
CHAPTER 5

5.1. ACKNOWLEDGEMENTS

First, I would like to express my devotion and utmost thankfulness to almighty God for giving me the opportunity to start this project and providing the facilities to terminate it successfully. Second, peace be upon Prophet Muhammad who said 'An hour's contemplation is better than a year's adoration'. The project of this magnitude involves many more people than just the writer. The completion of this work would not have been possible without the help of many people. The author wishes to express sincere thanks to:

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- I express my greatest appreciation to my husband Mr Ramin Zare for his advices to pursue science as my life career and my adorable daughter Kimia for her support and patience throughout the period of the study.

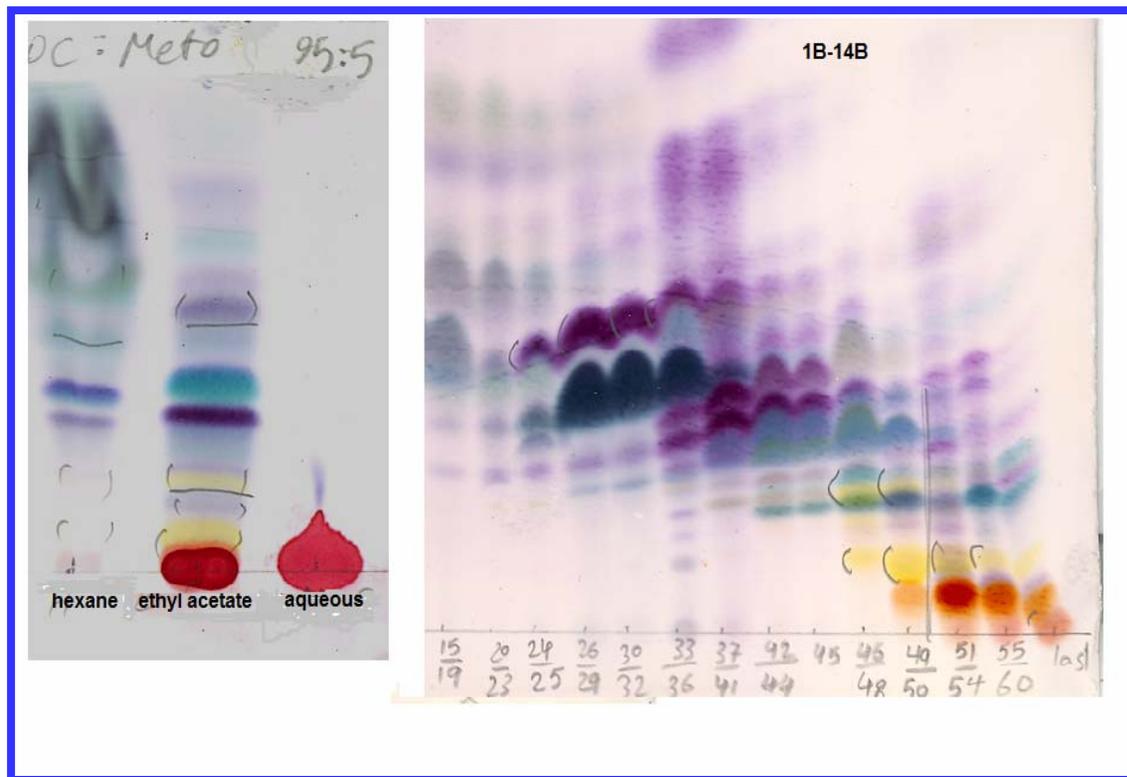
- To my parents for their everlasting supports and encouragements; my dear father Mr Kazem Momtaz and my sweetheart mother Mrs Khadijeh Khonveh. Also, my elder brother Mohammad Reza Momtaz and his wife Mrs Haniyeh Monfared, my youngest brother Hamid Reza Momtaz, and my delightful aunt Nazanin Khonveh for their encouragement during this study.

Additionally I would like to thank:

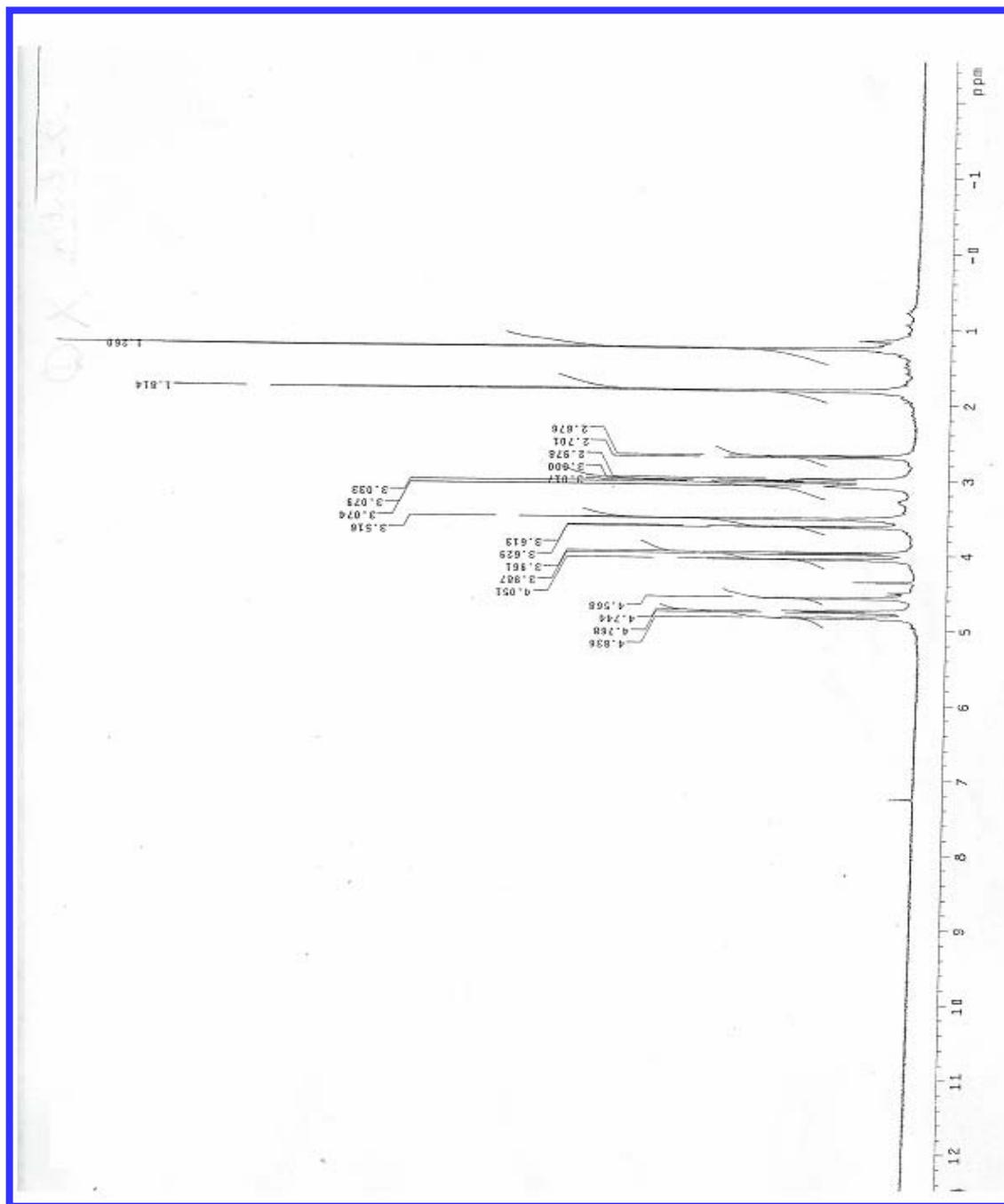
- Mrs Shohreh Tavajohi for her invaluable friendship and support through the complicated times. She was a symbol of reliability, responsibility, and patience to me.
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- To my colleagues in Faculty of Pharmacy, and Pharmaceutical Sciences Research Center for their friendships and supports in University of Medical Sciences, Tehran, Iran.

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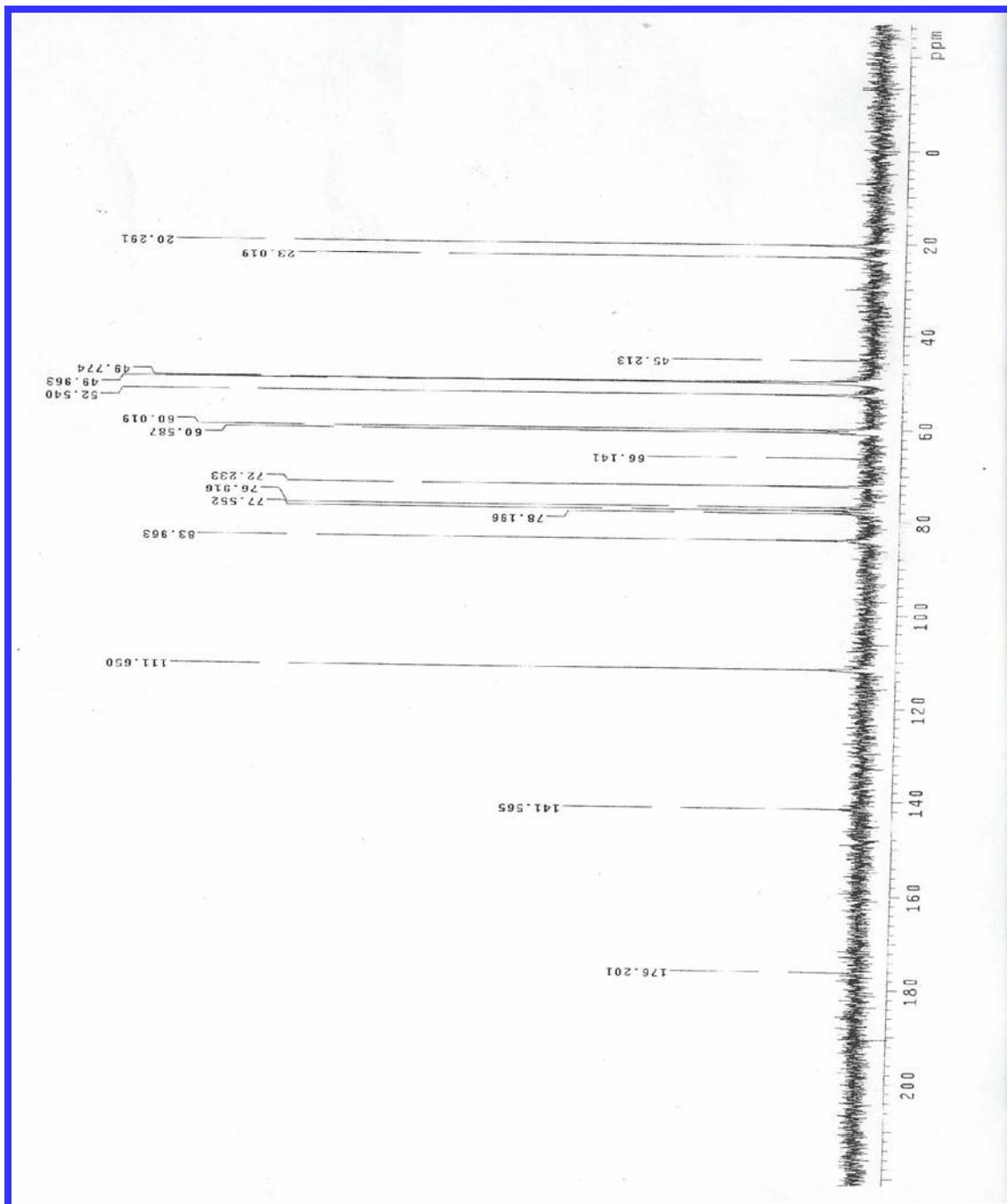
APPENDIX A: *HYAENANCHE GLOBOSA*



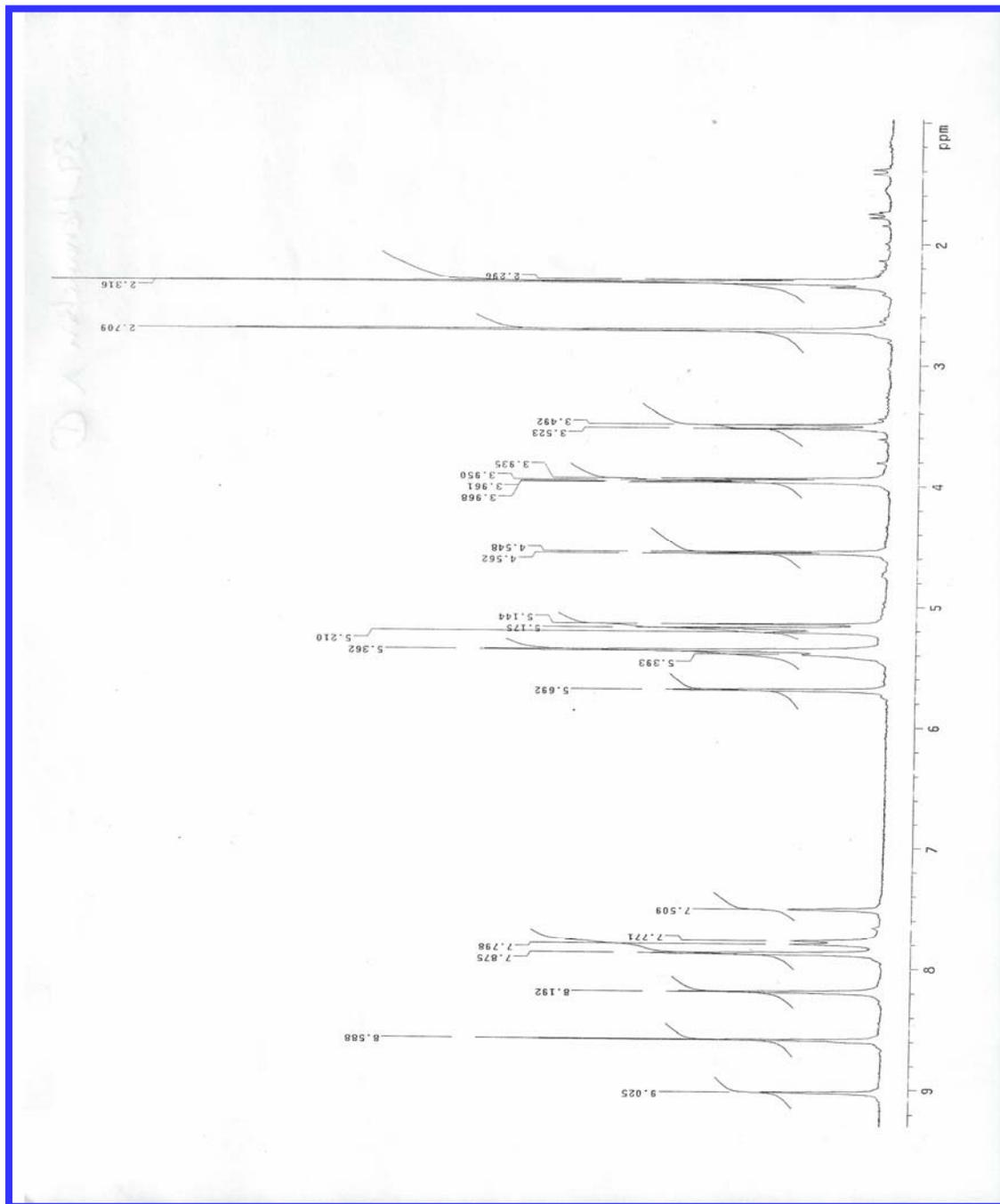
Appendix A.1: Hexane, ethyl acetate and aqueous fractions of the fruits of *H. globosa* (F. E) and 14 main fractions (1B-14B) of the hexane fraction.



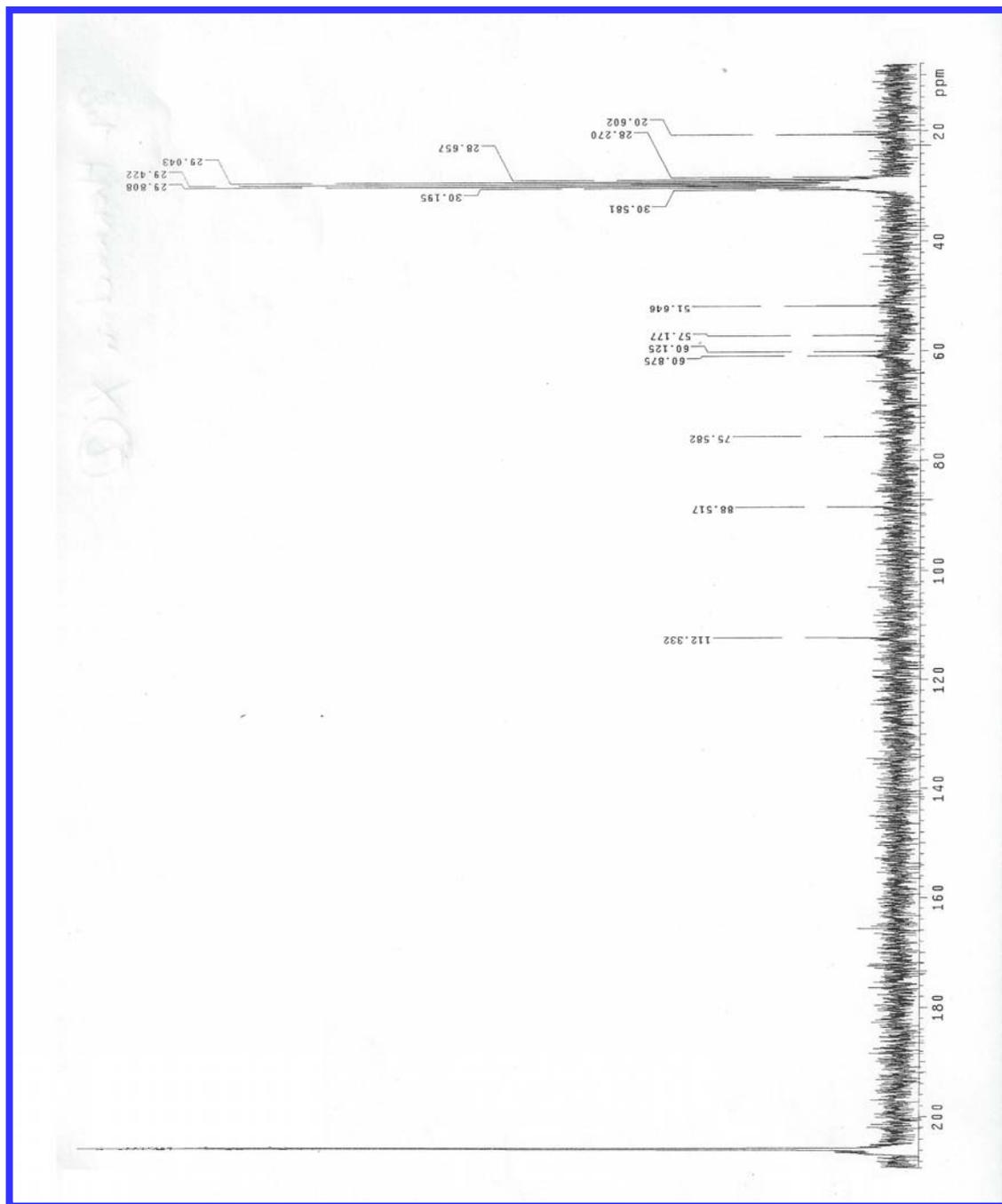
Appendix A.2.1: The ^1H NMR spectra of 'tutin 1'.



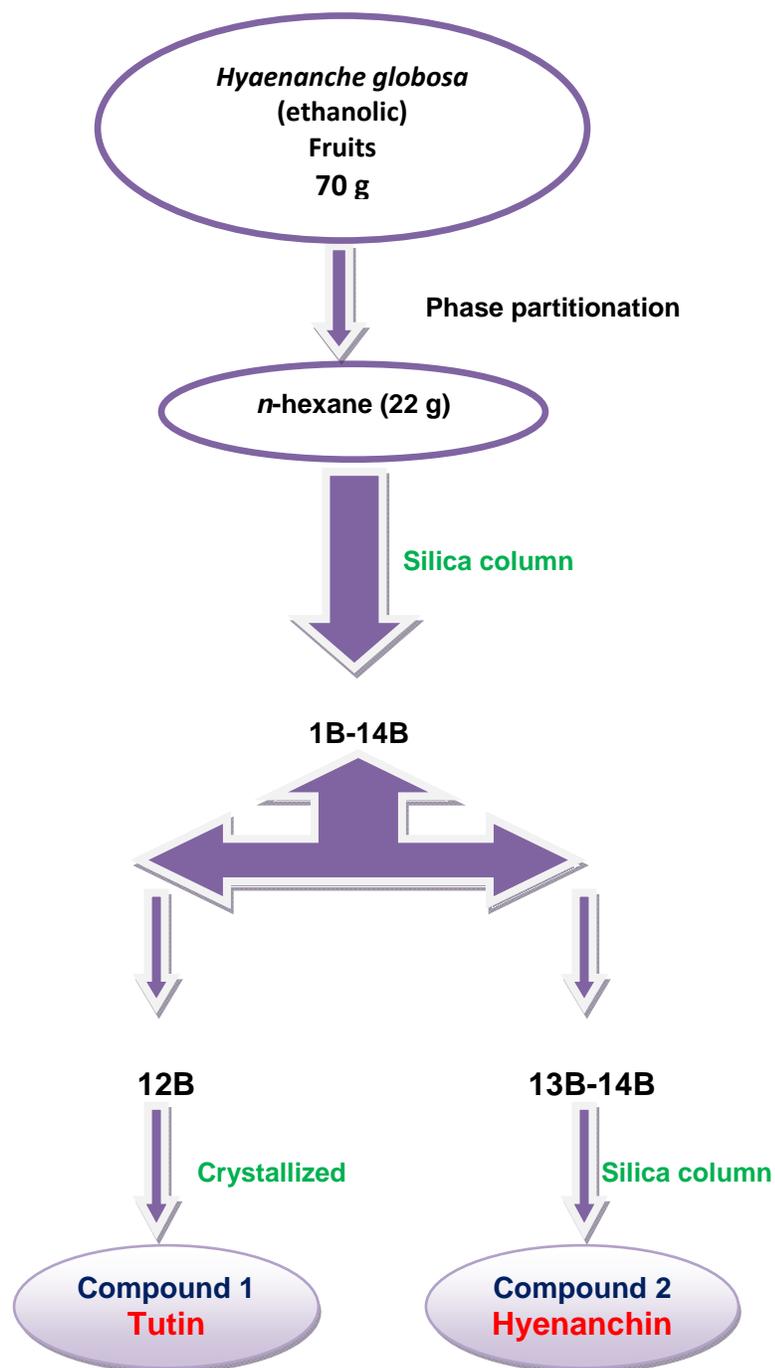
Appendix A.2.2: The ^{13}C NMR spectra of 'tutin 1'.



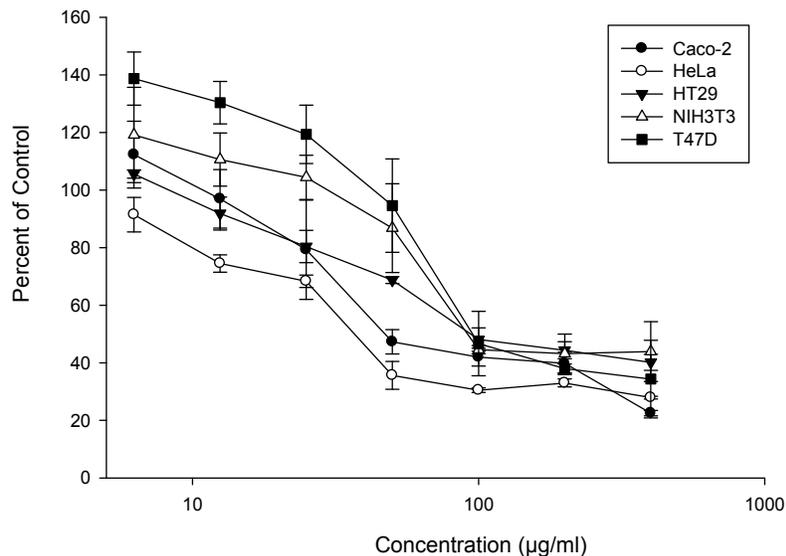
Appendix A.3.1: The ^1H NMR spectra of 'hyenanchin 2'.



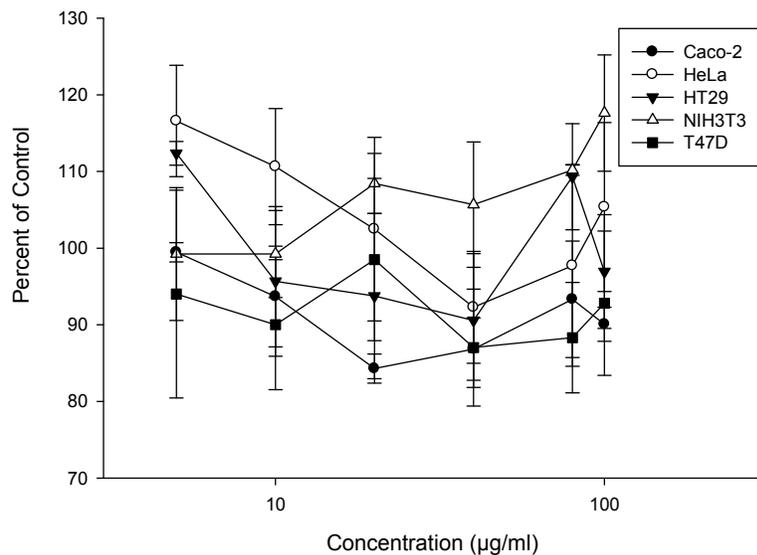
Appendix A.3.2: The ^{13}C NMR spectra of 'hyenanchin 2'.



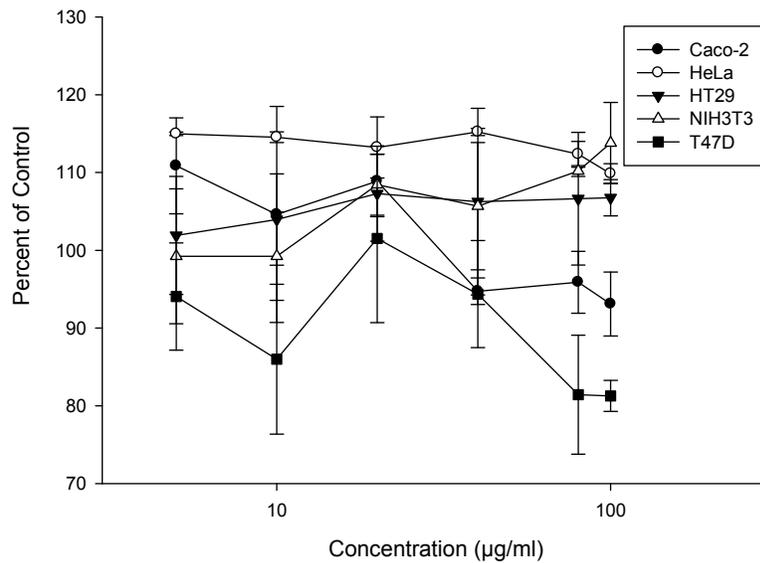
Appendix A.4: The schematic presentation of the isolation steps of *H. globosa* ethanolic extract of the fruits (F.E).



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 \pm SD, n=3).



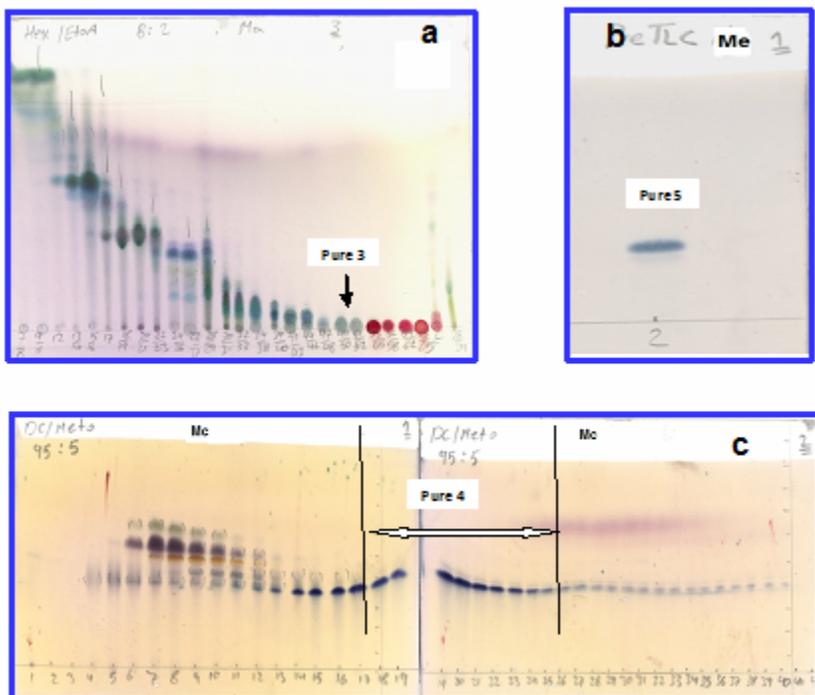
Appendix A.6: The cytotoxicity effects (MTT assay) of 'tutin 1' on different cell lines, (mean \pm SD, n=3).



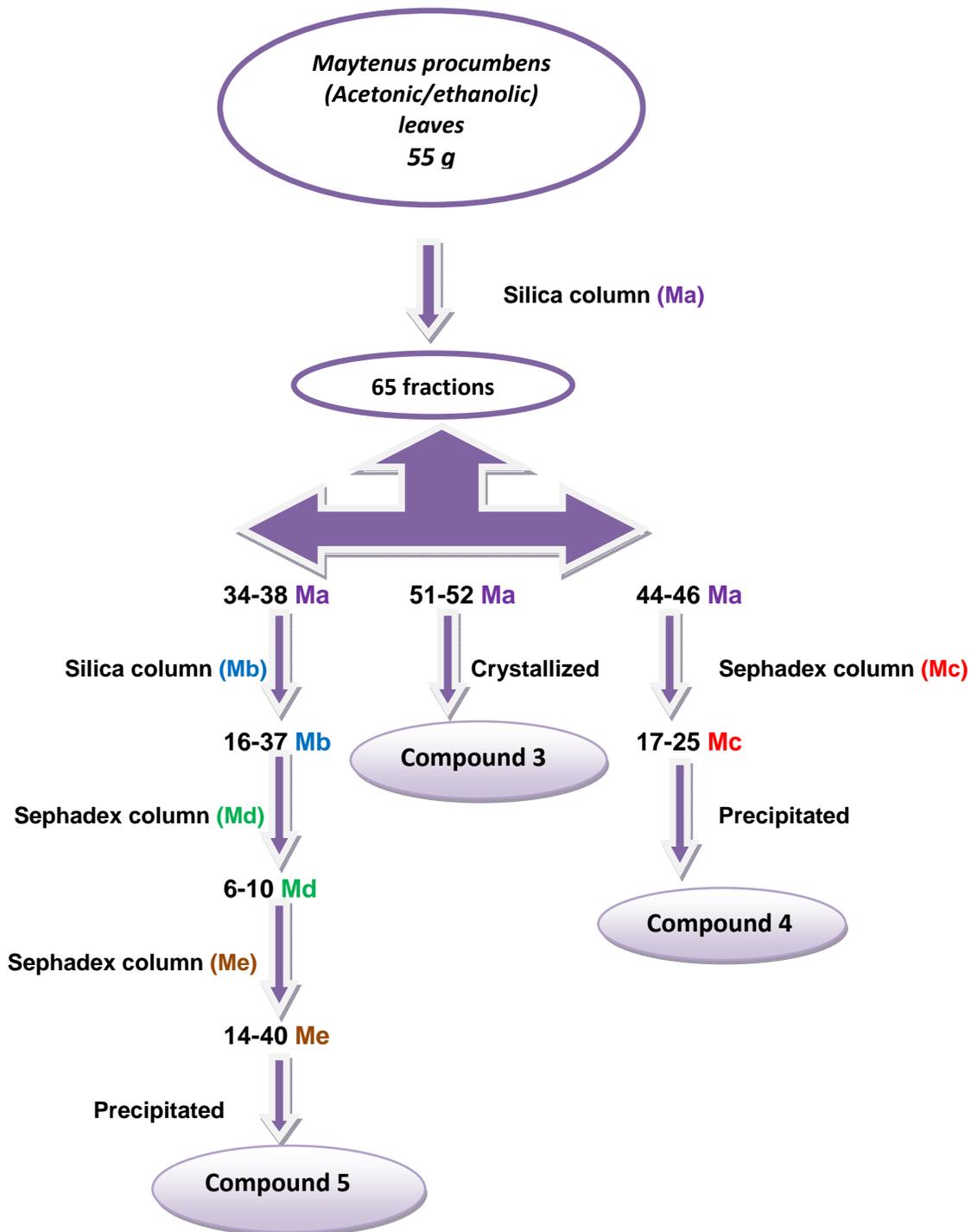
Appendix A.7: The cytotoxicity effects (MTT assay) of 'hyenanchin 2' on different cell lines, (mean \pm SD, n=3).

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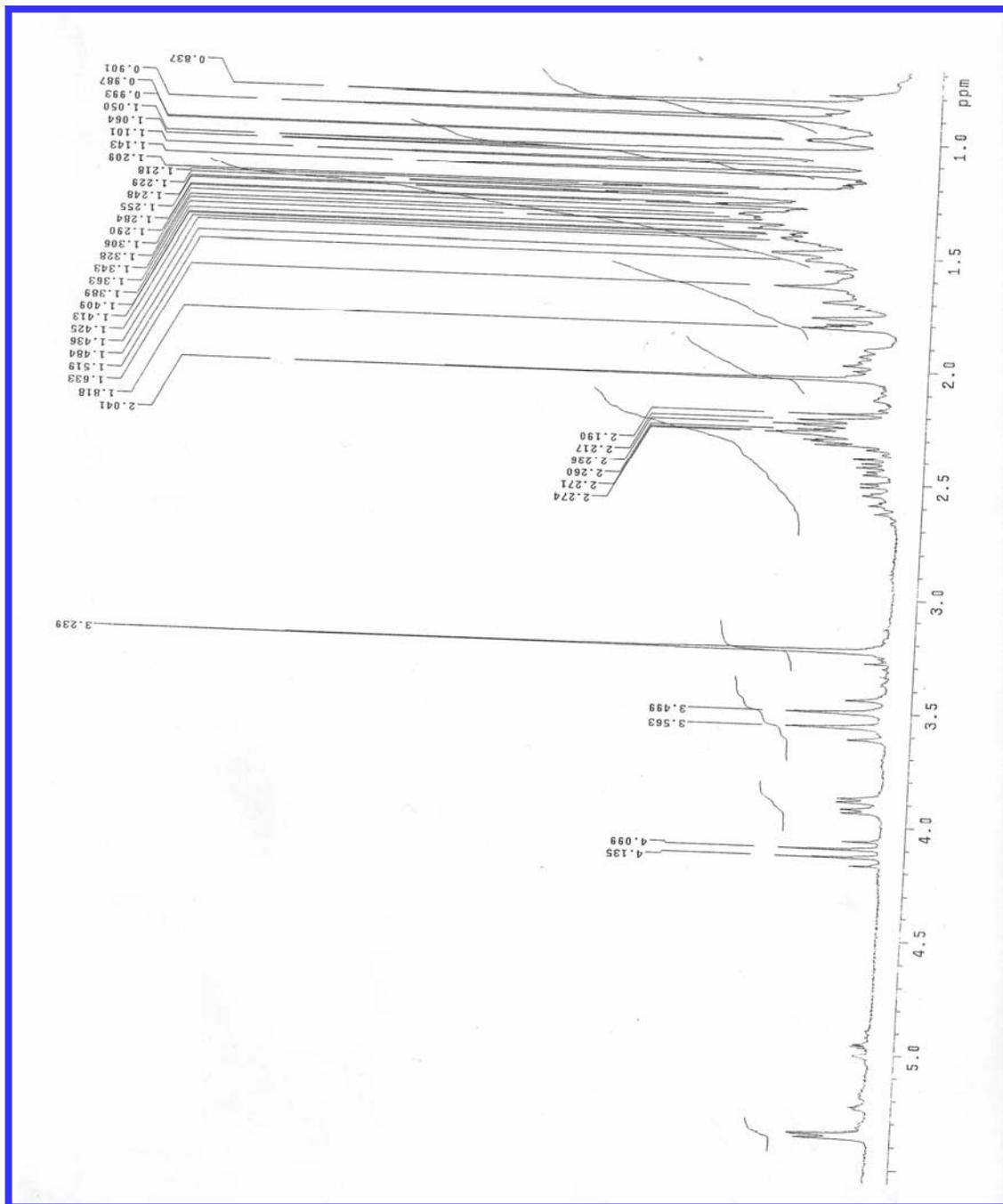
APPENDIX B: *MAYTENUS PROCUMBENS*



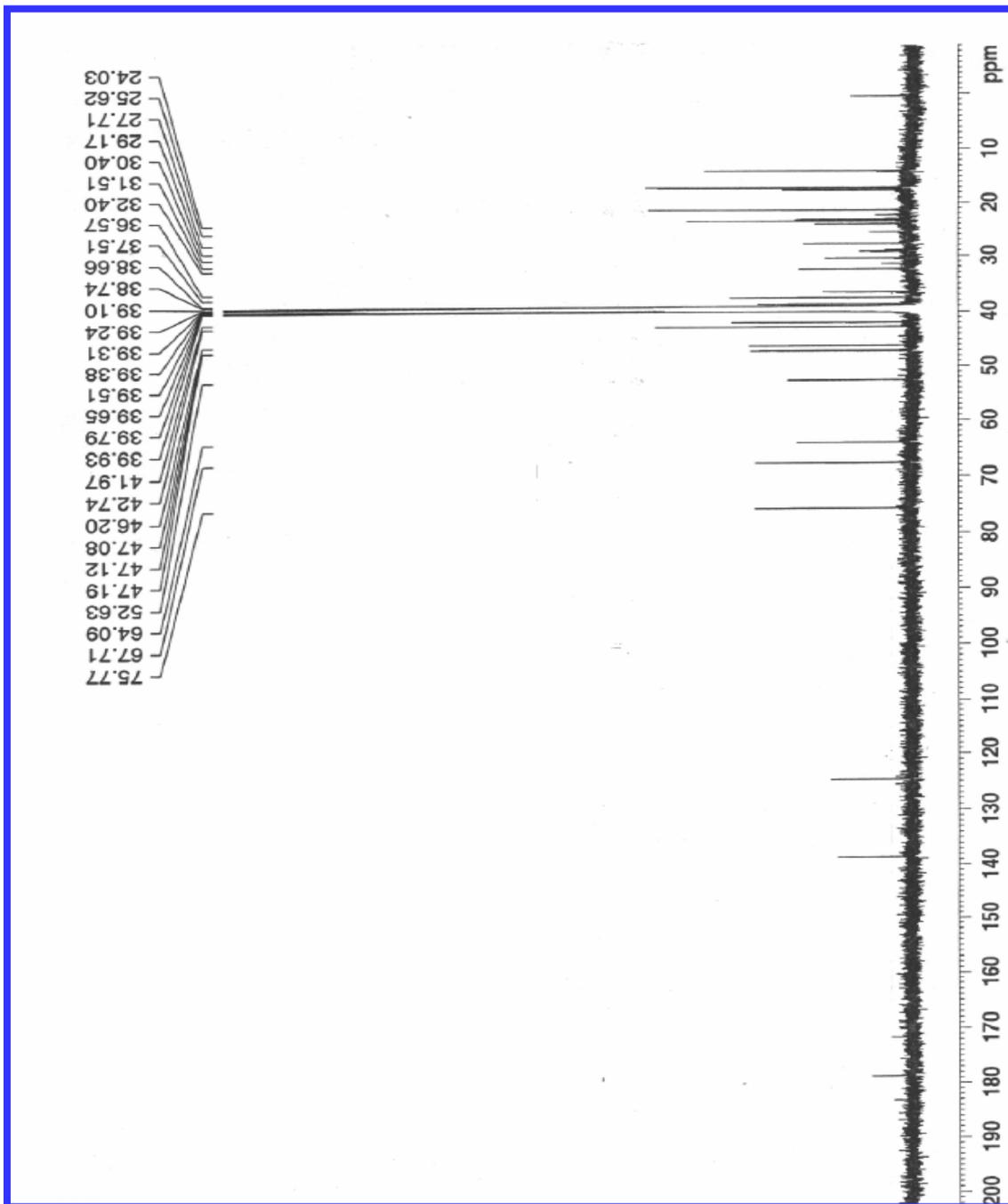
Appendix B.1: Twenty six main-fractions collected from **Ma** of which a pure powder was crystallized from 51-52**Ma** (compound **3**) (a), a pure compound was precipitated from fractions 16-37**Mb** (compound **5**) (b), and collected fractions 17-25**Mc** were combined, which resulted in a pure powder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



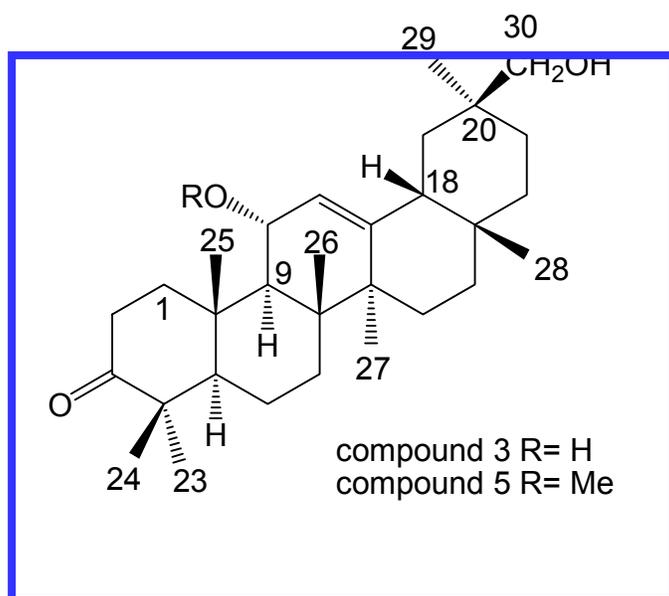
Appendix B.2: The schematic presentation of the isolation steps of *M. procumbens* total extract (L.M.P).



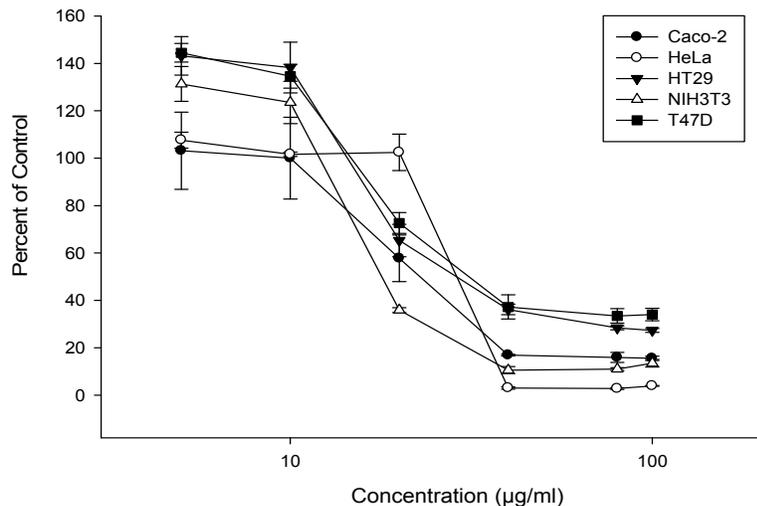
Appendix B.3: The ^1H NMR spectra of 'asiatic acid 4'.



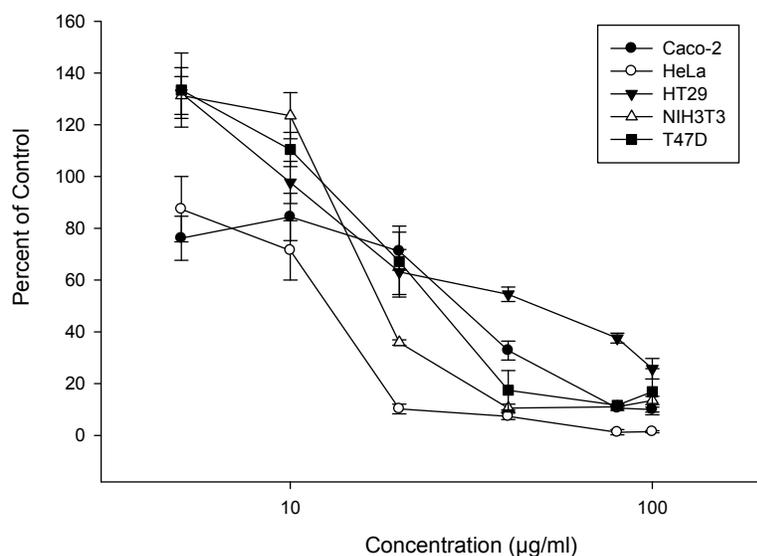
Appendix B.4: The ^{13}C NMR spectra of 'asiatic acid 4'.



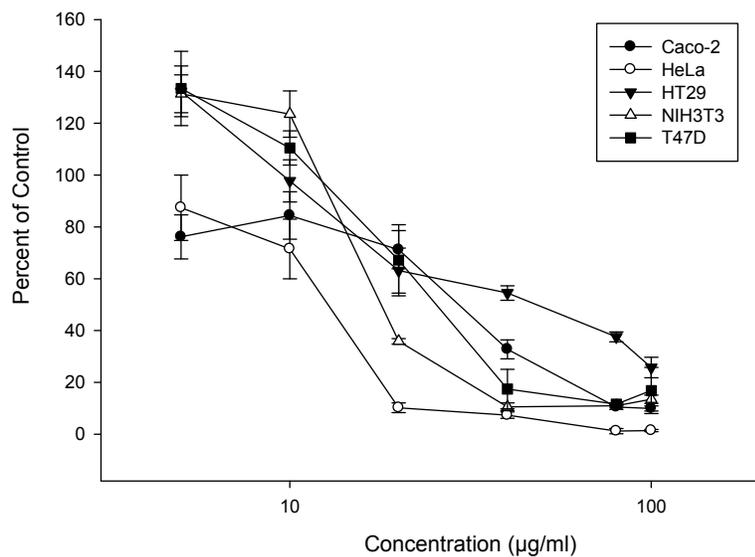
Appendix B.5: A methoxy group at position C-12 of 'compound 5'.



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-11 α -methoxy-18 β -olean-12-en-3-one 5' on different cell lines, (mean \pm SD, n=3).

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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 cm², (Nunc, Denmark) with the confluent cells.
- The confluent culture was washed with 5 ml of PBS (Phosphate Buffer Saline, without Ca²⁺ or Mg²⁺).
- 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% CO₂ 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 $\times 10 \times 10,000$

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 predictor 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 chromophore 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.

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 (dH₂O) , 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 dH₂O.

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 IC₅₀. 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% CO₂) 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

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 $\mu\text{g}/\text{ml}$) 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).

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 $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1 was prepared freshly. Briefly, 100 μ l of cultured cells containing different concentration of samples were added to 600 μ l freshly prepared and pre-warmed ($37^\circ C$) FRAP reagent and incubated at $37^\circ C$ 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 μ l FRAP reagent + 100 μ l 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 ($FeSO_4 \cdot 7H_2O$) 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:

$$\frac{\Delta A_{5953 \text{ nm test sample}} \times \text{FRAP value of standard (mM)}}{\Delta A_{593 \text{ nm standard}}}$$

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 μ l of TCA 20% (m/V) was added into 250 μ l of the samples (cultured HeLa cells containing different concentration of samples), which was then centrifuged at 1,500 rpm for 10 minutes. Thereafter, 500 μ l of sulfuric acid (0.05 M) and 400 μ l TBA (0.2%) were added to the sediment, shaken and incubated for 10 minutes in a boiling water bath. Subsequently, 800 μ l *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 (BIOTEK, 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 °C 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.