 µl cells in 20 µl trypan blue) in an eppendorf tube was prepared and mixed. Ten microlitres (10 µl) of this dilution was transferred to two chambers of the hemacytometer. A hand-held tally was used to count the cells under the microscope. The number of cells was counted in the number of squares. The concentration of cells was determined by using the following formula:

Cell concentration (cells/ml) = Number of cells per square × 10 × 10,000

A cell suspension of the given concentration was determined using the following formula:

$$\text{Volume (cell suspension)} = \frac{\text{Cell concentration wanted} \times \text{volume wanted}}{\text{Concentration of cells in suspension}}$$

C.1.3. Mechanism of MTT assay

The MTT assay is suitable for measuring cell proliferation, cell viability of cytotoxicity. Cells were exposed to varying concentrations of the experimental plant samples for a period of time depending on the cell type. After contracting of cells with experimental samples for a while, the percentage of viable cells in each well of culture plates was determined by adding yellow dye MTT. Metabolically active cells have the ability to convert the tetrazolium salts to dark blue (purple) formazan crystals by cellular enzymes presented in the mitochondria of a metabolic active cell. The amount of formazan that forms correlates with cell viability and can be measured by means of spectrophotometry. These enzymes are rapidly inactivated when a cell dies, and hence the activity of these enzymes can be used to monitor the viability of a cell.

Cells were grown in a microtitre plate, and incubated with yellow MTT solution for 3 to 4 hours. After this incubation period, the blue formazan crystals are formed which are soluble in DMSO. An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample. This increase, directly correlates to the amount of formazan formed, and is monitored by the absorbance at wavelength of 570 nm. *In vitro* toxicity can be predicator of the *in vivo* activity. Although a high level of cytotoxicity dose not always predict a high degree of antitumor activity *in vivo*, a low level of cytotoxicity dose correlate with marginal or no activity *in vivo*.

C.1.3. Mechanism of trypan blue

Trypan blue is a vital dye used to selectively color dead tissues or cells blue. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue is not absorbed. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable; therefore, this staining method is also described as a dye exclusion method.

Appendix C.2: The flow cytometry assay

C.2.1. Mechanisms of Annexin-V and PI

Propidium iodide (PI) is fluorescent dye with impermeability through the cell membrane of viable cells, and can be used as fluorescent indicator of dead cells. PI is known as an intercalating agent and a fluorescent molecule with a molecular mass of 668.4 Da that can be used to stain cells. PI is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. Generally, PI is used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. PI is the most commonly used dye to quantitatively assess DNA content.

PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted ~30–40 nm to the red and the fluorescence emission maximum is shifted ~15 nm to the blue. Although its molar absorptivity (extinction coefficient) is relatively low, PI exhibits a sufficiently large Stokes shift to allow simultaneous detection of nuclear DNA and fluorescein-labeled antibodies, provided the proper optical filters are used. PI excites at 530 nm and emit at 620 nm. PI is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin-V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS.

Based on this phenomenon, a method using extrinsically applied hapten (i.e., FITC or biotin) labeled Annexin-V to detect apoptosis. Hapten-labeled Annexin-V binds in the presence of millimolar Ca^{2+} to PS residues that are exposed at the outer leaflet of the plasma membrane of apoptotic cells. Annexin-V is not able to bind to normal vital cells since the molecule is not able to penetrate the phospholipid bilayer. In dead cells, however, the inner leaflet of the membrane is available for binding of extrinsically applied Annexin-V, since the integrity of the plasma membrane is lost. To discriminate between dead and apoptotic cells, a membrane impermeable DNA stain, such as PI can be added simultaneously to the cell suspension. In this way vital, apoptotic and dead cells can be discriminated on basis of a double-labeling for Annexin-V and PI, and analyzed either by flow cytometry or fluorescence microscopy.

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Appendix C.3: The alkaline comet assay

C.3.1. Preparation of buffers and agarose

Lysing solution: (100 mM EDTA, 10 mM Tris/HCL, 2.5 M NaCl, 1% Triton-X100, pH = 7.5) in 1,000 ml distilled water (dH_2O) , kept refrigerated and cold.

Final lysing solution or alkaline solution: (300 mM NaOH, 1 mM EDTA, pH > 13), kept refrigerated and cold.

Electrophoresis buffer: (300 mM NaOH, 1 mM EDTA, pH = 13.5), kept refrigerated and cold.

Neutralization buffer: (0.4 M Tris/HCL, pH = 7.5), stored at room temperature.

Staining solution: Ethidium Bromide (EtBr; 10X Stock-10 µg/mL), stored at room temperature. For 1X stock 1 ml EtBr was mixed with 9 mL dH_2O.

Low melting point agarose (LMPA) 0.5%: 250 mg of agarose powder was dissolved in 50 ml of PBS and microwaved until near boiling and the agarose dissolved.

Normal melting agarose (NMA) 1%: 500 mg of agarose powder was dissolved in 50 ml Milli Q water and microwaved until near boiling and the agarose dissolved.

C.3.2. Cell culture preparation for comet assay

HeLa cells (2×10^6) were seeded in each well of a 6 well culture plate and were incubated for 24 hours. On second day, pure compounds were added to the cells at concentrations of their IC_{50}. Cells without any treatment (only treated with RPMI) were considered as control group (4 wells). The plates were incubated in culture condition (37°C and 5% CO_2) for 72 hours. Subsequently, cells were resuspended in PBS and the cell viability was assessed using trypan blue dye-exclusion staining. HeLa cells were scraped very gently (avoiding DNA damage), centrifuged (1,500 rpm, 5 min, 4°C), and

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washed with PBS. A suspension was prepared including 10 µl of cells (2.4×10^5 cell/ml) and 75 µl of 0.5% LMPA (cooled to 45°C).

C.3.3. Preparation of slides

Fully frosted microscope slides were covered with a thin layer of normal agarose (NMA). While NMA was hot (50-60°C), frosted slides were dipped up to one-third the frosted area and gently removed. The undersides of the slides were wiped to remove agarose and placed in a tray on a flat surface to dry. Pre-coated microscope slides were covered by 85 µl of prepared suspension cells (2.4×10^5 cell/ml in 0.5% LMP). The slides were placed on the ice-cold surface for 10 minutes to allow solidification of the agarose. The slides were immersed in lyses buffer for 1 hour at 4°C in dark to lyse the cells and to permit DNA unfolding. Thereafter, the slides were placed once more in cooled alkaline solution for additional 40 minutes at 4 °C.

C.3.4. Electrophoresis

The slides were kept horizontally in electrophoresis chamber with a cold (4°C) high pH electrophoresis buffer until the liquid level completely covers the slides for 20 minutes). The slides electrophoresis was performed at 25 Volts and 300 milliamperes (mA) for 20 minutes (Horizontal Gel Electrophoresis Apparatus, GIBCO BRL, Life Technologies). After electrophoresis, the slides were rinsed with neutralization buffer at room temperature for 15 minutes (3 times, each time for 5 minutes). The slides were stained with 80 µl of ethidium bromide (20 µg/ml) for 5 minutes and dipped in chilled distilled water to remove excess stain. To prevent additional DNA damage, all the steps were performed in the dark. The cover slips were placed over the slides and the results were scored immediately. For visualization of DNA damage, the slides 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).

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Appendix C.4: Measurement of radical scavenging capacity (RSC)

C.4.1. Preparation of experimental samples for DPPH antioxidant assay

Final concentrations of 7.8-1000 µg/ml were prepared for crude extract (L.M.P) for DPPH antioxidant assay. Pure compounds (**3** and **5**) were tested at the concentration ranging from 0.78-100 µg/ml by re-dissolving the dried samples in 100% ethanol (regarding to the insufficient amount of 'asiatic acid **4**', this compound was not tested). For each sample, a dilution series was prepared in a 96 well ELISA plate by adding distilled water as a dilution medium. All the samples were prepared in triplicate. 'Vitamin C' was used as control and was tested at the concentrations ranging from 7.8-1000 µg/ml. Its stock solution was prepared using distilled water.

C.4.2. DPPH scavenging antioxidant assay

Distilled water (100 µl) was added as medium to the wells of 96 well ELISA plates. In plate (I), 10 µl of each extract (stock concentration 29 mg/ml for total extract and 2.9 mg/ml for pure compounds) was added into the wells in triplicate. For each extract, 8 dilution series (doubling dilutions) were prepared separately. Vitamin C was prepared in the same way as extract and used as standard control. Subsequently, 90 µl (90 µM) of methanolic DPPH was added to each well. In plate (II) (reference plate), 10 µl of each extract was added in to the wells in triplicate. A doubling dilution series was prepared for each sample and 90 µl of distilled water was added in all wells. The plates were left in the dark to develop at room temperature. 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 and calculated as:

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Absorbance of solvent}}{\text{Absorbance of sample}}\right] \times 100$$

Appendix C.5: Ferric-reducing antioxidant power (FRAP) of HeLa cells

The FRAP reagent consists of 300 mM/L acetate buffer (3.1g $C_2H_3Na_2$, $3H_2O$ in 16 CC glacial acetic acid, pH = 3.6), 10 mM/L TPTZ in 40 mM/L HCl and 20 mM/L $FeCl_3.6H_2O$ in the ratio of 10:1:1 was prepared freshly. Briefly, 100 µl of cultured cells containing different concentration of samples were added to 600 µl freshly prepared and pre-warmed (37°C) FRAP reagent and incubated at 37°C for 10 minutes. Only medium treated cells were used as negative control. The absorbance of the blue colored complex was read against a reagent blank (600 µl FRAP reagent + 100 µl distilled water) at 593 nm using a Synergy4 BIOTEK multi-well plate reader (BIOTEK, USA). Standard solutions of Fe^{2+} in the range of 100 to 1,000 mM were prepared from ferrous sulphate ($FeSO_4.7H_2O$) in water. To report FRAP content; data were normalized by dividing the FRAP value on HeLa cells survival in related concentrations of samples. The absorbance change (ΔA) is translated into a ferric reducing/antioxidant power (FRAP) value (in mM) by known standard (FRAP) value, e.g., 1 M Fe^{2+} shown below:

$$\frac{\Delta A5953\ \text{nm test sample} \times \text{FRAP value of standard (mM)}}{\Delta A593\ \text{nm standard}}$$

Appendix C.6: Thiobarbituric acid reactive substance (TBAR) of HeLa cells

Assay of TBARS is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. To precipitate the cell's proteins, 500 µl of TCA 20% (m/V) was added into 250 µl of the samples (cultured HeLa cells containing different concentration of samples), which was then centrifuged at 1,500 rpm for 10 minutes. Thereafter, 500 µl of sulfuric acid (0.05 M) and 400 µl TBA (0.2%) were added to the sediment, shaken and incubated for 10 minutes in a boiling water bath. Subsequently, 800 µl *n*-butanol was added and the solution was centrifuged, cooled, and the supernatant absorption was recorded at 532 nm, using a Synergy4 BIOTEK multi-well plate reader (BIOTECK, USA). The calibration curve was obtained using different concentrations of 1,1,3,3-tetramethoxypropane as a standard to determine the concentration of TBA-MDA adducts in samples. Data were normalized by dividing the TBA content on HeLa cells survival in related concentrations of samples.

Appendix C.7: Antibacterial assay

C.7.1. Bacterial culture

All bacteria strains were maintained as stock strains in microbank™Cryovials and kept at -80 ºC until used. Each organism was maintained on a nutrient agar slant and was recovered for testing by growing them in fresh nutrient broth for 24 hours. Prior to sensitivity testing, the bacteria inocula were prepared by suspending overnight colonies (the tubes containing bacteria were incubated at 30-35°C for 24 h and those containing fungi were incubated at 20-25°C) from Mueller Hinton (MH) broth media in 0.9% saline. The *C. albicans* and *A. niger* inocula were prepared by suspending colonies from 48 h and 72 h old Sabouraud dextrose (SD) broth cultures in 0.9% saline, respectively. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to 0.5 McFarland standards (1.5×10^8 CFU/ml). The suspensions were then vortexed and swapped to the plates.

C.7.2. Preparation of experimental samples for antibacterial assay

Plant extract and pure compounds were diluted in DMSO to produce a stock concentration of 8 mg/ml. The isolated pure compounds were dissolved in DMSO resulted a stock solution of 400 µg/ml.

C.7.3. Agar well diffusion assay (Agar-based cup–plate method)

Aliquots of the stock solution were mixed with melted-autoclaved agar to produce a series of concentrations (0.5-8 mg/ml for crude extract) and (5-400 µg/ml for pure compounds) in Petri dishes in triplicate. The assay was performed by means of the agar-based cup–plate method. The surface of Petri dishes containing 25 ml of MHA/SDA were seeded individually with bacterial suspensions (equivalent to 0.5 McFarland standard, 1.5×10^8 $CFUml^{-1}$) using a sterile cotton swab. Wells were created by punching a stainless steel cylinder onto the agar plates and removing the agar to form a well. Aliquots of 50 µl of each sample were placed individually in the wells. The plates containing bacteria were incubated at 30-35°C for 24 hours and those containing

fungi were incubated at 20-25°C for 48 hours. Plates containing only nutrient agars and 100% DMSO were included, although no antibacterial and antifungal activity has been noted. In addition, 'streptomycin sulfate' at concentrations of 10, 50 and 100 µg/ml served as antibacterial positive control. 'Amphotricin B' (5 mg/ml) were served as positive antifungal control. 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.

Appendix C.8: Publications and conference presentations

Saeideh Momtaz, Namrita Lall, Ahmed Hussein, Seyed Naser Ostad and Mohammad Abdollahi. Investigation of the possible biological activities of a poisonous South African plant; *Hyaenanche globosa (Euphorbiaceae)* (Published, 2010, Pharmacognosy Magazine).

Saeideh Momtaz, Namrita Lall, Ahmed Hussein, Seyed Naser Ostad and Mohammad Abdollahi. Growth inhibition and induction of apoptosis in human cancerous HeLa cells by *Maytenus procumbens* (Accepted, 2012, Journal of Food and chemical Toxicology).

Momtaz, S, Abdollahi, M. Ostad, SN. Lall, N. Cytotoxicity effects of *Hyaenanche globosa* and *Maytenus procumbens*. May 2009, 10th Iranian Toxicology Congress, Tehran (Poster presentation).

A second paper (Cytotoxicity effects of *Hyaenanche globosa* and *Maytenus procumbens)* was accepted at an international conference "47th Congress of the European Societies of Toxicology 2011' for poster presentation.