ORAL PRESENTATION

Assessment of Antioxidant activity and Antiproliferative Effects of *Hypericum scabrum* L. on Human Colon Cancer HT-29 Cells

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Abstract

In this study, antioxidant activity and antiproliferative effects of various extracts (hexane, water and methanol) from the aerial parts of *Hypericum scabrum* L. (*H. scabrum*) were investigated. Total phenolic content of the extracts was determined by Folin Ciocalteu assay. Radical scavenging and metal reducing capacity of the extracts were determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. The antiproliferative power of the extracts were evaluated on human colon cancer (HT-29) and healthy continuous (GF-1) cell lines via 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) assay. Folin Ciocalteu results showed that the methanol extract (ME) was the richest in phenolic content (13.20 mg GAE/g extract), followed by water extract (WE) and hexane extract (HE). ME also exhibited much higher DPPH radical scavenging (81.57 µg/mL) and iron ion reducing capacity (48.06 mgTE/g extract) than WE and HE. XTT results indicated that we and ME and WE showed dose-dependent antiproliferative effects between 100 and 250 µg/mL on HT-29 cells, while they did not exhibit any cytotoxicity on GF-1 cells up to 500 µg/mL. In conclusion, all these preliminary results assume that *H. scabrum* creates the impression that it can be considered as a potential source in the treatment of colon cancer with its rich phenolic content, moderate antioxidant capacity, and remarkable antiproliferative power.

Keywords: *Hypericum scabrum* L., Phenolic content, Antioxidant activity, Cytotoxicity

INTRODUCTION

Colorectal cancer is one of the most frequently diagnosed cancer and the second leading cause of cancer death in the world (Siegel, R., et al., 2012; Lu X and Kang Y., 2007). Multiple factors are involved in the development of colorectal cancer, such as family history, lack of physical activity, excessive alcohol consumption, old age, and high-fat diets. The standard treatment for cancer, including colon cancer, is generally based on cytotoxic drugs, radiotherapy, chemotherapy, and surgery (Siegel, R., et al., 2012; Gurung RL, et al., 2010; Crohns, M., et al., 2009). Additionally, antiangiogenic agents have also been used for the treatment and control of cancer progression. Main problem in conventional anti-cancer treatment is the occurrence of side effects induced by the non-specific targeting of both normal and cancer cells. (Gurung RL, et al., 2010; Head J and Johnston SR., 2004). Most of the drugs used in chemotherapy have many side effects. Many studies have reported that chemotherapeutic drugs can lead to an increase in oxidative stress and also a loss of antioxidants in patients undergoing chemotherapy (Crohns, M., et al., 2009; Weijl, N. I., et al., 1997). Chemotherapy increases lipid peroxidation products, decreases free radical scavenging capacity in blood, and decreases in plasma antioxidant levels such as vitamins A, E, and C. It has been reported that antioxidant levels of cancer patients are important in terms of chemotherapy response (Block, K. I., et al., 2007; Simone, C. B., et al., 2007). After the 1900s, the demand for natural products increased as people discovered the side effects of synthetic drugs and became aware of the harms of synthetic substances in food and beverages to human health (KUTLUTÜRKAN, S., & KARATAŞ, T. 2014).

Medicinal and aromatic plants have been used for various purposes including cosmetics, drugs, perfumery, spices and food since the beginning of human history (Baytop, T. 1999). Today, many herbs are being still used for treatment of many diseases all around the world. According to the Food and Agriculture Organization (FAO), approximately 30% of the drugs used in the world consist of different bioactive compounds obtained from plants (Acıbuca, V., Bu et al., 2018). It has been widely known that various diseases such as diabetes, jaundice, wounds and burns have been treated by means of extracts or phytochemical secondary metabolites obtained from these plants (Abdullahi, A. A. 2011). Demand for medicinal plants; It is increasing in both developed and developing countries due to its low cost, no side effects, less toxic effects and being naturally produced (Sekar and Kandavel, 2010).

Hypericaceae family contains more than 400 species in different parts of the world (Cirak C, et al., 2014; Crockett SL, et al., 2011), 80 species of which are grown in Turkey. Many members of this family include the naphthodiantrone derivatives hypericin and pseudohyperiin. They are found in glandular tissues that appear as black, orange, or translucent dots or streaks on leaves and other parts of the plant. Composition of hypericum; It carries tannin, essential oil, flavone species and hypericin (Baytop, 1999). *Hypericum scabrum* L. is a perennial herbaceous plant that grows on dry rocky slopes in Turkey. This herb has been used to treat hepatitis, cystitis, chronic gastritis, ulcers, wounds, hemorrhoids, and constipation. It has been reported to have antispasmodic, anti-inflammatory, sedative and antidepressant properties (Serbetci, 2002; Kizil et al. 2004; Ayan et al. 2009). *Hypericum scabrum* L. has been used for treatment of some biological diseases such as antidepressant (Butterweck V, et al., 2002; Nogueira T, et al., 1998), antimicrobial (Crockett SL.2010; Dall'Agnol R, et al., 2003; Jayasuriya H, et al., 1989), anticancer (Guclu, G., et al., 2019; agostinis P, et al., 2002), antioxidant (Cakir A, et al., 2003), antiviral (Meruelo D, et al., 1988), antifungal (Cakir A , et al., 2005; Fenner R, et al., 2005).

According to our best knowledge, there are no or limited information about the antioxidant and cytotoxic activity regarding to *H. scabrum* species. Therefore, in this study, we aimed to determine the antioxidant capacity and antiproliferative activity of various extracts from *H. scabrum* L.

MATERIALS AND METHODS

Plant Material

The flowered branches of *H. scabrum* was collected from the Ardıçlı Lake, Erzincan region of Turkey during the flowering stage in 18.06.2019. The plant material was identified by Prof. Dr. Ali Kandemir and enumerated as 11031. The voucher specimens were deposited at the herbarium of the Department of Biology, Erzincan Binali Yıldırım University, Erzincan, Turkey.

Preperation of Crude Extracts

After the plant samples were collected, the leaves of the plant to be studied were cut into small pieces and powdered with the help of a mixer. To remove oily components, 200 g of the powdered sample was treated with 400 mL of petroleum ether for 12 h at room temperature two times. Methanol and water extracts were obtained respectively by sequential extractions from the remaining defatted powder part. For this, defatted powders was extracted with methanol solvent in a ratio of 1:3 (g/mL) for 12 h at room temperature by using a magnetic stirrer. For water extract, the same procedure was performed. All extracts were filtered over whatman paper (No.1) and the filtrates were concentrated using either a rotary evaporator at 60 ºC (Rotary evaporator, RE 100 Bibby, Stone Staffordshire, UK), or a freeze drier (FD-1000, Eyela, Japan). The obtained extracts were stored at -20°C in a glass bottle to be used for further experimental studies.

Determination of Total Phenolic Content

Total phenolic contents of the crude extracts were determined by Folin-Ciocalteu method (Altay, A., et al.,2019; Slinkard ve Singleton, 1977). Briefly, 20 µL of each plant extract at a certain concentration (0.1 mg/mL) was added into a 96-well plate, including 100 µL of Folin's reagent (20%) and mixed gently. Afterwards, 80 µL of sodium carbonate (Na_2CO_3) solution (10%) was added and the mixture was incubated for 30 min at room temperature. After the exact incubation time, the absorbance of the mixtures were monitored at 750 nm using a microplate reader (Epoch, BioTek, USA). The linear reading of the standard curve was obtained from 0 to 600 µg of gallic acid/mL. The TPC of the extracts, obtained from the linear reading of the

standard curve (y = 0,0009X + 0.0208, R^2: 0.999) of gallic acid were expressed as milligrams of gallic acid equivalent (GAE)/gram extract.

Antioxidant Activity

DPPH· Free Radical-Scavenging Activity

The DPPH radical scavenging activity of the extracts was determined according to the method of Blois with some modifications (Liyana-Pathirana ve Shahidi, 2005; Altay, A., ve ark.,2019). Briefly, 140 µL of DPPH solution (0.05 mg/mL) was mixed with 10 µL of each extract at varying concentrations (5-100 µg/mL) in a 96-well plate and incubated 20 min. After the incubation, the absorbance of the reaction mixture was monitored spectrophotometrically at 517 nm using a microplate reader (BioTek, USA). Trolox, was used as an antioxidant standard. The percentage of DPPH scavenging activity was calculated with the following equation; [1 − (test sample absorbance/blank sample absorbance)] × 100(%). The IC_{50} values (the concentration of an antioxidant scavenging 50% of DPPH radical) were calculated from the RSA% versus final concentrations of each sample.

Ferric Ions Reducing Power (FRAP)

Ferric ions reducing antioxidant power of the extracts was evaluated to the procedure described by Oyaizu (Altay, A., ve ark.,2019; Altay, A. 2018). Accordingly, 250 µL of each plant extract at a final concentration of 0.2 mg/mL and mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6). Then, 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%) was added and the reaction mixture was incubated at 50°C for 20 min. At the end of the incubation, the reaction mixture was cooled to room temperature and 1.25 mL of trichloroacetic acid (10%) and 1.25 mL of $FeCl_3$ (0.1%) were added to the reaction mixture, respectively. Finally, the absorbance readings were taken immediately at 593 nm spectrophotometerically (Perkin–Elmer, USA). Trolox was used as positive control and FRAP results of the extracts, determined from the linear reading of the standard curve (y= 0.0041x + 0.1289, R^2 = 0,999) of were expressed in terms of mg trolox equivalents (TE)/g extract.

Cytotoxicity

Cell lines and Culture

Human colon cancer (HT-29) cells and GF-1 were purchased from the ATCC (American Type Culture Collection, LGC Promochem, UK). HT-29 cells were grown in McCoy′s 5A Medium and GF-1 cells were grown in Eagle's minimal essential medium (EMEM) including L-Glutamine and 25 mM HEPES (BI, USA), integrated with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% Na-pyruvate (BI, USA). Cells cultures were incubated at 37°C with 5% carbon dioxide and 95% humidity in incubator (NUVE, Turkey). Studies were performed in Metisafe Class II Safety Cabinet.

Measurement of Cell Survival

The cytotoxic activity of the extracts were evaluated over HT-29 and GF-1 cancer cell lines by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide (XTT) assay, based on the extracellular reduction of tetrazolium salt XTT by NADH produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator (De Logu ve diğerleri, 2003; Scudiero et.al.1988). Accordingly, the cells were seeded at a concentration of $1x10^5$ cells/mL (100 µL) into 96-well culture plates and incubated overnight in CO_2 incubator. After incubation, the cells were treated with hexane, methanol and water extracts seperately at varying concentrations (25, 50, 100, 250 and 500 µg/mL) for 24 h. The complete medium comprising dimethyl sulfoxide (0.5% DMSO) was used as negative control. When incubation times were over, the cellular metabolic activity of cancer cell line was measured at 470 nm (Epoch Microplate Reader, BioTek, USA). All tests were performed in triplicate. The cytotoxicity results of the extracts were expressed in terms of their half maximal scavenging concentration (IC_{50}), calculated by nonlinear regression curve. The dose-response curve was obtained by plotting the percentage of inhibition versus the concentrations. Cisplatin was used as a standard reference drug.

Statistical Analysis

Statistical analyses were performed for assessment of antioxidant and cytotoxic activity results by unpaired Student's t-test by using statistical program of GraphPad Prism 6 (GraphPad, La Jolla, CA) Software 7.0). All results were denoted as means with their standard deviation (SD) $p<0.05$ was taken as the minimum level of importance.

RESULTS AND DISCUSSION

Total phenolics content (TPC)

Phenolic compounds, which constitute a diverse group of phytochemicals in medicinal plants, have various biological activity in living organisms (Altay, A., et al., 2017; Altay, A., 2018). In the present study, TPC of HE, ME, and WE of *H. scabrum* was determined and the results were shown in Table 1. The results revealed that ME contained the highest level of phenolics (13.20 mg GAE/g extract), followed by WE (8.30 mg GAE/g extract), and HE (5.15 mg GAE/g extract), respectively. These results approve that the polar extracts comprise much more phenolics in their structures compared to non-polar extracts such as hexane. In the literature, there are some studies conducted with different Hypericum species possessing different phenolic amounts depending on the part of the plant used, type of the solvent, as well as the extraction procedure. For instance, Karatoprak et al. reported that the TPC of the aerial parts of *Iranian Hypericum scabrum,* extracted with water and methanol were 93.00 ± 0.22 mg CA/g extract and 186.94 ± 2.66 mg GAE/g extract, separately (Karatoprak et al., 2019). Zengin et al. outlined the TPC of methanol extract obtained from the aerial parts of *Hypericum lanuginosum* as 168.56 mg GAE/g extract (Mahomoodally et al., 2019). These data indicate that *H. scabrum* extracts contain moderate level of phenolic substances compared to the literature.

Table 1. Total phenolic content, DPPH and FRAP results of the extracts of *H. scabrum* L.

Extracts	TPC (mg GAE/g extract)	DPPH IC_{50}(µg/mL)	FRAP (mg TE/g extract)
Hexane	5.15±0.42^{a}	>100^{d}	N.D.
Methanol	13.20±1.95^{c}	81.57^{c}	48.06±1.60^{c}
Water	8,30±0.12^{b}	97.69^{b}	17.00±0.46^{b}
Trolox		12.80±0.54^{a}	-

*Data marked with different superscripts within the same column (a-d) express the significant difference ($p< 0.05$).

N.D: Not determined

DPPH radical scavenging

DPPH assay is remarkably used method for evaluation of antioxidant capacity of the natural and synthetic compounds because of their vital roles in scavenging of free radicals from biological systems (Sridhar and Linton, 2019). Accordingly, the dose dependent DPPH radical scavenging activity of the extracts and standard antioxidant trolox was studied and their IC_{50} values were represented in Table 1. These results showed that DPPH radical scavenging activity of the ME was superior to that of all extracts tested with the lowest IC_{50} value of 81.57 µg/mL, followed by the WE (97.69 µg/mL) and HE (>100 µg/mL). ME was also observed to have the closest IC_{50} value to that of trolox (12.80 µg/mL). When compared with the literature, *H. scabrum* extracts appear to exhibit closely similar radical scavenging activity. For instance, Zengin et al. reported the IC_{50} values of ethyl acetate, methanol and water extracts of Hypericum lanuginosum for DPPH radical scavenging activity as 73.49, 105.93, and 354.60 µg/mL, respectively (Mahomoodally et al., 2019). The

present results indicate that when the solvent polarity changes, its capability of dissolving the selected antioxidant compounds alter, and eventually influence the antioxidant activity.

Ferric ions reducing power (FRAP)

Metal ion reduction ability of a compound can serve as an important indicator to estimate of its antioxidant power via donating electrons to radicals making them more stable and unreactive species ALTAY, A. (2018; Altay, A., et al.,2017; Em, 2001). In this line, in the current study, ferric ion reducing powers of the H. *scabrum* extracts were tested and the results were calculated in terms of trolox equivalent (TE) (Table 1). The results revealed that similar to the DPPH results, the ME showed the highest iron reducing capacity (48.06 mg TE/g extract), followed by WE (17.00 mg TE/g extract). The ferric ion reduction capacity of both extracts of *H. scabrum* are greater than those of *H. lanuginosum* reported by Zengin et al. (2019). The results from the DPPH and FRAP assays reflect that *H. scabrum* extracts can be considered as an important source for prevention of radical formation that may lead to oxidative stress.

Cytotoxic activity

Cytotoxic activities of the ME and WE of *H. scabrum* against human colon (HT-29) cancer and normal GF-1 cell lines were assessed using XTT assay. The cytotoxic activity of water and methanol extracts from the aerial parts of the *H.scabrum* plant against the human colon (HT-29) cancer cell line and normal (GF-1) cell lines were evaluated using XTT analysis. The results showed that both ME and WE of *H.scabrum* significantly inhibited the proliferation of the HT-29 cell line. The IC_{50} values belonging to the extracts and cisplatine were calculated and tabulated in Table 2. As seen in Table 2, WE displayed slightly higher cytotoxic activity against HT-29 cells with an IC_{50} value of 0.440 mg/mL than ME (IC_{50}= 0.519 mg/mL). And, the IC_{50} value of cisplatine on HT-29 cells line was determined as 0.101 mg/mL. These results reveal the moderate cytotoxic activity on HT-29 cancer cells, compared to cisplatin. As for GF-1 normal cells, both extracts could not show cytotoxicity up to 1 mg/mL. These results confirm the selective powers of the extracts between the the cancer and normal cells.

In the literature, some studies related with the cytotoxic effect of the extracts from the different parts of *H. scabrum* obtained different regions were reported. For instance, water and ethanol extracts obtained from the flowers of *H.scabrum* were tested on MCF-7 and HTC-116 cancer cell lines with IC_{50} values ranging from 0.53 to 1.79 mg/mL (Keser, S., et al., 2020). In another study, the IC_{50} values of methanol extract from the flowers of *H. scabrum* against MCF-7 and HT-29 cell lines were reported 0.546 and 0.464 mg/mL, respectively (Naghibi F., et al., 2014). The data obtained in this study on cytotoxicity are very close to the results in the literature. In addition, as a result of this study, the importance of the polarity of the solvent used in the preparation of the extract obtained from the plant was once again demonstrated. As a result, all these data make it important in terms of taking the *in vitro* cytotoxicity studies of the plant one step further to *in vivo* studies.

The results we obtained as a result of our study show similar results with the extract-based studies in the literature.

Table 2. The IC_{50} values of methanol and water extracts of *H. scabrum* on HT-29 and GF-1 cell lines

Samples	HT-29 IC_{50} (mg/ml)	GF-1 IC_{50} (mg/ml)
ME	0.519	>1
WE	0.440	>1
Cisplatine	0.101	-

*ME; Methanol Extract, WE; Water Extract
IC_{50}; Concentration of the test compound inhibiting the cell proliferation by 50%.

CONCLUSION

In the present study, *Hypericum scabrum* L. was investigated for its phenolic content, antioxidant capacity, and antiproliferative effects on human colorecatal adenocarcinoma HT-29 and normal GF-1 cell lines. All the results of TFC, DPPH, FRAP, and XTT assays demonstrated that the polarity of the solvent used in the preparation of the plant extract and therefore the type of phytochemical contained in the extract highly affect the biological activity of the plant, including antioxidant and anticancer properties.

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