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

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).

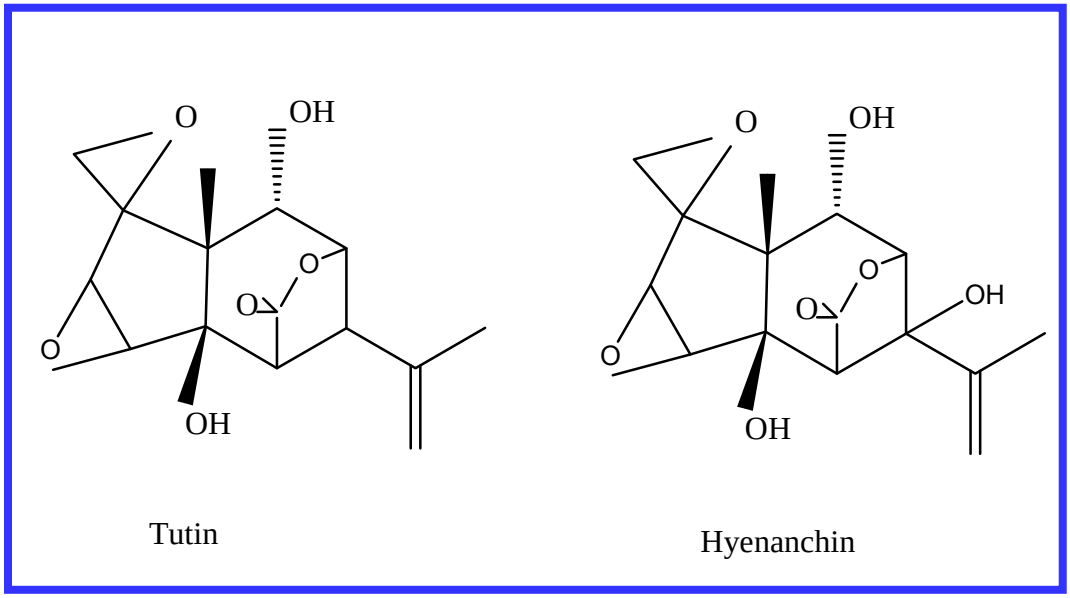


Figure 2.1: Chemical structures of the isolated compounds from the ethanolic extract of F.E (fruits, ethanol extract) of *H. globosa*.

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

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

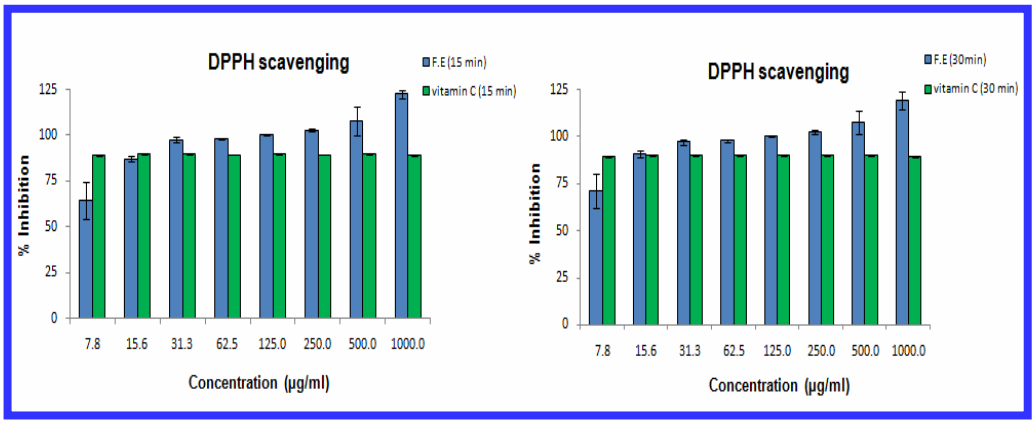
30 minutes. F.E showed DPPH discoloration between almost 70% and 120% at concentrations 7.8-1000 µg/ml after 30 minutes (Fig 2.2.a). 'Tutin **1**' showed the highest antioxidant activity (50% inhibition of DPPH at the highest concentration tested after 30 minutes) which was followed by 'hyenanchin **2**' (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.

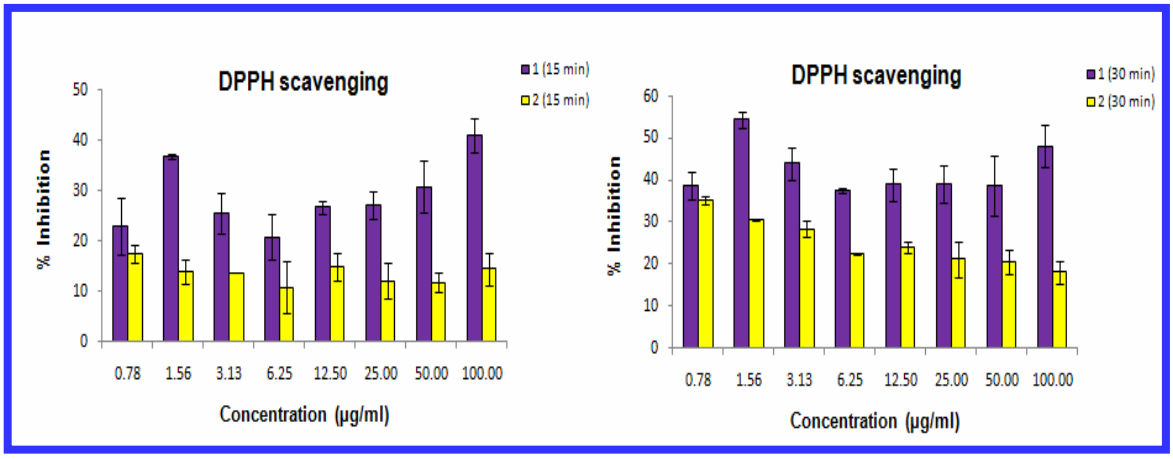
2.5. CONCLUSION

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).

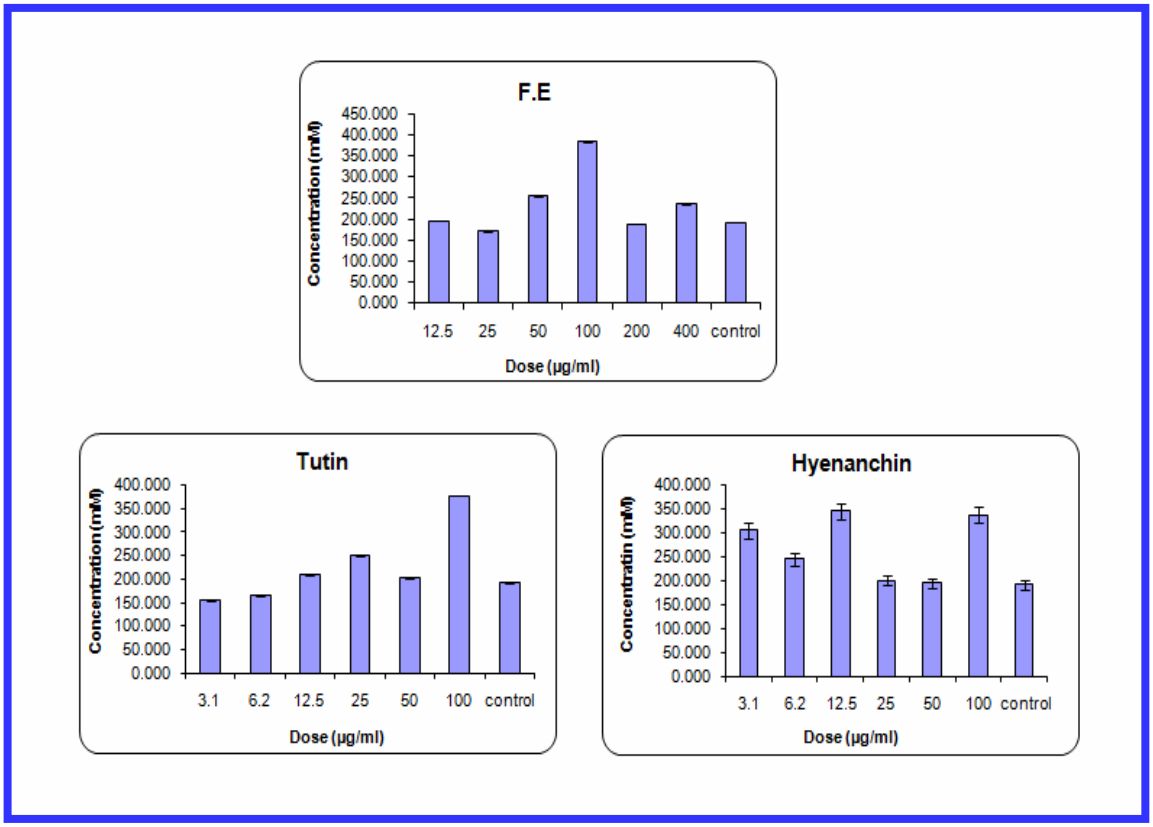


Figure 2.3: Ferric-reducing antioxidant power (FRAP) potential of the F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**' in cultured HeLa cells.

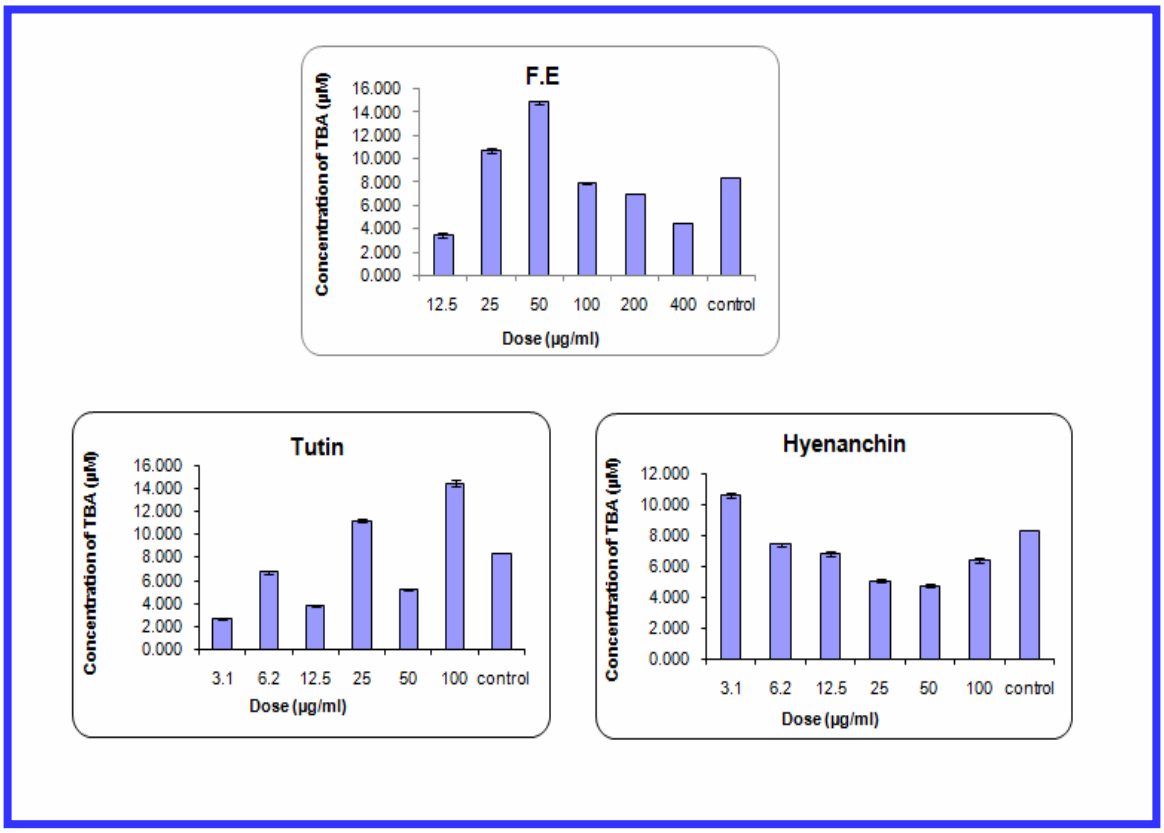


Figure 2.4: Lipid peroxidation thiobarbituric acid reactive substance (TBARS) potential of the F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**' TBARS in cultured HeLa cells.

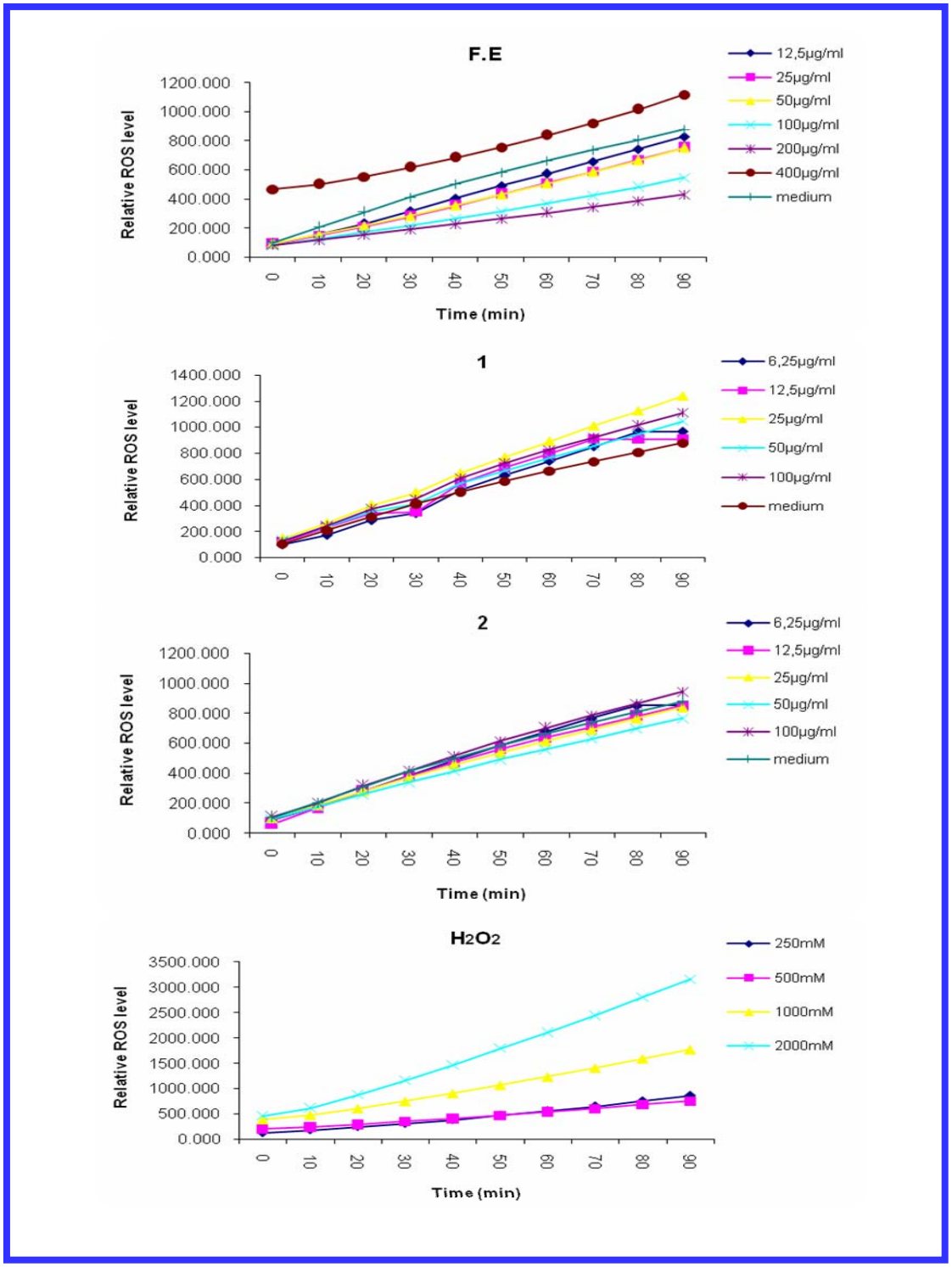


Figure 2.5: Time-response curve for DCF fluorescence in HeLa cells after 90 min exposure to various concentrations of F.E (fruits, ethanol extract), 'tutin **1**' and 'hyenanchin **2**'. Each data point represents the mean of data from three wells (n= 3).

2.6. REFERENCES

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

Ames, B.N., Gold, L.S. and Willett, W.C., 1995. The causes and prevention of cancer. Proceedings of the National Academy of Sciences USA., 92: 5258-65.

Bjelakovic, G., Nikolova, D., Simonetti, R.G. and Gluud, C., 2004. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. The Lancet., 364: 1219-1228.

Corbella, A., Jommi, G., Rindone, B. and Scolastico, C., 1969. Structures of pretoxin and lambicin. Tetddna., 25: 4835-4841.

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

Curto, E.V., Kwong, C., Hermersdofer, H., Glatt, H., Santis, C., Virador, V., Hearing Jr, V.J. and Dooley, T.P., 1999. Inhibitors of mammalian melanocyte tyrosinase: *In vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochemical Pharmacology., 57: 663-672.

Dehghan, G., Shafiee, A., Ghahremani, M., Ardestani, S. and Abdollahi, M., 2007. Antioxidant potential of various extracts from *Ferula szovitsiana* in relation to their phenolic content. Pharmaceutical Biology., 45: 691-699.

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

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

Fuentealba, J., Guzman, L., Manriquez-Navarro, P., Perez, C., Silva, M., Becerra, J. and Aguayo, L.G., 2000. Inhibitory effects of tutin on glycine receptors in spinal neurons. European Journal of Pharmacology., 559: 61-64.

Hall, R., 1978. Naturally occurring toxicants and food additives: Our perception and management of risks. Marabou Symposium on Food and Cancer, Sundbyberg, Sweden: US8045967.

Halliwell, B., 1996. Commentary: vitamin C: antioxidant or pro-oxidant in vivo? Free Radical Research., 25: 439-454.

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

Kinoshita, T., Itaki, N., Hikita, M., Aoyagi, Y., Hitotsuyanagi, Y. and Takeya, K., 2005. The isolation and structure elucidation of a new sesquiterpene Lactone from the poisonous plant *Coriaria japonica* (Coriariaceae). Chemical and Pharmaceutical Bulletin., 53: 1040-1042.

Lee, K.T., Kim, B.J. and Kim, J.H., 1997. Biological screening of 100 plant extracts for cosmetic use (I): Inhibitory activities of tyrosinase and DOPA auto-oxidation. International Journal of Cosmetic Science., 19: 291-298.

Lewis, W.H. and Elvin-Lewis, M.P., 1995. Medicinal plants as sources of new therapeutics. Annals- Missouri Botanical Garden., 82: 16-24.

Mativandlela, S.P., Meyer, J.J., Hussein, A.A. and Lall, N., 2007. Antitubercular activity of compounds isolated from *Pelargonium sidoides*. Pharmaceutical Biology., 45: 645-650.

Mativandlela, S.P., Meyer, J.J., Hussein, A.A., Houghton, P.J., Hamilton, C.J. and Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M. tuberculosis* by extract of South African medicinal plants. Phytotherapy Research., 22: 841-845.

Momtaz, S., Basson, A. and Lall, N., 2008. Inhibitory activities of mushroom tyrosine and DOPA oxidation by plant extracts. South African Journal of Botany., 74: 577-582.

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

Nerya, O., Vaya, J., Musa, R., Israel, S., Ben-Arie, R. and Tamir, S., 2003. Glabrene and isoliquiritigenin as tyrosinase inhibitors from Licorice roots. Journal of Agricultural and Food Chemistry., 51: 1201-1207.

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

Perry, N.B., Aiyaz, M., Kerr, S., Lake, R. and Leach, M.T., 2001. NOESY on Neurotoxins: NMR and conformational assignments of picrotoxins. Phytochemical Analysis., 12: 69-72.

Porter, L.A., 1969. Picrotoxinin and related substances. Chemical Reviews., 67: 441-464.

Poulsen, H.E., Prieme, H. and Loft, S., 1998. Role of oxidative DNA damage in cancer initiation and promotion. European Journal of Cancer Prevention., 7: 9-16.

Sarkhail, P., Rahmanipour, S., Fadyevatan, S., Mohammadirad, A., Dehghan, G., Amin, Shafiee, A. and Abdollahi, M., 2007. Antidiabetic effect of *Phlomis anisodonta*: effects on hepatic cells lipid peroxidation and antioxidant enzymes in experimental

diabetes. Pharmacological Research., 569: 261-266.

Satho, K., 1978. Serum lipid peroxidation in cerebrovascular disorders determined by a new colorimetric method. Clinica Chemica Acta., 90: 37-43.

Sayyah, M., Valizadeh, J. and Kamalinejad, M., 2002. Anticonvulsant activity of the leaf essential oil of *Laurus nobilis* against pentylenetetrazole and maximal electroshock-induced seizures. Phytomedicine., 9: 212-216.

Sutherland, M.D., 1992. New Zealand toxic honey and the actual story. Analytical Proceedings., 29: 112-115.

Sylvestre, M., Pichette, A., Longtin, A., Nagau, F. and Legault, J., 2006. Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. Journal of Ethnopharmacology., 103: 99-102.

Tabanca, N., Ma, G., Pasco, D.S., Bedir, E., Kirimer, N., Baser, K.H., Khan, I.A. and Khan, S.I., 2007. Effect of essential oils and isolated compounds from *Pimpinella* species on NF-B: A target for anti-inflammatory therapy. Phytotheraphy Research., 21: 741-745.

Van Wyk, B.E., Van Heerden, F. and Gericke, N., 1997. Medicinal plants of South Africa. South Africa: Briza Publications.

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

Yong Sun, S., Li, W., Yue, P., Lippman, S.M., Hong, W.K. and Lotan, R., 1999. Mediation of N-(4-Hydoxyphenyl) retinamide-induced apoptosis in human cancer cells by different mechanisms. Cancer Research., 59: 2493-2508.

Zhang, S., Won, Y.K., Ong, C.N. and Shen, H.M., 2005. Anti-cancer potential of sesquiterpene lactones: Bioactivity and molecular mechanisms. Current Medicine

Chemistry., 5: 239-249.

Zhou, H., Tang, Y.H. and Zheng, Y., 2006. A new rat model of acute seizures induced by tutin. Brain Research., 1092: 207-213.