Anticancer, antioxidant properties and phenolic, flavonoid composition of *Heracleum platytaenium* plant methanolic extracts

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ABSTRACT: *Heracleum platytaenium* is an edible herb and strongly aromatic plant that is endemic to Turkey. Although, a number of studies have been conducted on the components and composition of essential oils of the different parts of this species, its antioxidant, antiproliferative and / or cytotoxic properties have never been explored. The present study aimed to evaluate total phenolic content (TPC), total flavonoid content (TFC), the antioxidant activity and anticancer properties of the methanolic extracts of *H. platytaenium*. The results showed that the total phenolic and flavonoid contents were found to be higher in the methanolic extracts from the flowers of the plant. The methanolic flower extract of *H. platytaenium* scavenged about 86% and stem extract of *H. platytaenium* scavenged about 83% of free DPPH radicals. The best inhibitory profile for GST was observed with the methanolic extract of the plant from flowers. In addition, the methanol extract of *H. platytaenium* from the stem increased GPx activity with the 1.25 mg/mg plant extract. The maximum dose of the flower and stem extracts of the plant inhibited 10% and 8% CAT activity. Then, MTT assay was used for the study of the cytotoxic effect of the plant extracts. Both methanolic flower and stem extract of *H. platytaenium* showed moderate anticancer effect on T47-D cell lines, but no cytotoxic effect was observed on MDA-MB-231 cells. In conclusion, methanolic extracts of *H. platytaenium* flower and stem showed apparent antioxidant and cytotoxic effects leading the way for their potential use as food additives to help to fight against the growth and spreading of cancerous cells. However, for a definitive conclusion, further studies on other cell lines as well as animal models and subsequent clinical studies are warranted.

KEYWORDS: Antioxidant enzymes; breast cancer cell line; cytotoxicity; flavonoids; *Heracleum platytaenium*; phenolics.

1. INTRODUCTION

The genus *Heracleum*, one of the broader classes of the Apiaceae family, is represented by approximately 125 species worldwide and are known to contain flavonoids, coumarins and triterpene saponins [1]. *Heracleum* is represented by 109 species in Asia with 23 species found in Turkey among which 9 are endemic [2]. Some *Heracleum* species are commonly used as painkillers, antipyretics, diaphoretic, antiseptics, carminatives (digestive gut), digestive facilitators [3]. Apart from that, it is also consumed as a flavoring agent and spice in meals [4].

*H. platytaenium* is a plant with a 1-2 m length, sharp aromatic odor that blooms once a year. The body of the plant is at least 2 cm in diameter and has a deep grooved, almost regular long and soft pile shape. The flowers are white in color. The fruits are wide, inverted egg and kidney shaped and average 8-14, 6-11 mm in size. *H. platytaenium* is endemic to Turkey and it is distributed in the Kaz mountains and also in North West and Central Anatolia. In Turkish folk medicine *H. platytaenium* is known as ‘Baldırgan’ or ‘Tavşancık otu’ [5]. Various parts of this plant produce essential oils (mainly aliphatic esters and monoterpenes) with a wide range of biological activity [1]. In the last few years, new compounds such as p-cymene (33.9%), terpinolene (14.3%), γ- terpinene (7.1%), elemicine (3.1%) and myristicine (2.9%) as the major constituents have been isolated from the roots of the *H. platytaenium* plant [6]. It was demonstrated that terpinolene has antioxidant activity and it is a potent antiproliferative agent for brain tumour cells [7]. In addition, myristicine has been implicated as responsible for anticancer activities of some medicinal plants [8].
Cancer is one of the most important health problems seen all over the world. Due to the increase in life span and changes in lifestyle, there has been an increase in the number of cases all over the world in recent years. According to the statistics of the World Health Organization, about 12 million people were diagnosed with cancer in 2008 and this number is expected to increase to at least 26 million by 2030 [9].

Breast cancer is the most common cancer in women worldwide, which represents one in four of all cancers in women [10, 11]. Breast cancer incidence and mortality rates have been increasing rapidly in Turkey as in the most of the developing countries. The studies show the frequency of breast cancer has had a two-fold increase in Turkey over the last 20 years [12]. Chemotherapy is one of the strategies commonly used in the treatment of breast cancer. However, this therapy causes side effects such as nausea, bone marrow failure, and the development of multiple drug resistance (MDR) [13, 14]. It is necessary to develop new anticancer natural drug leads with fewer side effects.

Under normal conditions, there is a balance between activities and intracellular levels of antioxidants [15]. During cancer therapy, chemotherapeutic agents generate free radicals that facilitate the apoptosis of cancer cells. Studies have shown that antioxidant enzymes can reduce free radicals and reduce the effectiveness of therapeutic agents [16, 17]. Thus, the inhibition of antioxidant enzymes may improve the treatment of proliferative diseases. Plant-derived compounds play vital roles in disease prevention and are important sources of novel pharmacologically active compounds that serve to discover new naturally occurring enzyme inhibitors [18].

The aim of this study was to investigate the chemical and biological activities of *H. platytaenium* stem and flower extracts. For this purpose *H. platytaenium* extracts were evaluated for phenolic chemical composition, flavonoid contents and antioxidant activity. In addition, anticancer properties of the extracts were investigated on estrogen receptor positive (T47D) and estrogen receptor negative (MDA-MB-231) breast cancer cell lines.

2. RESULTS AND DISCUSSION

Medicinal plants are important sources of medical agents for thousands of years. In recent years, there has been a growing interest in the alternative therapies and the therapeutic use of natural products and their derivatives. This study was designed to study the phenolic chemical composition, flavonoid contents and antioxidant activity of methanolic extract of *H. platytaenium* from flower and stem. In addition, the anticancer properties of the extracts were also investigated in breast cancer cell lines.

Phenolics and flavonoids, a group of natural substances with variable phenolic structures, are broadly found in the plant kingdom [19]. These compounds have been reported to have anticancer, antioxidant and multidrug resistance reversal activities and reduce the risk of metabolic syndromes [20, 21]. This study is the first to reveal the profiles of phenolic acids and flavonoids of the *H. platytaenium* plant. In this study, the flower extract of *H. platytaenium* has shown to have higher amounts of total phenolic and flavonoid compounds than the stem extract of the plant. Phenolics and flavonoids were reported to play an important role in antioxidant activity, and higher phenolic contents led to stronger antioxidant activity.

The present study was performed to evaluate total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity and anticancer properties of methanolic extract from the stem and flower parts of *Heracleum platytaenium*. Total phenolics were determined using the Folin Ciocalteu method. The results showed that the flower extract of *H. platytaenium* was richer in phenolic compounds (36.89 ±0.027 mg GAE/100g dried sample) than that of the stem extract of the plant (18.38 ±0.0048 of GAE/100g dried sample). The determination of total flavonoids was performed according to the aluminum chloride colorimetric method based on quercetin standard curve \( y = 0.0429x +0.153, R^2 =0.998 \). The highest TFC was found in the methanol extracts from the flower part of *H. platytaenium*, which was 20.26 ±0.039 whereas the TFC of the stem extract, was 7.07 ± 0.0005 mg QE/100g of dried sample.

Glutathione-S-transferase (GST) is an important phase II enzyme that plays a critical role in cellular enzymatic detoxification processes [18] present in every cell and in every living species. GSTs catalyze the reactions in which reduced glutathione is conjugated to toxic oxidizing compounds [22]. These compounds are produced either as a result of a normal cellular activity or as an exposure to endogenous molecules, carcinogens, drugs and other environmental pollutants in order to detoxify the cells by reducing their ability to react with cellular macromolecules. It has been described that the expression of GST in cancer cells is representative of a more aggressive and resistant phenotype [23]. Therefore, the inhibition of GST activities is important in preventing drug resistance of cancer cells against chemotherapeutic agents. In this study, the final concentration of plant extracts within concentration range of 0.312-10 mg/mL was used to determine the
inhibition percentage of GST activity and IC₅₀ values. It was observed that at 10mg/mL, the methanolic extract of *H. platytaenium* flower inhibits GST activity by 47% and methanolic extract of *H. platytaenium* stem inhibits GST activity by 38% with IC₅₀ values 0.1021±0.0072 mg/mL, 0.0572±0.0013 mg/mL, respectively (Figure 1). For this study, it has been concluded that both the flower and stem extract of *H. platytaenium* showed some inhibitory effect on GST activity.

![Figure 1](image1.png)

Figure 1. The inhibitory effect of methanol extracts of *Heracleum platytaenium* from flower and stem on GST activity with IC₅₀ values 1021±0.0072 mg/mL and 0.0572±0.0013-mg/mL.

Glutathione peroxidase enzyme (GPx) is a cytosolic enzyme that eliminates hydrogen peroxides by using reduced glutathione. Thus, GPx plays a significant role in protecting cells against oxidative damage. Several studies have reported the association of dysfunctional GPx with higher cancer risk [24, 25]. The flower and stem extracts of *H. platytaenium* plant were used at concentrations varying from 0.312 to 10 mg/mL to calculate the percentage of GPx activity. Results showed that at 1.25 mg/mL dose, the methanolic flower extract activates 14% and the methanolic stem extract activates 33% of GPx enzyme activities compared to the control (Figure 2).

![Figure 2](image2.png)

Figure 2. The effect of *Heracleum platytaenium* methanol extracts from flower and stem on the glutathione peroxidase (GPx) activity.

Catalase is an antioxidant enzyme that plays a major role by detoxifying H₂O₂. Catalase expression is also altered in cancer cells and it can constitute a future therapeutic target in the context of cancer [26]. The result of catalase specific activities from methanolic flower and stem extracts of *H. platytaenium* was shown in Figure 3. At a concentration of 10 mg/mL, CAT activity was inhibited by 10% in the flower extract and by 8% in the stem extract of the plant (Figure 4). It can be concluded that the methanolic flower and stem extracts of *H. platytaenium* have a moderate inhibitory effect on CAT activity.
Figure 3. Effect of methanol extracts of *Heracleum platytaenium* from flower and stem sections on CAT activity.

Figure 4. Effect on free radical scavenging activity of methanolic extracts *Heracleum platytaenium* from flower and stem. The data represents mean values (±SD (n=3), P<0.05)

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is an oxidative assay, which has been widely used to quantify the free-radical scavenging ability of samples [27]. DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The bleaching of the DPPH solution increases linearly with increasing amounts of extract in a given volume. DPPH results are presented as DPPH radical scavenging activity of plant extracts from different plant sections (µg/mL) according to inhibition curves and IC$_{50}$ values. We measured the percentage of antioxidant activity of methanolic extracts from different parts of the plant using DPPH). Figure 1 showed that the methanolic flower extract of *H. platytaenium* presents higher radical scavenging activity compared to the stem extract of the plant (Flower IC$_{50}$=0.336±0.0068, Stem IC$_{50}$= 0.876±0.007µg/mL). Erkan et al. [28] reported that there is a close correlation between radical scavenging activity, total phenolic content and total flavonoid content of extract from various natural sources. Our results showed that the DPPH antioxidant activity of *H. platytaenium* increased 86% for flower and 83% for stem extract of the plant that are consistent with Erkan et al findings [28].

*Heracleum* genus typically contains coumarin compounds with many biological and pharmacological activities. Coumarins possess a variety of biological properties, such as antioxidant, and enzyme inhibitory activity [1]. So, it might be responsible for bioactivity of the different parts of the methanolic extracts of the *H. platytaenium* plant.
Until now, the scientific literature does not report about the anti-proliferative activity of the methanolic extracts of the *H. platytaenium* plant. Thus, we evaluated the cytotoxic effects of the methanolic extracts from different parts of *H. platytaenium* on estrogen receptor positive, T47D, and estrogen receptor negative, MDA-MB-231, breast cancer cell lines. To investigate the growth inhibitory effects of the methanol extract of *H. platytaenium*, human breast cancer cell lines (T47D, MDA-MB 231) were incubated with final concentrations (8-500 µg/mL) of the methanolic extract of the plant. The potential toxic effect of these extracts was assessed using the MTT assay. The inhibitory activity was shown as IC\textsubscript{50} values. As shown in Table 1, methanol flower extract of *H. platytaenium* inhibited growth of T47D human breast cancer cell lines with an IC\textsubscript{50} value of 1.461±0.34 mg/mL after 24 hours and an IC\textsubscript{50} value of 0.443±0.127 mg/mL after 48 hours. The growth inhibitory effect of methanol stem extract of the plant on T47D human breast cancer cell line was measured as 1.318±0.350 mg/mL IC\textsubscript{50} after 24 hours incubation and 0.582±0.177 mg/mL IC\textsubscript{50} after 48 hours incubation. No significant growth inhibitory effect of extracts was observed on MDA-MB 231 cell lines. The lower the IC\textsubscript{50} value and the higher the anticancer effect suggests that the methanolic flower extract of *H. platytaenium* has better anticancer effect on T47D human cancer cell line with 0.443 mg/mL IC\textsubscript{50} value for 48 hours (Table 1) than the stem extract.

We concluded that estrogen receptor positive, T47D, cell lines were significantly sensitive treatment of the to methanolic extract *H. platytaenium* plant in a time and dose dependent manner. Interestingly, we did not observe any anti-proliferative effect on MDA-MB-231. Estrogen receptor (ER) expression is the main indicator of potential responses to endocrine therapy of human breast cancers [29]. This study highlights a potential use for *H. platytaenium* plant extracts as food additives to help preventing the growth and the spread of estrogen receptor positive breast cancer. The molecular mechanisms underlying the anti-proliferative activity of *H. platytaenium* plant extracts on estrogen receptor positive cells have not been very clear. So, more detailed studies with other cell lines and animal models are required to explore the clinical applicability of *H. platytaenium*.

| Table 1. The anti-proliferative effect of *Heracleum platytaenium* extracts against T47D breast cancer cell lines at different time points (24 hours and 48 hours) |
|-----------------|------------------|------------------|
|                 | Flower           | Stem             |
| 24 h            | 1.461±0.34       | 1.318±0.350      |
| 48 h            | 0.443±0.127      | 0.582±0.177      |

3. CONCLUSION

In conclusion, the results of this study show that *Heracleum platytaenium* plant is rich with phenolic compounds and flavonoids, and it has a cytotoxic effect in ER+ breast cancer cell lines (T47D) in a dose and time dependent manner. Total phenolic and flavonoid contents and antioxidant properties are highest for methanolic flower extract of the plant. There is a correlation between antioxidant activity, TPC and TFC. *H. platytaenium* plant is a natural antioxidant source that can prevent many diseases and potentially be used in the food and pharmaceutical industries.

4. MATERNALS AND METHODS

4.1. Chemicals

Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), sodium azide (Na\textsubscript{3}N), and 4-aminoantipyrine (4-AP) were purchased from (Acros,USA). Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, reduced glutathione (GSH), glutathione reductase (GR), horse raddish peroxidase (HRP), catalase (CAT), gallic acid and quercetin hydrate were supplied from Sigma Chemical Company (Sigma Aldrich, Germany). Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was acquired from Gerbu (Germany). All other chemicals used were obtained from Sigma-Aldrich (Germany).

4.2. Collection and identification of plant material

Dr. Mustafa Alkan collected plant materials from Trabzon Akçabaat, Turkey in June-July 2010. Assistant Professor Dr. Bedrettin Selvi from the Department of Biology at Tokat Gaziosmanpaşa University, botanically
identified the plant samples. A voucher specimen has been deposited at the Tokat Gaziosmanpaşa University Herbarium of Faculty of Science (GOPU-3019).

4.3. Plant material extraction and isolation

The plant parts were dried in shade and crushed into very small pieces with mortar and pestle. Then, these samples were extracted with methanol for 24 hours at room temperature with sample to solvent ratio of 1:10 (w/v). The plant extracts were concentrated with a rotary evaporator at 40°C under reduced pressure (337 mbar) until dryness. The obtained product was dissolved in DMSO and stored in a dark and cool (-20°C) place to be used for further analysis.

3.4. Determination of total phenolic contents

Total phenolic contents in the extracts were determined by using the Folin Ciocalteu method with some modifications [30]. According to the method, the TPCs of extracts were calculated using the equation obtained from the standard curve of gallic acid (y = 0.0749x + 0.0866, R²= 0.9898). Gallic acid was used as reference (0.05, 0.1, 0.15 and 0.2 mg/mL) and results were expressed as gallic acid equivalents in milligrams per 100 g dried sample (mg GAE/100g). The absorbance of mixture was measured at 765 nm using a spectrophotometer.

3.5. Total flavonoids content

The amount of total flavonoid in H. platytaenium was determined by aluminum chloride colorimetric method. For this method; ethanol (95 %), aluminum chloride (10 %), 1M sodium acetate and DMSO were used as described previously [31]. 0.5 mL of each plant extract was mixed separately with 1.5 mL of ethanol (95 %), 0.1 mL of aluminum chloride (10 %), 0.1mL of 1M sodium acetate and 2.8 mL of DMSO. Then, samples were incubated for 30 min in the dark at room temperature the absorbance of the reaction mixture was read at 415 nm with a spectrophotometer. The standard curve used was prepared with 0.025, 0.05, 0.1, 0.15 and 0.2 mg/mL of quercetin (Q) in DMSO. The total flavonoid content of extracts was expressed as milligrams of quercetin equivalents per 100 g dried sample (mg QE/100g dried sample).

3.6. Determination of anti-oxidant activity by DPPH assay

Radical scavenging activities of H. platytaenium methanolic extract were determined by using the 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, according to the method reported by of Sharma and Bhat [32]. DPPH antioxidant assay revisited with some modifications. The antioxidant activities of the plant extract were measured at 517nm absorbance using UV-vis spectrophotometer while Gallic Acid (GA) was employed as positive control. Anti-oxidant capacity of each sample was expressed as the half maximal inhibitory concentration (IC50) value and calculated from the dose-response inhibition curve.

3.7. Preparation of cytosol from bovine liver

A bovine liver was obtained immediately after butchering from a slaughterhouse in Kazan, Ankara, Turkey. 20-25 g of liver tissue was homogenized in 10mM potassium phosphate buffer (pH 7.0), containing 0.15M KCl, 1mM EDTA, and 1mM of DTT, using homogenizer and centrifuged at 10,000 × g for 20 min. The supernatant was filtered through cheesecloth and filtrate was centrifuged at 30,000 × g for 60 min. The collected supernatants were filtered again and the resultant filtrate was referred as cytosol. The prepared homogenates were kept in -80°C for future analysis.

3.8. Measurement of glutathione-s-transferase (GST) activity

GST activities were measured with UV-vis spectrophotometer against the substrates 1-chloro-2,4- dinitrobenzene (CDNB) by monitoring the thioether formation at 340 nm [33]. The assay mixture consisted of plant extract solution (the final concentration was in the range of 0.312 to 10 mg/mL), 100mM potassium phosphate buffer (pH 6.5) with 2.4mM CDNB, and 3.2mM GSH; and bovine liver cytosolic fractions at a final concentration of 1.996 mg protein/mL were prepared and used as the enzyme source to measure GST activity. The conjugation of GSH and CDNB was monitored in 1 mL total volume at a wavelength 340 nm for 2 min. Initial rate of enzymatic reactions were defined as the nanomoles of the conjugation product of GSH and reported as nmole/ minute/ mL.

3.9. Measurement of glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was measured according to an established procedure [34]. The activity of the enzyme was defined as the amount of enzyme that converts 1 mmol of NADPH per minute in
1 mL, and expressed as U/mg of total protein. The assay mixture consisted of plant extract solution (0.312 to 10 mg/mL), 0.4 U/mL glutathione reductase, 0.2mM NADPH, 1.6mM GSH, 0.07mM Cumyl hydroperoxide (Cum-OOH), and 50mM Tris-HCl with pH 8.0. The contents were mixed well and incubated at room temperature for 2 min. The reaction was initiated by adding bovine liver cytosolic fractions used as the GPx enzyme source and the change in the absorbance was recorded at 340 nm for 5 min.

3.10. Measurement of catalase (CAT) activity

Catalase activity was measured colorimetric at 520 nm using purified CAT (20 unit/mL) from the bovine liver (Sigma) as an enzyme source and plant extracts (0.312 to 10 mg/mL) against 10mM hydrogen peroxide substrate, in 50mM potassium phosphate buffer (pH 7.0) according to the previously described method [35]. The principle of the method is based on the measure of a decrease in absorbance of the plant extract by the induced decomposition of H₂O₂ in the presence of catalase enzyme. The calibration curve was constructed in the range of 9.61–307.6 mM hydrogen peroxide and CAT inhibition was determined by monitoring a red quinoneimine dye at 520 nm corresponding to the remaining hydrogen peroxide.

2.11. Cell culture conditions

MDA-MB-231 and T47D cells were grown in DMEM (Invitrogen) and incubated at 37°C in an atmosphere of 5% CO₂ and 95% air. Each medium contained 10% fetal bovine serum (Invitrogen) supplemented with 50 units/mL of penicillin (Invitrogen) and 50 µg/mL of streptomycin (Invitrogen).

3.12. Cytotoxicity assay

Colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used for measuring toxicity as previously described [36]. In this assay, the human breast cancer cell lines (MDA-MB-231 and T47D). All the treatments were done using 2×10⁴ cells per well in 96 wells plate and incubated overnight. Then, cells were treated with the plant extract samples (500, 250, 125, 62.5, 31.25, 15.62, 7.81 µg/mL) for 24 and 48 h at 37 °C under 5% CO₂. After incubation, 10 µL MTT (5 mg/mL dissolved in phosphate-buffered saline) was added to each well and plate was incubated for 4 h. MTT containing media were removed, and the purple formazan crystals were dissolved in 100 µl dimethyl sulfoxide DMSO. The absorbance was recorded on a microplate reader at the wavelength of 570 nm.

3.13. Statistical analysis

Student’s t-test was used to analyze inter-group differences. The experiments were performed in at least triplicate, and data are represented as the mean ± standard deviation (SD). A p-value of less than 0.05 was considered significant.

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