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LC-MS/MS profiles, multielement levels and biological activities of *Hypericum heterophyllum* Vent.

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Plants belonging to the genus *Hypericum* L. are widely used in traditional folk medicine due to their hypericin and pseudohypericin contents. In this study, we investigated the biological activity, phenolic and elemental content of methanol and acetone extracts of sheepskin grass, *Hypericum heterophyllum* Vent. a species that that lacks both hypericin and pseudohypericin. The total antioxidant status of the extracts was determined by commercial kits. Antibacterial effect of extracts was investigated on seven bacterial strains. Cytotoxic effects of the extracts on lung cancer cell lines were determined by MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide) method. Phenolic content was determined by LC-ESI-MS/MS. Elements in the plant were determined by ICP-OES. The total phenolic content and antioxidant status of the species' methanol extract were found to be higher than the acetone extract. Both of the extracts at a concentration of 20% have an antibacterial effect, especially the antibacterial effect of acetone extract. It was determined that acetone extract has an anticarcinogenic impact depending on the dose. Chlorogenic acid, miquelianin and isoquercitrin are the most abundant flavonoids in methanol and acetone extract. The plant contains Ca, K, and Mg elements in high concentrations. The phenolic substances and elements in *H. heterophyllum*, widely used in our country, have been presented for the first time in the literature. Besides, it can be said that the plant has antioxidant, anticarcinogen, and antimicrobial activities due to the crucial flavonoids and elements it contains.

Keywords: Anticancer, Antimicrobial, Antioxidant, Cytotoxic, Folk medicine, ICP-OES, Phenolic content, Sheepskin grass, Traditional medicine

Hypericum (Guttiferae or Hypericacea) is a traditionally used family that grows widely in the world's temperate regions. *Hypericum*, which has approximately 400 species, is represented as 89 species in our flora. Fourty-five of them are endemic[1]. *Hypericum* L. (Hypericaceae) is characterized by different secretory structures, including translucent glands, black nodules, and secretory ducts. Secretory structures, which are sites of synthesis and/or accumulation of biologically active substances, are essential to distinguish between taxa[2]. *Hypericum heterophyllum* Vent., is endemic to northwest and west-central Anatolia in Turkey. As it is grown in its natural environment, its culture is also made. *H. heteropyhllum* is an endemic species that grows in arid, stony or rocky calcareous areas. It has the form of a bush. The stem of the plant is 20-60 cm long, erect or branched from the base. It is semi-deciduous and glabrous and lacks dark glands. The leaves on the main stem are 5-13 mm, narrow and short, on the shoots 0-5 mm, broadly oval. Sepals 2-3-5 mm, oblong to lanceolate, acute, entire. Its yellow flowers are numerous and unspotted, similar to leaves[3]. As the name of the species suggests, it contains leaves in different shapes. These are located between the permanent lower leaves in the stem and the upper leaves falling[4].

Plants have been used in the composition of drugs, and many positive results have emerged in human health. They take place in the pharmaceutical industry because they are the basis of drug production, which are used naturally in production and used as drugs in treatment. Anti-inflammatory, antiviral, antimicrobial,

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antifungal, antidepressant, and antioxidant activities have been described in different species belonging to the genus *Hypericum*. Also, it was reported the cytotoxic activities of the species, tumor invasion and metastasis inhibition, antitumor, and apoptosis-inducing effects in recent studies[5-7]. There are numerous studies with different species, especially *Hypericum perforatum*, but there is little research with *Hypericum heterophyllum*. Biological activities, the type, and content of phenolic substances, and elements were determined for *Hypericum heterophyllum* endemic species in the presented research.

Material and Methods

Plant material and extraction protocol

Arial parts of the *Hypericum heterophyllum* plant were collected from Köroğlu Beli, Bayat, Afyonkarahisar (38° 58′ 59″ N, 30° 55′ 29″ E), in July 2018. It was identified by Dr. Mustafa Kargıoğlu. The aerial parts of the *H. heterophyllum* plant consisting of branch, leaf, flower, and stem parts was dried and ground in darkened environment. Methanol and acetone extracts of the dried plant were prepared to be used in the analysis. The maceration method was used to obtain methanol and acetone extracts. For this purpose, the arial parts of the plant taken in certain amounts were kept at room temperature (21ºC) for 24 h with methanol and acetone. The filtration process was carried out and the solvents were removed with a rotary evaporator. Dry extracts were stored at 4ºC for qualitative, quantitative, and biological activity analysis.

Biological activities of extracts

Total antioxidant status, antimicrobial and anticarcinogenic properties were determined to assess the biological activities of *H. heterophyllum* methanol and acetone extracts. Total antioxidant capacity of plant's acetone and methanol extracts were measured using commercial kits (Rel Assay, Gaziantep, Turkey). The working principle of the kit is based on the oxidation of 2,2-azino bis-3-ethyl benzo thiazoline-6-sulfonic acid ($ABTS^{\cdot}$) radical to ABTS by reacting with H_2O_2. The absorbance was measured spectrophotometrically at 660 nm. Results are given as mmol Trolox Equiv./L[8].

The antimicrobial activity of acetone and methanol extracts of *Hypericum heterophyllum* was determined by the disk diffusion method. *Listeria monocytogenes* (ATCC 7644), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25292), *Escherichia coli O157* (ATCC 43894), and *Salmonella typhimurium* (ATCC14028) were used as references bacterial strains. Reference strains were counted by the nutrient liquid serial dilution method. Suspensions were prepared to a final bacteria concentration of 10^6-10^7 cfu/mL. A bacteria culture was added to each petri dish containing Mueller-Hilton agar (Oxoid CM337). Methanol and acetone extracts added to the discs in different concentrations (2.5, 5, 10 and 20%). Gentamicin (Oxoid CT0024B) and ciprofloxacin (Oxoid CT0425B) were used as controls. After 24 h of incubation, the zones formed around the discs were measured[9].

Lung cancer cell line (A549) cells were used to determine the anticarcinogenic effect of acetone and methanol extracts of *H. heterophyllum*. Cytotoxicity levels of the extracts were determined by MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide) method. MTT is a positively charged compound that can easily cross the membrane of eukaryotic cells and be reduced inside the cell. Still, the formazan formed due to the reduction is insoluble in water and therefore precipitates in crystalline form. Living cells perform the color change by reducing the relevant compounds and converting them into purple-colored formazan. The number of living/dead cells was determined by measuring the colour change with spectrophotometric method. Optical densities were measured at 540 nm in an ELISA microplate reader (Biotek, ELx800). The group that was not treated with plant extracts was determined as the control group. The control group's cell viability was accepted as 100%, and the following formula calculated each dose's on cell viability.

$$\text{Cell viability (\%)} = \frac{(100 * \text{Absorbance of the Sample})}{(\text{Absorbance of the Control})}$$

The viability of cells is expressed as a percentage for each dose. The anticarcinogenic effect on acetone and methanol extracts of *H. heterophyllum*, percent viability data obtained in MTT tests were used[10].

Qualitative and quantitative analysis of extracts content

Qualitative analysis of the phenolic content of methanol and acetone extracts of *H. heterophyllum* aerial parts werefirst performed. The phenolic substances and elements contained in the species were determined within the scope of quantitative analysis. The total phenolic substance of the extracts and caffeic acid were determined according to the Folin-Ciocalteu method defined by Blainski *et al.*[11]. Folin-Ciocalteu

reagent was added to the extract and caffeic acid solution, followed by Na_2CO_3. After the prepared mixture was kept at room temperature (21ºC) in a dark place for 30 min, samples' absorbances were measured spectrophotometrically at 760 nm. Results are given as mg gallic acid equivalent (GAE)/g extract.

Reverse-phase UHPLC system used in LC-MS/MS system preferred for quantitative analysis; It consists of an autosampler (SIL-30AC model), a column oven (CTO-10ASvp model), a gradient pump system (LC-30AD model) and, a degasser (DGU-20A3R model). Chromatographic separation (Agilent Poroshell 120 EC-C18 model) was performed using a column (150 mm×2.1 mm, 2.7 µm). The column temperature is set to 40°C. The elution gradient consisted of mobile phase A (ultrapure water + 5 mM ammonium formate + 0.1% formic acid) and mobile phase B (ultrapure water + 5 mM ammonium formate + 0.1% formic acid). The gradient elution profile used was as follows: 20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min). The mobile phase flow rate and injection volume were determined as 0.5 mL/min and 5 µL, respectively[12]. Shimadzu LCMS-8040 model sequential mass spectrometer was used equipped with an electrospray ionization source operating in both positive and negative modes for the mass spectrometer detection of the LC-MS/MS system. LC-ESI-MS/MS data was taken and processed with LabSolutions software (Shimadzu). MRM (multiple reaction monitoring) modes has been used for the quantitation of phytochemicals. The MRM method is optimized for selective detection and quantitation of phytochemicals based on screening for specific major ion-fragmentation ion transitions. The collision energies (CE) are optimized to achieve optimum phytochemical fragmentation and maximal passage of desired cleavage ions. MS operating conditions applied: drying gas (N_2) flow, 15 L/min; nebulizer gas (N_2) flow, 3 L/min; DL temperature, 250°C; the heat block temperature is set at 400°C and the interface temperature as 350°C.

Sample was taken from the homogenizer containing the flower, leaf, branch, and stem parts of the plant to determine their elements and concentrations. It was measured by the method based on the excitation of the sample by the argon plasma, which is reached to high temperatures by electro-magnetic induction.The degradation of organic components was carried out by adding perchloric acid, nitric acid, and hydrogen peroxide to the sample and applying certain temperatures (90-150ºC) by microwave (Speed Wave, Erghof) method[13]. After the samples were ready for analysis, the plant elements' qualitative and quantitative analysis were determined by ICP-OES (Spectro Genesis, Kleve, Germany).

Statistical analysis

The extracts used in the study were prepared in triplicate, and the measured results were expressed as mean ± standard deviation (mean ± SD). SPSS package program (17.0, USA) was used for the statistical analysis of the data in this study. Differences between groups were determined by one-way analysis of variance (one-way ANOVA). The distribution between groups was determined at P <0.05 significance value according to Duncan multiple range test.

Result and Discussion

Turkey is an important center for *Hypericum* species. *Hypericum heterophyllum* is one of these endemics and is widely used locally for medical purposes. Antioxidants can delay or prevent the oxidation of lipids and/or other molecules by preventing the initiation or progression of oxidative chain reactions. Chemicals with antioxidant activity are found naturally and in large amounts in many fruits, vegetables, and medicinal plants. Medicinal plants contain numerous free radical scavenging compounds such as polyphenols, flavonoids, quinones, coumarins, lignans, alkaloids, amines (nitrogen compounds such as betalains; terpenoids including vitamins and carotenoids, and some other endogenous metabolites with high antioxidant activity[14]. The free radical scavenging and antioxidant activity of phenolic compounds, which constitute an important group of phytochemicals, generally depends on the number and position of hydrogen donor -OH groups in the aromatic ring of phenolic molecules, and glycosylation of aglycones and other H donor groups (-NH, -SH). This feature plays a vital role in absorbing and neutralizing free radicals, trapping singular and ternary oxygen, or separating peroxides. Thanks to this feature, phenolic compounds are reducing agents, hydrogen donors, singular arrows. They serve as oxygen traps and metal chelators[15,16]. Flavonoids and other plant phenolic compounds have scavenging radicals, such as superoxide, alkoxyl, peroxyl and nitric oxide, iron, and copper chelation, α-tocopherol regeneration. The reason for the antioxidant activity of phenolic compounds is their redox property, which is closely related to the compounds' chemical structure[17].

There are many antioxidant activity studies done with *Hypericum* species. DPPH (1,1-diphenyl-2-picrylhydrazyl) was used to determine the radical scavenging effect of *H. triquetrifolium* and *H. scabroides* ethanol extracts. The ethanol extract of both *Hypericum* species exhibited a high reducing power, indicating that the extracts have strong electron-donating capacity. It was determined that the total antioxidant activity of ethanol extract of both *Hypericum* species was comparable to vitamin E. As a result, *H. triquetrifolium* and *H. scabroides* ethanol extracts show a potential source of natural antioxidants[18]. Unal *et al.* (2008) investigated the antimicrobial and antioxidant activities of chloroform, acetone, ethanol and water extracts of 25 plants used in Turkish tradition. Three *Hypericum* species (*H. scabrum*, *H. hyssopifolium*, and *H. heterophyllum*) have found that they have strong antioxidant potential due to their high phenolic content[19]. The total antioxidant status of the extracts in the study is given in Table 1. Methanol extract of *H. heterophyllum* plant (0.79±0.08) was found to have higher antioxidant capacity than acetone extract (0.47±0.25). In addition, the yield of methanol and acetone extracts obtained by maceration method is (12.38±3.6%) and (14.71±2.09%), respectively. The yields are also shown in Table 1.

Antibacterial activity is provided by compounds that kill bacteria or slow their growth rate without being overly toxic to surrounding tissues. While antibiotics can be entirely synthetic, purely natural products can also show this effect. The most recently discovered antimicrobial agents are natural compounds and modified natural compounds[20-22]. In a study, antimicrobial activity of extracts of some *Hypericum* species (*H. rupestre*, *H. vacciniifolium* and *H. imbricatum*) with different solvents was investigated. It was determined that methanol and chloroform extract of *H. vacciniifolium*, *H. rupestre* and *H. imbricatum* methanol, butanol and chloroform extracts showed good antimicrobial activity against Gram positive and Gram negative bacteria. Methanol extracts of these *Hypericum* species were also found to have no antifungal activity[23]. The antifungal effect of the essential oils of *H. linarioides* was investigated in the study. Five *Fusarium* species (*F. oxysporum*, *F. culmorum*, F. *sambucinum*, *F. solani* and *F. acuminatum*) and five anastomotic groups of *Rhizoctonia solani* (AG-3, AG-4, AG-5, AG-9 and AG-11) were tested for antifungal activity against ten agricultural pathogenic fungal culture using *in vitro* microbial growth inhibition assays[24]. In the present study, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *E. coli O157* and *Salmonella typhimurium* were used as reference bacterial strains. The antibacterial effect of methanol and acetone extracts of the species is shown in Table 2. Neither dose of methanol and acetone extracts appear to be as effective an antibiotic as ciprofloxacin. However, there is no statistically significant difference (P <0.05) between the plant's 10% acetone extract, 5% methanol extract and gentamicin on the antibacterial effect to the *L. monocytogenes* bacteria. Moreover, it is seen that the antibacterial effect of 10 and 20% methanol extract and 20% acetone extract are statistically significantly higher (P <0.05) from gentamicin. Concentrations of 20% of both extracts appear to be effective against *S. aureus* bacteria, but

Table 1 — Total antioxidant status, yield and total phenolic substance and standard substance

Extract/Standart	HHM	HHA	Caffeic acid
Total Antioxidant Status (mmol Trolox Equiv./ L)	0.79±0.08	0.47±0.25	
Yield (%)	12.38±3.6	14.71±2.09	
Total Phenolic Substance (mg GAE/g ekstract)	129.93±14.56^{b}	101.73±5.64^{a}	243±7.5^{c}

[HHM; *Hypericum heterophyllum* methanol extract, HHA; *Hypericum heterophyllum* acetone extract, GAE; Gallic Acid equivalent. Different superscripts on the same line indicate statistical difference (P <0.05)]

Table 2 — Antibacterial effects of *Hypericum heterophyllum* methanol and acetone extracts

	Gentamicin (10µg)	Ciprofloxacin (10µg)	The dose of applied to HHM				The dose of applied to HHA			
			2.5%	5%	10%	20%	2.5%	5%	10%	20%
L. monocytogenes	16.67±0.58^{b,c}	30.33±1.15^{f}	-	15.66±0.58^{b}	19.33±0.6^{d}	19.67±0.58^{d,e}	-	10.33±0.58^{a}	17.33±0.58^{c}	20.33±0.58^{e}
B. cereus	27.33±0.58^{b}	30.66±1.15^{c}	-	-	-	-	-	-	10.33±0.58^{a}	11.33±0.58^{a}
S. aureus	19.66±2.51^{c}	22.66±2.08^{d}	-	-	10.33±0.58^{a}	12.66±0.58^{b}	-	-	-	12.66±0.58^{b}
E. faecalis	14.66±2.08^{a}	21.33±1.15^{b}	-	-	-	-	-	-	-	-
E. coli	18.33±0.6^{a}	27.66±0.58^{b}	-	-	-	-	-	-	-	10.33±0.58^{c}
E. coli O157	15.33±1.15^{a}	27.66±1.15^{b}	-	-	-	-	-	-	-	-
S. typhimurium	15.66±1.52^{a}	31.33±0.6^{b}	-	-	-	-	-	-	12.33±0.6^{c}	16.33±0.58^{a}

[HHM, *Hypericum heterophyllum* methanol extract; HHA, *Hypericum heterophyllum* acetone extract. Different superscripts on the same line indicate statistical difference (P <0.05)]

not as much as synthetic antibiotics. It is seen that 20% concentration of acetone extract against *S. typhimurium* bacteria is as effective as gentamicin. It can be said that the application of both extracts at a concentration of 20% has an antibacterial effect. Especially, acetone extract is more prominent as an antibacterial effect.

Investigation of the effects of plant-based substances on cancerous cells is a current approach that continues today. In addition to the methods and drugs currently used in the treatment of cancer in modern medicine, it is known that many compounds found in medicinal plants and foods are effective protective properties in some experimental carcinogenesis models as well as anti-tumor agents. However, the mechanism of action of most of them is not well known. Therefore, the main target in researching new cancer drugs from plants should be cancer cells directly without damaging the healthy target cells. Considering the basically similar properties of cancer cells and normal cells, reducing the cancer cells' viability of (cytotoxicity) without damaging normal cells is not a very simple event. Cytotoxicity imaging models are an important parameter that provides preliminary information to help select plant extracts with potential antineoplastic properties for future studies[25,26]. Plant chemicals demonstrate anticancer or antitumor activity in different ways. Among the phytochemicals found in plants, especially alkaloids and phenolic compounds, have neoplastic or anti-tumor activity. Alkaloids act on the spindle threads, preventing the progression of cancer cells through the cell cycle. In contrast, phenolic compounds act more on cell cycle control proteins and the stimulation of the apoptosis mechanism. Carotenoids also stand out in terms of their cancer inhibiting effects.

Plant chemicals' action mechanisms can be investigated at the cell cultures and molecular targets, such as receptors, receptor proteins, matrix proteins, growth factors, transcription factors, etc. In particular, cytotoxicity imaging models such as cancer cell cultures provide initial data for selection of herbal extracts with anticancer and cytotoxic properties[27,28]. In a genotoxicity study with *H. heterophyllum*, human lymphocytes were incubated with aqueous extracts. Increasing extract concentrations of *H. heterophyllum* were found to induce micronucleus. The rise of micronucleus shows suggested that *H. heterophyllum* at high concentrations could become carcinogenic and genotoxic[28,29]. In the presented study, cytotoxicity levels of methanol and acetone extracts of *Hypericum heterophyllum* species in the A549 cell line are shown in Fig. 1. In order to determine the cytotoxicity levels, acetone and methanol extracts of plant at seven different concentrations (25, 50, 100, 200, 500, 1000 and 2000 µg/mL) were applied to the cells. Applications were repeated six times for each dose. When the obtained data were examined, it was determined that acetone extract of *H. heterophyllum* species showed more cytotoxic effects in A549 cell line than methanol extract. While acetone extract showed cytotoxic effects in the dose range of 25-500 µg/mL, it was determined that methanol extract was not cytotoxic at these doses. On the contrary, it increased cell viability. Therefore, it can be said that *H. heterophyllum* acetone extract to have anti-carcinogenic activity against A549 cells, but methanol extract did not show anticarcinogenic activity.

Hypericum species contain many metabolites that belong to at least 11 different classes, including naphrodiantrons, flurogonol derivatives, flavonoids, organic acids, essential oils, amino acids, xanthones, tannins, proxyanidins and other water soluble compounds[30,31]. *Hypericum origanifolium* and *H. montbretii* chemical content and activity relationship were investigated in a study to determine the total polyphenolic content of water, ethyl acetate and methanol extracts and evaluate their antioxidant activity. The results were compared with butyl hydroxy toluene (BHT), a synthetic antioxidant, and *H. perforatum*. It was observed that there was a correlation between antioxidant activity and total phenolic substance content in the extracts. Antioxidant activity analysis, it was found that leaf extracts of *H. origanifolium* exhibited much higher antioxidant activity than BHT, *H. perforatum* and *H. montbretii* extracts[32]. In the study where the total phenolic substance content of the leaf, flower and stem parts of the methanol extract of the *H. heterophyllum* plant was examined, it was found

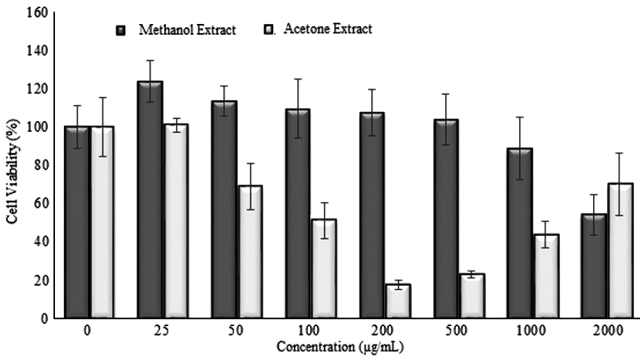

Fig. 1 — Effects of *Hypericum heterophyllum* methanol and acetone extracts different concentrations on A549 cell viability (%)

that it was the highest in the leaf (60.5 mg GAE/g extract) and the lowest in the root (34.1 mg GAE/g extract) part[32]. In the presented study, the total phenolic content of methanol and acetone extracts of *Hypericum heterophyllum* is shown in Table 1. The total phenolic content of the methanol extract (129.93±14.56 mg GAE/g extract) was higher than the acetone extract (101.73±5.64 mg GAE/g extract), but the methanol extract was statistically significantly lower (P <0.05) than reference substance caffeic acid.

The main group in the *Hypericum* species chemical composition' are naphtodiantrons, including hypericin, pseudohypericin and their precursors, protohypericin and protopeudohyperis. Hyperforin, adhyperforin and their oxygenated derivatives are also very important. Besides, there are xanthones, flavonoids (rutin, hyperoside, quercitrin, isoquercitrin), biflavonoids, tannins, proanthocyanidins, phenolic acids and essential oil in their chemical composition[33]. In a study, Ayan & Çirak[34] examined hypericin/pseudohypericin content of *H. heterophyllum* and stated that the aerial parts of the species (flower, leaf and stem) do not contain hypericin and pseudo-hypericin. In the presented study, LC-ESI-MS/MS chromatograms of standards chemicals and *H. heterophyllum* methanol/acetone extracts are given in Fig. 2. Also, phenolic substance content and analytical

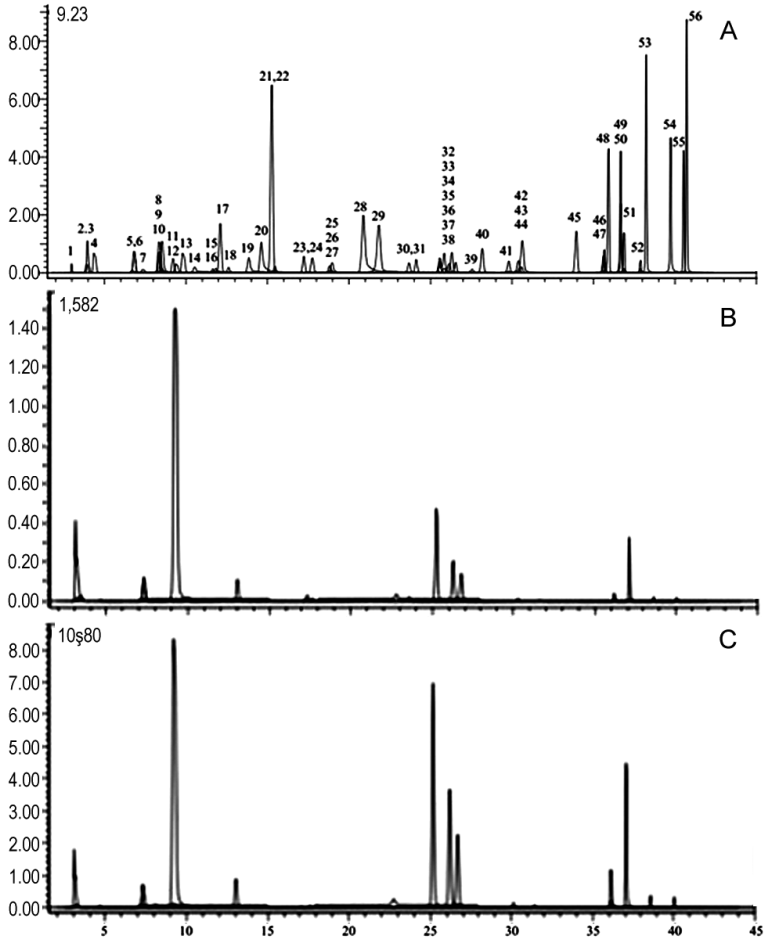

Fig. 2 — LC-ESI-MS/MS chromatograms of standards chemicals and Hypericum heterophyllum methanol/acetone extracts. (A) Standard phenolic compounds chromatogram analysed by the developed LC-MS/MS method (1:Quinic acid, 2: Fumaric acid, 3: Aconitic acid, 4: Gallic acid, 5: Epigallocatechin, 6:Protocatechuic acid, 7: Catechin, 8: Gentisic acid, 9: Chlorogenic acid, 10: Protocatechuic aldehyde, 11: Tannic acid, 12: Epigallocatechin gallate, 13: 1,5-dicaffeoylquinic acid, 14: 4-OH Benzoic acid, 15: Epicatechin, 16: Vanilic acid, 17: Caffeic acid, 18: Syringic acid, 19: Vanillin, 20: Syringic aldehyde, 21: Daidzin, 22: Epicatechin gallate, 23: Piceid, 24: p-Coumaric acid, 25: Ferulic acid D3, 26: Ferulic acid, 27: Sinapic acid, 28: Coumarin, 29: Salicylic acid, 30: Cynaroside, 31: Miquelianin, 32: Rutin, 33: Rutin D3, 34: isoquercitrin, 35: Hesperidin, 36: o-Coumaric acid, 37: Genistin, 38: Rosmarinic acid, 39: Ellagic acid, 40: Cosmosiin, 41: Quercitrin, 42: Astragalin, 43: Nicotiflorin, 44: Fisetin, 45: Daidzein, 46: Quercetin D3, 47: Quercetin, 48: Naringenin, 49: Hesperetin, 50: Luteolin, 51: Genistein, 52: Kaempferol, 53: Apigenin, 54: Amentoflavone, 55: Chrysin, 56: Acacetin); (B) LC–MS/MS chromatogram of Hypericum heterophyllum methanol extract; and (C) LC–MS/MS chromatogram of Hypericum heterophyllum acetone extract

characteristic by LC-ESI-MS/MS of *H. heterophyllum* methanol and acetone extractsare shown in Table 3. Quinic Acid (56.430 mg/g extract), chlorogenic acid (38.948 mg/g extract), fumaric acid (26.156 mg/g extract), miquelianin (18.551 mg/g extract) and isoquercitrin (10.289 mg/g extract) were found in the highest amount in methanol extract. In acetone extract, the five components are also at their highest concentration, but the amounts and order are different. Acetone extract contains miquelianin (24.601 mg/g extract), quinic acid (20.064 mg/g extract), chlorogenic acid (20.005 mg/g extract), isoquercitrin (16.582 mg/g extract) and fumaric acid (3.271 mg/g extract). Naringenin and kaempferol found in acetone extract could not be identified in methanol extract.

Quinic acid is the starting material of the synthesis of Oseltamivir, which is used for the treatment of influenza A and B, an antiviral drug. As a result of dehydrogenation and oxidation reactions, gallic acid, an important antioxidant, is synthesized[35]. Chlorogenic acids are a phenolic compound commonly found in plants. Studies have shown chlorogenic acid's radical scavenger, antioxidant and anti-apoptotic activity. It has been reported that the expression of chlorogenic acid cyclooxygenase-2 (COX-2) and tumor necrosis factor-α (TNF-α) decreased, and in parallel, renal oxidative stress and inflammation decreased. Chlorogenic acid has also been shown to have anticancer activities. It has been reported that chlorogenic acid regulates the expression of apoptosis related genes and self-regenerative stem cell markers in cancer cells. In one study, lung cancer cell line A549 was cultured with and without chlorogenic acid. The presence of chlorogenic acid was determined to decrease cell proliferation as measured by MTT activity. These results show that chlorogenic acid affects the expression of genes encoding stem cell markers and genes associated with apoptosis, which are part of oxidative stress and p38 MAP-dependent pathways. As a result, chlorogenic acid may contribute to the polyphenolic anticancer effect related to consuming vegetables and fruits[36]. Fumaric acid activates the Nrf2/antioxidant response pathway, which is the primary cellular defense against the oxidative stress' cytotoxic effects. Especially in neurodegenerative situations, therapies activate Nrf2/antioxidant response element signaling, which regulates the expression of antioxidant, anti-inflammatory and cytoprotective genes[37]. Miquelianin is one of the flavonoids in *Hypericum*

Table 3 — Phenolic substance content and analytical characteristic by LC-ESI-MS/MS of *Hypericum heterophyllum* methanol and acetone extracts

Analytes	HHM (mg analyte/g extract)	HHA (mg analyte/g extract)	Equation	r^{2d}	LOD/LOQ (μg/L)[f]
Piceid	0.226	0.134	y=25.42x+0.008	0.999	13.8/17.8
Hesperidin	0.248	0.262	y=13.28x+0.14	0.999	19.0/26.0
Quinic acid	56.430	20.064	y=2.98x-0.013	0.996	25.7/33.3
Fumaric aid	26.159	3.271	y=1.035x-0.082	0.995	135.7/167.9
Aconitic acid	0.158	0.064	y=32.99x-0.70	0.991	16.4/31.4
Gallic acid	0.132	0.140	y=20.82x+0.055	0.999	13.2/17.0
Protocatechuic acid	4.055	2.389	y=12.86x+0.21	0.997	21.9/38.6
Gentisic acid	1.225	0.731	y=12.15x-0.024	0.997	18.5/28.2
Protocatechuic aldehyde	0.199	0.317	y=25.47x+0.26	0.996	15.4/22.2
Chlorogenic acid	38.948	20.005	y=36.39x+0.29	0.995	13.1/17.6
Caffeic acid	0.640	0.519	y=95.46x+0.12	0.999	7.7/9.5
Salicylic acid	0.130	0.091	y=153.66x+0.24	0.999	6.0/8.3
Cynaroside	0.686	0.123	y=6.13x+0.28	0.997	12.1/16.0
Miquelianin	18.551	24.601	y=5.50x-0.01	0.999	10.6/14.7
isoquercitrin	10.289	16.582	y=4.11x-0.11	0.998	8.7/13.5
Rutin	0.351	0.303	y=-0.08+2.90x	0.999	15.7/22.7
o-Coumaric acid	0.049	0.020	y=0.008+11.21x	0.999	31.8/40.4
Astragalin	0.309	0.677	y=0.008+3.51x	0.999	6.6/8.2
Quercetin	0.819	3.192	y=3.39x+0.006	0.999	15.5/19.0
Luteolin	0.703	1.169	y=-0.054+30.74x	0.999	2.6/4.1
Hesperetin	0.068	0.302	y=6.072x+0.044	0.999	7.1/9.1
Apigenin	0.029	0.055	y=34.87x+0.12	0.998	1.3/2.0
Amentoflavone	0.023	0.051	y=33.37x+0.727	0.992	2.8/5.1
Naringenin	nd	0.019	y=14.64x-0.004	0.999	2.6/3.9
Kaempferol	nd	0.047	y=3.14x-0.005	0.999	10.2/15.4

[HHM, *Hypericum heterophyllum* methanol extract; HHA, *Hypericum heterophyllum* acetone extract. LOD, Limit of detection; LOQ, limit of quantification; nd, not detected]

perforatum L., which is the most known and used *Hypericum* species. Miquelianin is an important antioxidant that significantly suppresses the consumption of lycopene, β-carotene and α-tocopherol. Treatment with 0.1 μM miquelianin is noted to suppress ROS formation, cAMP, and RAS activation, phosphorylation of ERK1/2 and expression of HMOX1, MMP2, and MMP9 genes. Miquelianin suppresses the invasion of MDA-MB-231 breast cancer cells and MMP9 induction. Miquelianin may function to suppress invasion of breast cancer cells by controlling the 2-adrenergic signal, and it has also been reported that it may be a chemopreventive factor in the diet for stress-related breast cancer[38,39]. Isoquercitrine (quercetin-3-O-β-D-glucopyranoside) is found in fruits, vegetables and plant-derived foods and beverages. It has higher bioavailability than quercetin and exerts a range of chemoprotective effects both *in vitro* and *in vivo* against oxidative stress, cancer, cardiovascular disorders, diabetes and allergic reactions. Investigation of its antiradical activity revealed the ability of iso-quercitrine to scavenge superoxide anion radicals, hydroxyl radicals, peroxyl radicals and peroxynitrite ROS and RNS. Isocersitrine has also been found to scavenge superoxide radicals produced by a xanthine/xanthine oxidase system and inhibit xanthine oxidase activity. Isoquercitrine has also been found to attenuate lipopolysaccharide (LPS) induced inducible nitric oxide synthase (iNOS) expression[38,39].

Herbal medicines continue to evolve as an alternative to synthetic medicines. However, due to their complex structure, interactions with other drugs, allergic reactions, and poisoning due to the metals it contains can also be seen. Therefore, besides secondary metabolites in plant material, characterization of element types and element profiling have become important for quality assessments and control measures[40]. Bioelement content study with *H. heterophyllum* has not been found in the literature. In a study, 11 elements (Al, B, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr and Zn) was found in *H. perforatum* andCa, Mg and Fe had the highest concentration[40]. The elements determined in the presented study and their concentrations are shown in Table 4. Al, B, Ba, Be, Bi, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn are qualitatively determined elements. Among these, the elements with the highest concentration were determined as Ca (4958.32±27.79 mg/kg), K (3583.57±47.30 mg/kg) and Mg (1538.72±12.03 mg/kg). It has been determined that *Hypericum heterophyllum* contains the same elements in high concentrations with *H. capitatum*, which is also the endemic species.

Table 4 — Qualitative and quantitative analysis of the multielements in *Hypericum heterophyllum* by ICP-OES

Bio-element	Concentration (μg/g dry plant)	Detection wavelenght (nm)	R^2	LOD (μg/g dry plant)
Al	114.80±6.22	309.401	0.9986	0.00734
B	47.11±2.31	249.773	0.9994	0.00197
Be	0.024±0.003	313.042	0.9991	0.000064
Bi	13.75±1.89	223.061	0.9977	0.0222
Ca	4958.32±27.79	214.438	0.9999	0.0008
Cd	0.12±0.041	267.716	0.9999	0.000427
Cr	0.24±0.004	317.933	0.9997	0.00734
Cu	9.14±0.26	324.754	0.9998	0.000293
Fe	98.21±3.31	259.941	0.9997	0.000609
K	3583.57±47.30	766.491	0.9999	0.0275
Mg	1538.72±12.03	279.553	0.9993	0.000074
Mn	106.77±3.96	257.611	0.9989	0.000558
Na	117.76±7.67	330.237	0.9996	0.0645
Ni	0.88±0.03	231.604	0.9997	0.000939
Pb	2.94±0.81	220.353	0.9988	0.0109
Zn	43.14±0.69	213.856	0.9999	0.00131

[Multielement analyses of *Hypericum heterophyllum* quantified by ICP-OES. Concentrations are given as mean ± standard deviation. LOD; Limit of detection]

Conclusion

The above study on the endemic *Hypericum heterophyllum* species is significant as it can be widely used for its beneficial effects of the flavonoids, particularly their antioxidant and anti-inflammatory activities. Its antioxidant and/or antiradical effects may be mediated by direct clearance of reactive oxygen/nitrogen species (ROS/RNS), inhibition of pro-oxidant enzymes, or induction of antioxidant enzymes. It is thought that the antioxidant effect of flavonoids and elements involved in the antioxidant enzyme structure may play a positive role in various oxidative stress-related diseases such as inflammation and cancer. It was observed that the phenolic content and antioxidant status of the methanol extract of the *H. heterophyllum* was high. It was found that acetone extract showed more effective antibacterial and anticarcinogenic properties. Since the most common phytochemicals in both extracts are chlorogenic acid, miquelianin and isoquercitrin, it can be said that they are antioxidant/anticarcinogenic. In the mineral analysis, the fact that the plant contains Ca, K and Mg elements in high concentrations, as well as the high concentrations of Fe and Mn elements included in the antioxidant enzyme (CAT and GPx) structure may have contributed to the antioxidative effect of the species. As a result of

the demonstration of the biological effects of *H. heterophyllum* extracts as well as the mechanisms to achieve this effect, it will enable the development of more effective and targeted protective and therapeutic formulations.

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Conflict of interest

Authors declare no competing interests.

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