In the Name of God, Most Gracious

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An Evaluation of Anti-cancer Activities of *Hyaenanche globosa* Lamb. (Euphorbiaceae) and *Maytenus procumbens* (L.F.) Loes. (Celastraceae)

by

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I DEDICATE THIS DISSERTATION TO

MY BROTHER EHSAN; TO HIS

BLESSED SOUL

MY COMPASSIONATE HUSBAND

RAMIN

& MY DEAREST DAUGHTER KIMIA

I declare that the thesis/dissertation, which I hereby submit for the degree of PHILOSOPHIAE DOCTORAL of science at the University of Pretoria and Tehran University of Medical Sciences, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Saeideh Momtaz

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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

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

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

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

SUMMARY

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

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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

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

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

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

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.

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.

'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

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*.

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.