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Antioxidant Capacity and Cytotoxicity of *Aesculus hippocastanum* on Breast Cancer MCF-7 Cells

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ABSTRACT

Aesculus hippocastanum L. is a native tree of Asia. Its leaves, seeds and flowers have long been used in folk medicine and in traditional food ingredients. In this study, the bark, seeds, leaves and flowers as aerial parts of *A. hippocastanum* were extracted in ethanol. The antioxidant capacity of each part was determined for its 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging capacity, microsomal lipid peroxidation inhibition capacity and total phenolic content. Among all the parts examined, the bark extract of *A. hippocastanum* revealed the highest antioxidant capacity with an IC_{50} value of 0.025 mg/mL and 0.014 mg/mL for the inhibition of lipid peroxidation and for the scavenging of DPPH radical, respectively. The bark extract was further examined for its cytotoxic effect on human breast cancer cells (MCF-7) and on healthy cells (3T3) using the MTT method. Cell viability was reduced to 30% upon the addition of 0.5 mg/mL bark extract for both cell lines.

Key words: *Aesculus hippocastanum* L., antioxidant capacity, cytotoxicity, MCF-7

INTRODUCTION

The *Aesculus* species in the family of Hippocastanaceae have found its use as a food supplement. After certain treatments, the seeds of *Aesculus turbinate* B. were used as ingredients in traditional foods and as constituents of herbal drugs in Japan(1).

Extracts of *A. hippocastanum* have been widely used for the treatment of chronic venous insufficiency(2-6). In particular, the seeds are used for the treatment of hemorrhoids, topical ulcers and cancer(7-9). The commonly known active component in the seeds, β-escin (aescin), was suggested to have anti-carcinogenic activity(10). Reparil(11) and Venostatin(12,13) are some of the drugs with aescin as the active ingredient and are known for anti-inflammatory, anti-oedematous(14-16) and anti-exudative activities(17).

Traditionally, the seeds, leaves and bark were used in medicinal and cosmetic preparations(18-20). The bark and leaves were used for their analgesic and astringent effects(21,22). The leaves have also been used in the treatment of arthritis and as a cough remedy(8). *A. hippocastanum* was quite effective against brain trauma and stroke, venous congestion(8,21,23,24) and thrombophlebitis(25).

Many of the beneficial effects of medicinal plants are attributed to their high polyphenolic content. These molecules are proposed to reduce cellular oxidative stress, which plays an important role in the pathogenesis of a number of diseases including cancer and cardiovascular diseases. In this study, the antioxidant activities of ethanolic extracts of *A. hippocastanum* parts (bark, seeds, leaves and flowers) were investigated by the methods of microsomal lipid peroxidation inhibition and DPPH radical scavenging, as well as the total phenolic content. The bark extract exhibited the highest antioxidant capacity. Therefore, further examination for cytotoxic effect was carried out against human breast cancer cells (MCF-7) using healthy cells (3T3) as a control.

MATERIALS AND METHODS

I. *Chemicals*

Ethylene diamine tetraacetic acid (EDTA), glycerol, bovine serum albumin (BSA), copper sulfate ($CuSO_4$),

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sodium potassium tartrate, sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), hydroxy methyl aminomethane (Tris), hydrochloric acid (HCl), iron salt ($Fe_2SO_4 \cdot 7H_2O$), glycerol, thiobarbituric acid (TBA), ortho phosphoric acid (H_3PO_4) alpha-tocopherol, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), gallic acid, quercetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphentyltetrazolium bromide (MTT) and RPMI-1640 were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulphoxide (DMSO) and Ciocalteu`s Folin phenol reagent were purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium with L-glutamine (DMEM/Ham's F12) were purchased from Biochrome AG (Germany). Isopropyl alcohol was purchased from Applichem (Germany). All other chemicals were of analytical grade and obtained from commercial sources at the highest purity available.

Human breast epitheloid adenocarcinoma cell line MCF-7 and 3T3 healthy fibroblastic cells were obtained from HUKUK Culture Collection (Institute of Foot and Mouth Disease, Turkey).

II. *Plant Materials*

Flower, leaf, seed and bark samples from *A. hippocastanum* were collected from the campus forestry of the Middle East Technical University in Ankara, Turkey in their appropriate seasons and the specimens were deposited in the Department of Chemistry. The plant specimens with their localities and the necessary field records were written and enumerated. They were pressed, dried according to herbarium techniques and identified in accordance with the Flora of Turkey[26].

III. *Preparation of Plant Extracts*

The collected plant specimens were air dried and ground using a Waring blender. Five grams of samples (in duplicates) were extracted in ethanol at 50°C for 24 h with a sample-to-solvent ratio of 1:10 (w/v). The crude extracts were filtered and dried under vacuum at 50°C using a rotary evaporator (Heidolph Laborota 4000). The resulting extracts were dissolved in suitable solvents for further studies. All samples were kept at 4°C until use.

IV. *Isolation of Sheep Liver Microsomes*

Microsomes used for lipid peroxidation study were obtained from the liver of slaughtered sheeps. Sheep livers, obtained fresh from the slaughterhouse of Kazan-Ankara, were homogenized with teflon glass attached to a multi-speed drill (Black and Decker, V850) in 20 mM of Tris-HCl (pH 7.4) containing 1.15% KCl (w/v), 1.0 mM of ethylenediaminetetraacetic acid (EDTA) and 1.0 mM of dithiothreitol (DTT), and centrifuged by Sorvall RC 5C Plus with SS-34 rotor at 10,800 ×g for 25 min and Sorvall Combi Plus Ultracentrifuge at 133,573 ×g for 50 min[27]. The protein content of the microsomal suspension was measured by Lowry's method[28].

V. *Lipid Peroxidation Inhibition Activity by Thiobarbituric Acid Test*

Microsomal lipid peroxidation was evaluated by the thiobarbituric acid (TBA) test using the method of Ohkawa, Ohishi & Yagi[29] with some modifications[30]. Lipid peroxidation was induced with 100 mM of ferrous sulphate solution ($Fe_2SO_4 \cdot 7H_2O$), in 0.2 M of Tris-HCl buffer at pH of 7.4. Malonedialdehyde (MDA), the end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) in acidic medium upon boiling and the resulting MDA-TBA adduct absorbed at 532 nm, which was monitored by a UV-VIS spectrophotometer (Cary 50 Bio).

The MDA values were calculated using the extinction coefficient $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmol/mg of protein. Alpha-tocopherol was used as the positive control. Microsomal lipid peroxidation inhibition was expressed as lipid peroxidation inhibition in percentage or as fifty percent inhibitory concentration IC_{50}.

Lipid peroxidation inhibition (%) $= [(OD^{532}_{control} - OD^{532}_{sample}) / OD^{532}_{control}] \times 100$ where $OD^{532}_{control}$ is the absorbance of control with ethanol and OD^{532}_{sample} is the absorbance of the extracts dissolved in ethanol. IC_{50} values were calculated after constructing the lipid peroxidation inhibition (%) *versus* log (extract concentration) curve and using *forecast function* of Microsoft Excel.

VI. *DPPH Radical Scavenging Activity*

A free radical forms a stable molecule by accepting an electron or hydrogen radical. Depletion of the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) in the solution reveals the radical scavenging capacity of the antioxidant molecule. This event was monitored by a decrease in absorption at 517 nm after 5, 10 and 30 min of reaction. One point four milliliters of 0.05 mg/mL of DPPH solution in ethanol and 0.1 mL of each extract were mixed and the reaction mixture was shaken vigorously. After 5 min of optimized reaction period, the depletion of DPPH was followed by a decrease in absorbance at 517 nm[31]. The radical scavenging activity in percentage was obtained from the following equation:

Radical scavenging activity (%) $= [(OD^{517}_{control} - OD^{517}_{sample}) / OD^{517}_{control}] \times 100$

The radical scavenging capacity was also expressed as the amount of scavenging material necessary to decrease the initial DPPH concentration by 50% (IC_{50}) and calculated by plotting radical scavenging capacity (%) *versus* log [extract concentration] and using the built-in function, forecast, in Microsoft Excel.

VII. *Determination of the Total Amount of Polyphenolics*

The total amount of polyphenolics found in the extracts were determined through the method of Singleton and Rossi[32]. A volume of 0.1 mL of each extract or gallic acid

solution was mixed with 2.0 mL of 2% Na_2CO_3 aqueous solution. After 3 min, 0.1 mL of 50% Folin-Ciocalteu's phenol reagent was added. Absorbance was monitored at 750 nm and the results were expressed as Gallic Acid Equivalents (GAE), that is, milligrams of gallic acid-like phenolic compounds per milligram of extract.

VIII. *Cell Culture and Treatments*

Cell cultures were obtained from HUKUK Culture Collection (Institute of Foot and Mouth Disease, Turkey) and they were allowed to grow in laminar flow chambers (Class II Holten, Denmark). HUKUK/3T3 and MCF-7 human breast epitheloid adenocarcinoma cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, containing 10% fetal bovine serum (FBS), 1% sodium carbonate and 10 μg/mL of gentamicin. The cells were passaged for 3 subcultures and seeded in T25 cell culture flasks (25 cm^2) at 37°C under a humidified 5% carbon dioxide (Heracell incubator, Germany). A suspension of cells was prepared in DMEM medium (2.5×10^4 cells/mL) and a 100 mL aliquats were added into each well of 96-well culture plates. After 24 h of incubation at 37°C, the cells were examined under an inverted cell culture microscope (Olympus, CK40, Japan) for growth and sterility. The medium was aspirated and the cells were treated with 100 mL/well of different concentrations of *A. hippocastanum* bark ethanol extract (0.01-0.5 mg/mL). Dry bark extracts were dissolved in DMSO before their application on cell cultures. The effect of the concentration of DMSO was examined as the negative control. The control data exhibited no effect on proliferation of either cell types, up to 1% of DMSO concentration. As a result, 1% DMSO concentration was utilized for the dissolution of extracts before the application on the cells.

IX. *MTT Cellular Cytotoxicity Bioassay*

The tetrazolium salt of MTT is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple reaction product and the assay was performed as described in the method of Mosman[33]. MTT was prepared in phenol red contained RPMI-1640 with a final concentration of 5 mg/mL. The media of cells which were incubated for 24 h in 96-well plates were decanted and 100 mL of DMEM medium and 13 mL of MTT solution were added to each well. After 4 h of incubation at 37°C in a humidified 5% CO_2 in the dark, the supernatant was removed and replaced with 100 mL/well of isopropyl alcohol. The absorbance value of each well was monitored at 570 nm by a microplate reader device (Molecular Device Corp, USA) and the results were compared with that of controls. The results were determined by the average of three independent experiments. The cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = [OD^{570}_{\text{treated cells}} / OD^{570}_{\text{control cells}}] \times 100$$

where $OD^{570}_{\text{treated cells}}$ is the absorbance for the cells treated with different extract concentrations and $OD^{570}_{\text{control cells}}$ is the absorbance for the cells treated with 1% DMSO concentration at 570 nm.

X. *Statistical Analysis*

All results are expressed as mean±standard deviation (SD). The statistical Q-test was used for deciding whether a suspected result should be retained or rejected.

Significant differences between the two sample means were checked by the of *t test statistic* and compared with the *critical value of t* obtained from statistical tables at 95% confidence level.

RESULTS

I. *Yield of A. hippocastanum Extracts*

Each 5 g of air-dried flower, seed, leaf and bark sample were extracted in 50 mL of ethanol at 50°C using a rotary evaporator. Extraction yields were in the range of 1.04-6.25% (w/w). The results are shown in Table 1.

Sample collections for the parts of *A. hippocastanum* were realized for two consecutive years during appropriate seasons. Experimentation was arranged with at least two 5-g samples and extraction was carried out in duplicate.

II. *Lipid Peroxidation Inhibition Activity of A. hippocastanum Extracts*

Lipid peroxidation inhibition was used as one of the methods to evaluate antioxidant capacity. Lipid peroxidation inhibition capacity of each extract was calculated in percentage and the results are shown in Figure 1. The final extract concentrations were prepared in the range of 0.05 mg/mL to 5 mg/mL during the lipid peroxidation inhibition experiments.

Fifty percent inhibition of lipid peroxidation (IC_{50}) was calculated for each of the extract in mg/mL to compare their antioxidant capacities, and the results are shown in Table 2.

The results indicated that the bark extract was a significantly more effective inhibitor of lipid peroxidation ($p < 0.05$), with an IC_{50} value of 0.025 mg/mL, compared to the seeds, leaves, flowers and even a well-known antioxidant standart, alpha-tocopherol (IC_{50} = 0.23 mg/mL).

Table 1. Ethanol extraction yields from the parts of A. hippocastanum

Parts	Yield of extract (mg)	Yield (%, w/w)
Bark	51.8 ± 14.1	1.04 ± 0.28
Seeds	103.8 ± 4.7	2.08 ± 0.09
Leaves	143.5 ± 31.1	2.87 ± 0.62
Flowers	312.5 ± 10.7	6.25 ± 0.22

III. *DPPH Radical Scavenging Activity of A. hippocastanum Extracts*

The free radical scavenging activity of *A. hippocastanum* part extracts were evaluated by using the DPPH radical scavenging method. The results were plotted as the radical scavenging activity (%) *versus* concentration of extracts (mg/mL)(Figure 2**)** and the representative IC_{50} values are shown in Table 2.

The bark extract of *A. hippocastanum* was found to be a significantly more effective ($p < 0.05$) radical scavenger, with an IC_{50} value of 0.014 mg/mL, compared to all the other parts. The next effective extract was the seed, with an IC_{50} value of 0.168 mg/mL.

IV. *Total Phenolic Contents of A. hippocastanum Extracts*

The total polyphenolic amount of each extract was determined as the effective bioactive constituents and the results are expressed in milligrams of gallic acid equivalents (GAE) per milligram of extract in Table 2. The extracts of leaves and bark revealed the highest amount of phenolics, 0.075 and 0.061 mg GAE/mg extract, respectively. The difference between the two values was insignificant. The flower extract was also considerably high in phenolic content (0.045 mg GAE/mg extract).

V. *Effect of Bark Extract on Cell 3T3 Healthy Fibroblasts and MCF-7 Breast Cancer Cell Lines*

Cytotoxicity studies were carried out using only the bark extract justified by its remarkable antioxidative properties. The effects of *A. hippocastanum* bark extracts were examined by monitoring the proliferation of breast cancer (MCF-7) and healthy fibroblast (3T3) cells in the presence of MTT solution.

At minimal concentrations of bark extract (0.01 mg/mL), the viability of MCF-7 cancer cells decreased to 90%, while that of 3T3 healthy fibroblast cells increased to 120%. Moreover, a 0.1 mg/mL of bark extract addition exerted a decrease on the viabilities of 3T3 and MCF-7 cells by 55 and 70%, respectively. Finally, about 30% of cell viabilities were accomplished upon the addition of 0.5 mg/mL of bark extract for both cell lines, as shown in Figure 3.

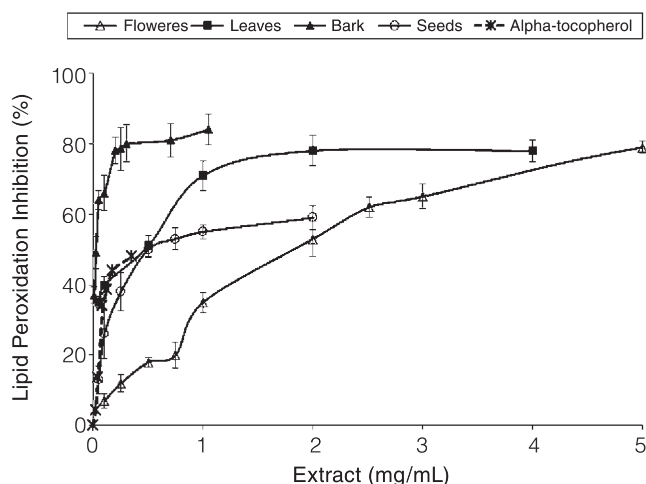

Figure 1. Lipid peroxidation inhibition of extracts of *A. hippocastanum* flowers, leaves, seeds, bark and alpha-tocopherol as a control. 1.0 mg/mL of microsomal suspension was used as the lipid source. Each data was obtained from 6 to 9 independent measurements.

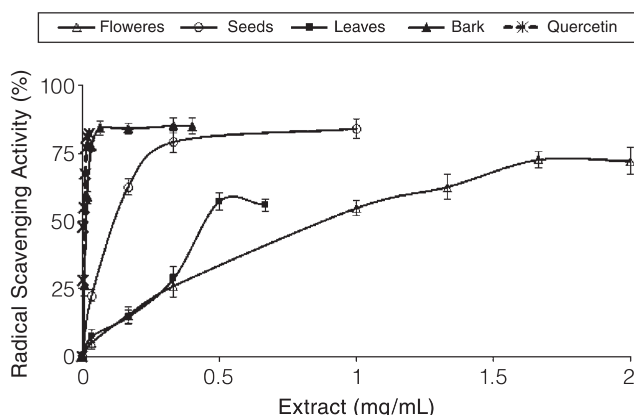

Figure 2. Free radical scavenging activity of *A. hippocastanum* part extracts. Each data was obtained from 6 to 9 independent measurements.

Table 2. Antioxidant capacities and the amount of total phenolic content in *A. hippocastanum* ethanol extracts.

A. HIPPOCASTANUM PARTS	LIPID PEROXIDATION IC_{50} (mg/mL)	DPPH RADICAL SCAVENGING IC_{50} (mg/mL)	TOTAL PHENOLIC CONTENT GAE (mg/mg extract)
Bark	0.025 ± 0.005*	0.014 ± 0.001*	0.061 ± 0.013
Seeds	0.886 ± 0.029	0.168 ± 0.074	0.021 ± 0.004
Leaves	0.666 ± 0.058	0.513 ± 0.017	0.075 ± 0.016
Flowers	3.103 ± 0.008	1.101 ± 0.061	0.045 ± 0.008
Alpha-tocopherol	0.230 ± 0.010	ND	NA
Quercetin	ND	0.009 ± 0.002	NA

ND: not determined.
NA: not applicable
*Significantly different data ($p < 0.05$). Statistical *t*-test was used to compare differences between means of different plant parts.

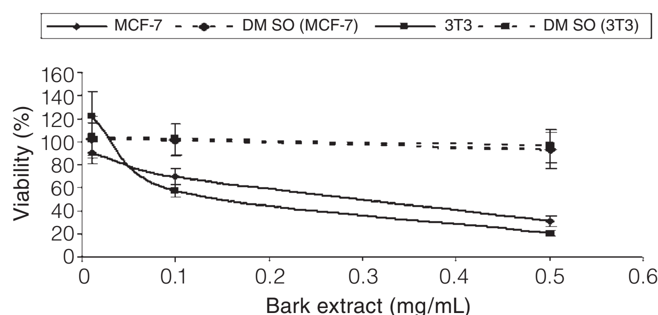

Figure 3. Effect of ethanol bark extract concentrations (0.01-0.5 mg/mL) on cell 3T3 healthy fibroblasts and MCF-7 breast cancer cell lines.

DISCUSSION

The presence of phenolic compounds(21,34-36) in *A. hippocastanum* had been reported in literature. The antioxidant activity of the seeds was attributed to the presence of flavonoids, as indicated in the report of Kapusta *et al*.(35). In 1999, Hübner, Wray and Nahrstedt demonstrated that the seeds of *A. hippocastanum* contained glycoside and acylated forms of quercetin and kaempferol(35,36). In numerous studies, *A. hippocastanum*, particularly the seeds(36-39), and its most common bioactive constituent escin(39-42) were investigated thoroughly. Escin was found to regulate inflammation mediators through the enhancement of endogenous antioxidative capacity(42), and was also known to exhibit hepatoprotective activity by decreasing lipid peroxidation(37,41). Incidentally, beta-escin was reported to be a natural inhibitor of cell proliferation and an inducer of apoptosis on acute myeloid leukemia (HL-60) cells(40). Similarly, beta-escin displayed antitumor activity on hepatocellular carcinoma *in vitro* and *in vivo*(41). Nevertheless, the scientific bioactivity search for the rest of this plant was, for the greatest part, left unexplored. On that account, all the aerial parts of *A. hippocastanum* besides the seeds, were investigated for their antioxidant activities. Particularly, lipid peroxidation inhibitory capacities were investigated by using sheep liver microsomes mimicking the physiological biomembranes for the first time in this study. The seeds were found to contain the lowest amount of phenolics, coinciding with its major constituent saponin(39) which was not phenolic in structure. The flowers were relatively low in phenolics and antioxidant capacities. Additionally, the leaves revealed a high amount of polyphenolic content, but it was relatively poor in antioxidant capacity. Consequentially, considering all the studied parts, the bark exhibited an outstanding antioxidant activity, consistent with its high phenolic content. In literature, plants were inquired as a source of cancer preventive characteristics in regard to their polyphenolic contents(40-43). This is the first report on the antiproliferative effects of the ethanol extract of *A. hippocastanum* bark on the human breast epitheloid adenocarcinoma (MCF-7) cells.

CONCLUSIONS

To the best of our knowledge, the aerial parts of *A. hippocastanum* were studied, for the first time, regarding their antioxidant capacities. For all the parts of *A. hippocastanum* extracts were found to have antioxidant properties. The bark extract particularly, revealed the highest antioxidant capacity; it was also effective to decrease the viability of MCF-7 breast cancer cells by about 70%. In the preliminary process of the natural drug discovery, some constituents of the ethanol extract of *A. hippocastanum* bark should also be considered for cytotoxicity studies of different cancer cell lines. Presently, these potentially active constituents of the bark extract are also under investigation in our laboratory.

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