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The Biological Activities of *Hypericum perforatum* L.

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Abstract

Representing the Hypericaceae family, *Hypericum perforatum* L. offers antidepressant, wound-healing, antioxidant, antiviral, and analgesic properties for burns, bruising, and edema. With the demise of many current treatment choices and the worrying levels of antibiotic resistance produced by dangerous bacteria, new therapy approaches are required. This study focused on *H. perforatum* plant's total phenolic content, antioxidant capability, and antibacterial activity. The fatty acid composition and phytochemical constituents in *H. perforatum* were also analyzed by GC-MS. The antibacterial activity of the plant's methanol extract was evaluated using disc diffusion method. Microorganisms utilized in the antibacterial activity assay included the fungus *Candida albicans*, bacteria strains *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. It was determined that *H. perforatum*'s methanol extract exhibited remarkable antagonistic activity against the examined pathogenic microorganisms with the lowest and strongest antibacterial effects against *S. typhimurium* and *P. aeruginosa*, respectively. The Folin-Ciocalteu phenol reagent method was used to quantify the phenolic compounds, and *H. perforatum* methanol extract was shown to have a total phenolic compound content of 1538.98±11.88 µg GAE mL^{-1}. The antioxidant capability was evaluated with three different methods. The methanol extract of the plant exhibited a 1552.74±3.85 µmol TE mL^{-1} DPPH radical scavenging activity while it showed 2876.92±15.46 µmol AAE mL^{-1} and 3307.84±45.87 µmol TE mL^{-1} PFRAP and CUPRAC values, respectively. The GC-MS analysis revealed that palmitic acid and linolenic acid were the predominant fatty acid species, along with the major phytochemical contituents were found as linalyl acetate and alpha-pinene.

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1. Introduction

Hypericum perforatum L. (St. John's Wort) is the most broadly grown plant among the *Hypericum* species, with yellow flowers, stemless, oval and linear, hairless and generally woody structure at the base (Burunkaya et al., 2021). Perennial *H. perforatum* is extensively found in temperate and tropical regions of America, Europe, Asia, Africa and Australia and grows naturally in areas up to 2500 m above sea level (Silva et al., 2021).

Hypericum is one of the oldest plant groups belonging to the Hypericaceae family, containing approximately 500 species that have been widely used in folk medicine for centuries (Crockett et al., 2011, Grafakou et al., 2022). This genus includes more than 100 taxa in Türkiye. They are classified into 19 sections, including 45 species endemic to Türkiye (Boga et al., 2016). Due to its many uses, such as treating skin injuries and burns, as well as stomach ulcers, bile disorders, bronchial and genitourinary tract inflammation, colds, migraines, headaches, obesity, and diabetes mellitus, it is a highly attended herbal folk medicine (Božin et al., 2013; Tokgöz et al., 2020).

The presence of bioactive substances, such as xanthones, phloroglucinols, flavonoids, biflavones, and naphthodianthrones is thought to be responsible for *H. perforatum*'s therapeutic effects. Additional biological activity of *H. perforatum* extracts include wound healing, antidiabetic, antiviral, antibacterial, antitumoral, and antioxidant properties (Kapoor et al., 2023). Pharmacological research has shown that several extracts made from the stem and flowers of *Hypericum* species have antibacterial, antidepressant, antioxidant, and antinociceptive properties (Eruygur et al., 2019). Plants, which are used medicinally, have recently attracted great attention due to the increasing need for raw materials as sources of organic bioactive compounds. These plants have been studied for antimicrobial activity against various microorganisms (Tala et al., 2015).

Phloroglucinol derivatives have demonstrated antifungal and antibacterial effects against some bacterial strains, such as *Bacillus cereus*, *Staphylococcus aureus*, *Nocardia garden*, and *Bacillus subtilis*. They are commonly found in the lipophilic fractions of several *Hypericum* species. The use of some *Hypericum* species in the treatment of microbiological diseases is becoming increasingly widespread. Recent research has shown that medicinal plants may have antibacterial properties, which makes it crucial to employ them in the treatment of both infectious and some chronic illnesses (Sherif et al., 2023; Tusevski et al., 2024).

Scientific research is required to determine the pharmacological and chemical characteristics of the bioactive chemicals found in *Hypericum* species, as well as to cultivate the plant in order to preserve and produce large quantities of these compounds (Çırak and Kurt, 2014). St. John's wort is consumed in different forms (tea, oil, etc.), but studies on the phenolic compound contents, antioxidant capacity and antimicrobial effects of *H. perforatum* grown in Türkiye remain limited.

The purpose of this study was to determine the biological activity of *H. perforatum*. For this purpose, methanol extract of *H. perforatum* was investigated in terms of total phenolic compounds, antioxidant, and antimicrobial activity. In addition, some phytochemical constituents and fatty acid composition of the hexane extract of the plant were determined by GC-MS.

2. Materials and Methods

H. perforatum plants used in this study were obtained commercially from Niğde Province and dried at 40 °C. The dried plant samples with a constant weight were then ground and stored under laboratory conditions before extraction.

2.1. Extract preparation

One gram of dried plant sample was mixed with 30 mL methanol and homogenized at 9000 rpm for 10 min by a Daihan HG-15D homogenizer. The mixture was then incubated

in an ultrasonic bath (Sonorex, Bandelin) at 40 °C for 30 min. All the plant debris was precipitated by centrifugation at 4000 rpm for 20 min. Next, the supernatant was passed through Whatman No. 1 filter paper. A rotary evaporator (HeiVap Value, Heidolph) was used to extract the solvent until it was completely dry. The residue was re-suspended in enough methanol to obtain an extract with a concentration of 100 mg mL^{-1}. The total phenolic compounds (TPC), antioxidant and antimicrobial effect assays were performed using this extract and its dilutions.

2.2. Content of total phenolics

Total phenolic compounds of *H. perforatum* methanol extract were determined by Folin Cocalteu's phenol reagent method (Singleton et al., 1999; Torunoğlu et al., 2024). Briefly; 100 µL extract was added to Folin Cocalteu's phenol reagent, shaken and incubated at ambient temperature for 10 min. Following the addition of 0.71 M Na_2CO_3 solution, the mixture has remained at ambient temperature for an additional 90 minutes in the dark. The absorbances of the blue color formed in the mixture and the blank solution without the extract were measured by a spectrophotometer (MultiSkan GO, Thermo) at 765 nm. All measurements were performed as triplicates. A gallic acid standard curve was established for the assessment of the results in µg GAE mL^{-1} extract.

2.3. Antioxidant activity

2.3.1. DPPH radical scavenging assay

DPPH (2,2 diphenyl-1-picrylhydrazyl) radical scavenging activity assay was performed to evaluate the antioxidant capability of the methanol extract of *H. perforatum*. DPPH has a maximum light absorption at 517 nm (Blois, 1958; Shimada et al., 1992). DPPH solution (0.1 mM) and a 5-step serial dilution of *H. perforatum* methanol extract (1, 2, 3, 4, and 5 mg mL^{-1}) were prepared with methanol. After adding 100 µL of extract from each dilution to 2.9 mL of DPPH solution, the mixture was agitated rapidly and remained at ambient temperature for 15 min in the dark. After the samples were incubated, the absorbances were measured at 517 nm using a spectrophotometer (MultiSkan GO, Thermo). The DPPH solution was used as a blank. A standard curve was established using the scavenging activity of various concentrations of Trolox. All the measurements were performed as triplicates and the results were presented as means and standard deviations in µmol TE mL^{-1} extract.

2.3.2. Potassium ferricyanide reducing antioxidant power (PFRAP) assay

The antioxidative activity of the sample, which reacts to generate potassium ferrocyanide ($K_4[Fe(CN)_6]$) by combining with potassium ferricyanide ($K_3[Fe(CN)_6]$), can be linked to a rise in the absorbance of ferric ferrocyanide, a complex with a maximum absorbance at 700 nm (Xiao et al., 2020). The PFRAP test was carried out in accordance with Mokrani et al. (2016) report. The ascorbic acid standard curve was established for data evaluation and the results were represented as µmol AAE mL^{-1} extract.

2.3.3. Cupric ion reducing antioxidant capacity (CUPRAC) assay

When substances that function as electron donors are present, the color of the neocuproine and $Cu(ii)Cl_2$ mixture changes from bright yellow to orange, signifying that the sample has the ability to reduce cupric (Cu^{+2}) ions to cuprous (Cu^{+}) ions. With minor adjustments, the CUPRAC assay was performed in accordance with the original technique of Apak et al. (2004). At 450 nm, each sample's absorbance was measured in relation to a blank solution. To assess the antioxidant capacity of *H. perforatum* extract at varying doses, the Trolox standard curve was developed, and the results were represented as µmol TE mL^{-1} extract.

2.4. Antimicrobial activity

The antimicrobial activity of *H. perforatum* methanol extract was evaluated by disc diffusion method against test microorganisms. *Pseudomonas aeruginosa* DSMZ 50071, *Salmonella typhimurium* SL1344, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus*

subtilis DSMZ 1971 and *Candida albicans* DSMZ 1386 were used as test microorganisms. The microbial strains were cultured for 24 hours at 36 °C in Luria-Bertani (LB) broth medium. The turbidity of the microorganisms was regulated to 0.5 McFarland standard before the assay (Taşkaya et al., 2023).

LB agar medium was used to plate 100 µL of microbial cultures. After that, 20 µL aliquots of the *H. perforatum* methanol extract with 25, 50, and 100 mg mL^{-1} concentrations were transferred to sterile paper discs that had a 6 mm diameter and were placed on the agar medium. The extraction solvent (methanol) was used as negative control and the discs containing vancomycin (30 µg/disc) and gentamycin (10µg/disc) were used as positive control. After a 24-hour incubation period at 36°C, the diameters of the inhibition zones that developed around the discs were measured on the plates. Each experiment was carried out in triplicate, and the measurements were given in millimeters.

2.5. GC-MS analysis

Although the main bioactive constituents such as hypericin, hyperforin and their derivatives of *Hypericum* species are generally determined and characterized by liquid chromatography (Nait-Si and Fourneron, 2004; Akdeniz et al., 2020; Zhang et al., 2022), GC-MS is preferred to investigate some aromatic compounds and fatty acids (FA). To determine aromatic compounds and FA composition of *H. perforatum* one g powdered plant sample was homogenized with 25 mL of hexane and incubated at room temperature in the dark overnight. Additional incubation in an ultrasonic bath for 30 min was also performed. The homogenate was then filtered through a filter paper and concentrated using a rotary evaporator. Methyl esterification was maintained by 2 M KOH in methanol and 1 N HCl. The upper layer of the extract was analyzed in GC-MS after being dried by anhydrous Na_2SO_4 and passed through a syringe filter.

GC-MS analysis was performed with a QP2010 Ultra GC-MS (Shimadzu) containing a Restek Rxi 5MS column (30 m x 0.25 mm x 0.25 µm). One µL extract was injected into the GC with a split ratio of 1:40 using AOC 2.0 autosampler. The temperature profile of GC was set according to Canpolat and Canpolat (2023). All compounds extracted from *H. perforatum* were identified by comparing their mass spectra with those from the Wiley mass spectra library (W9N11) and the Flavor and Fragrance Natural and Synthetic Compounds Library (FFNSC 1.2).

3. Results and Discussion

3.1. Total phenolic compounds and antioxidant activity

The biological activities of *H. perforatum* are well documented in terms of antidepressant (Bukhari and Dar, 2013), neuroprotective (Özdemir et al., 2016), antiepileptic (Ivetic et al., 2011), antimicrobial and antiviral (Chen et al., 2019), antitumor (Yi et al., 2015), and antioxidant (Radulović et al., 2007; Sagratini et al., 2008; Öztürk et al., 2009) activities which are frequently related with the content of secondary metabolites such as hypericin, hyperforin, and pseudohypericin along with the phenolic compounds (Zdunić et al., 2011; Budantsev et al., 2021). The total phenolic compound content of *H. perforatum* methanol extract at a concentration of 5 mg mL^{-1} was ascertained by Folin-Ciocalteu's phenol reagent method and found as 1538.98±11.88 µg GAE mL^{-1}.

Many scientific studies have shown that antioxidants reduce cellular damage, support defense and help prevent oxidative damage to cellular components (Wong et al., 2006; Çilesiz et al., 2023). The antioxidant activity of *H. perforatum* methanol extract was also investigated using three different techniques. In DPPH radical scavenging assay, DPPH can be transformed into its reduced form as DPPH-H by antioxidants acting as hydrogen donors which is determined by colorimetry with a decreased absorbance value at 517 nm. The highest DPPH scavenging activity in this study was determined as 1552.74±3.85 µmol TE mL^{-1} at the extract concentration of 5 mg mL^{-1}. The high antioxidant activity of *H. perforatum* is generally associated with the content of

phenolic compounds that donate H^+ to DPPH (Orčić et al., 2011).

Three *Hypericum* species, namely *H. perforatum*, *H. origanifolium*. and *H. scabrum*, were investigated in terms of total phenolic contents and antioxidant activities by Seyrekoğlu et al. (2022) indicating that the total phenolic contents of three species were found as 128.82±6.31; 137.03±9.67 and 148.31±4.57 mg GAE g^{-1} DW, respectively, while the IC_{50} values of ethanol extracts against DPPH were determined as 3.17±0.23; 3.79±0.27 and 3.65±0.4 µg mL^{-1}, respectively. They stated that the IC_{50} values of the ethanol extracts in the DPPH assay were higher than those of BHT and ascorbic acid, which were used as standard compounds, indicating the high antioxidant capability of the *Hypericum* species.

The DPPH radical scavenging activity of *H. perforatum* collected from İzmir, Türkiye was investigated along with some other biological activities. It was reported that the methanol extract at a concentration of 100 mg mL^{-1} exhibited a DPPH radical scavenging activity of 32 % (Okmen et al., 2017). However, the study conducted by Zheleva-Dimitrova et al. (2010) indicated that the DPPH radical scavenging activity of *H. perforatum* collected from Bulgaria was 77.6 %.

In another study investigating the effect of solvent concentration, temperature, and extraction time on extract yield, total phenolic content, and antioxidant activity of *H. perforatum*, it was indicated that the most effective DPPH radical scavenging activity was determined by the extract obtained using 100 % ethanol (EtOH) while it was followed by the extract obtained using 30 % EtOH (Seyrekoğlu and Temiz, 2020). The study also stated that 30 °C and 40 min were found as optimal temperature and extraction time values for the highest yield extracts with the highest DPPH radical scavenging activity.

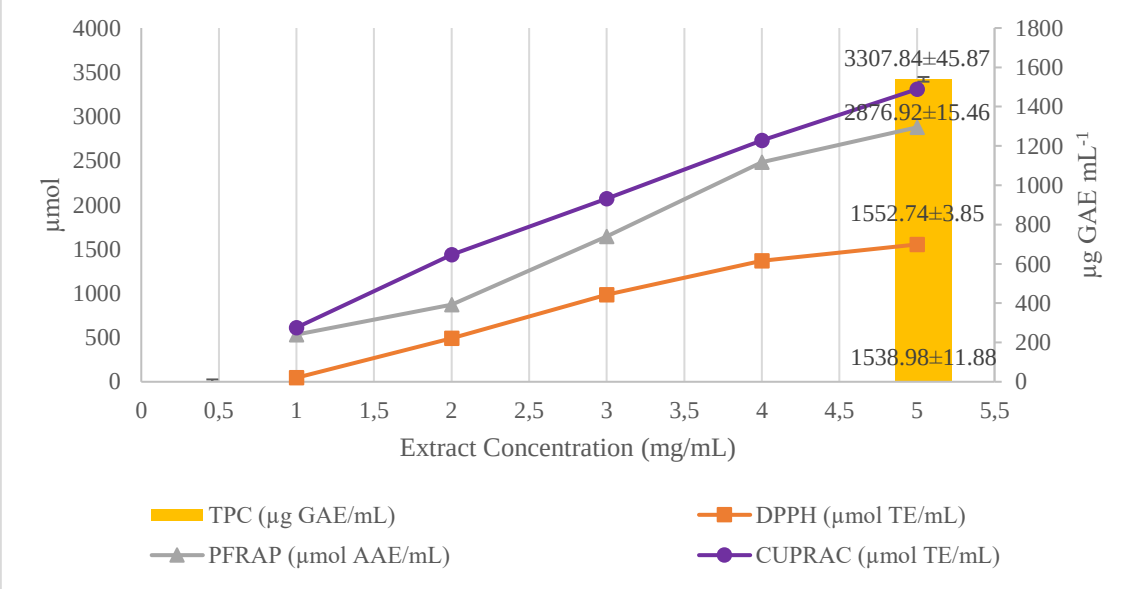

Fig 1. The total phenolic compounds and antioxidant activity (DPPH, PFRAP, and CUPRAC) of *H. perforatum* methanol extract with various concentrations

The antioxidant activity of *H. perforatum* was also evaluated in terms of metal ion reducing capability via potassium ferricyanide reducing power (PFRAP) assay and cupric ion reducing antioxidant capacity (CUPRAC) assay. PFRAP assay depends on the reducing power of the substances present in the extract which act as electron donors to reduce ferric (iii) ions to ferrous (ii) ions. Similarly in the CUPRAC assay cupric (ii) ions are reduced to cuprous (i) ions hydrogen donation by antioxidant substances that are present in the extract (Kırmızı Sönmez et al., 2022). Fig 1 indicated that methanol extract of *H. perforatum* at 5 mg mL^{-1} concentration exhibited a PFRAP value of 2876.92±15.46

µmol AAE mL^{-1} and a CUPRAC value of 3307.84±45.87 µmol TE mL^{-1} in this study. Maltaş et al. (2013) investigated the antioxidant and antimicrobial activities of the methanol extracts of three *Hypericum* species and reported that *H. perforatum* exhibited CUPRAC and FRAP values of 19.67±0.71 and 59.73±0.78 mg TEAC g^{-1} extract, respectively. It was also stated that antioxidant activity of *H. perforatum* was well correlated with the phenolic content of the sample which was determined as 10.01±0.89 mg GAE g^{-1} extract. In another study that investigated biological activities of the water extracts from some plants used as folk remedies in Türkiye, Sarıkürkçü et al. (2020) reported that *H. perforatum* water extract with a 181.02±1.47 mg GAE g^{-1} total phenolic content performed CUPRAC and FRAP values of 268.81±5.32 and 92.52±0.11 mg BHAE g^{-1} extract, respectively.

It was considered that the difference in the antioxidant activities of *H. perforatum* between the literature, and this study; might be related to the geographical, meteorological, and environmental conditions of the sampling area along with the investigated plant part, the growth phase of the plant, solvent, extraction conditions (time, temperature, technique, etc.) and the modifications in the experimental methods.

3.2. Antimicrobial activity

The antimicrobial activity of methanol extract of *H. perforatum* with different concentrations was investigated by disc diffusion method against six strains including one fungal strain, Gram-negative and Gram-positive bacteria.

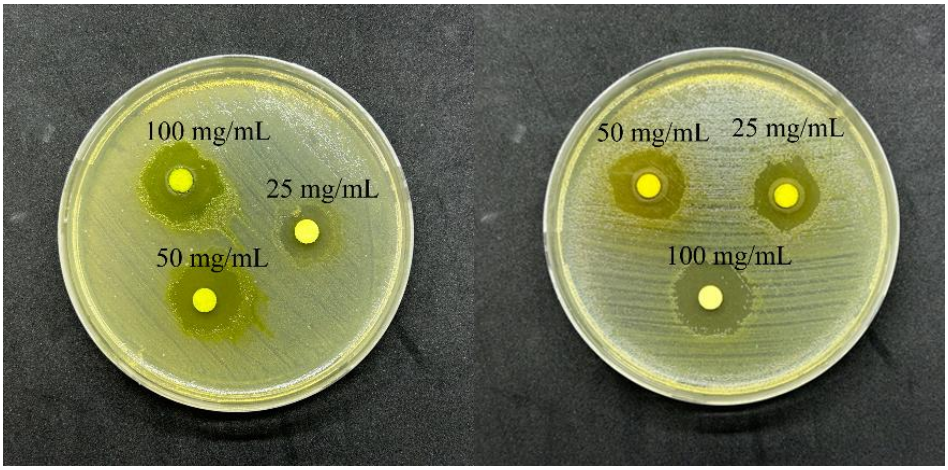

Figure 2. Antimicrobial activity of *H. perforatum* methanol extract against *P. aeruginosa* DSMZ 50071 (left) and *S. aureus* ATCC 25923 (right).

According to Table 1, the results revealed that the highest antimicrobial activity was exhibited by 100 mg mL^{-1} methanol extract against *P. aeruginosa*, a Gram-negative bacterium, followed by *S. aureus*, a Gram-positive bacterium. The findings are in agreement with studies reporting antimicrobial activity of all *Hypericum* extracts against *P. aeruginosa* (Barış et al., 2011; Gül et al., 2021). According to the studies conducted with pathogen bacteria including Gram negative (*P. aeruginosa*, *E. coli*) and Gram positive (*S. aureus, B. subtilis)* the extracts obtained with various solvents from *Hypericum* species exhibited remarkable antibacterial activity against these strains (Türker et al., 2018; Özkan et al., 2018).

Table 1. Inhibition zone diameters of *H. perforatum* methanol extract measured against test microorganisms

	Inhibition Zone Diameters (mm)				
	H. perforatum (mg mL^{-1})				
Test Microorganisms	**25**	**50**	**100**	**VA**	**CN**
E. coli ATCC 25922	8.67±0.47	11.33±0.47	13.67±0.47	12	20
S. typhimurium SL1344	7.33±0.47	8.33±0.94	12.00±0.82	8	21
P. aeuroginasa DSMZ 50071	13.33±1.25	16.67±1.25	18.67±0.94	nd	20
S. aureus ATCC 25923	10.33±0.47	13.67±0.47	17.33±0.47	17	22
B. subtilis DSMZ 1971	9.67±0.94	11.67±0.48	14.33±0.94	9	20
C. albicans DSMZ 1386	10.67±0.47	12.33±0.47	14.67±0.47	nd	nd

*****Results indicate means ± standard deviations of three independent experiments.
*VA: Vancomycin (30µg/disc), CN: Gentamycin (10µg/disc)

Table 1 also indicated that all concentrations of *H. perforatum* extract showed antimicrobial activity against all test organisms at varying levels. The antibacterial effects of *Hypericum* extracts have been the subject of numerous investigations showing different levels of antibacterial activity (Del Monte et al., 2015; Tusevski et al., 2016; Sherif et al., 2023). Saddiqe et al. (2020) reported that extracts of several *Hypericum* species obtained by various solvents, including methanol, have demonstrated similar antibacterial effects against some pathogen bacterial strains including *Enterobacter aerogenes*. The study also revealed that the antibacterial activity varied significantly depending on the plant species and solvent type.

According to a study examining antibacterial activity using antibiotic-resistant clinical isolates, *H. perforatum* extracts demonstrated superior antibacterial activity against a considerable fraction of Gram-positive bacteria than the susceptibility limits of broad-spectrum antibiotics (Kısa et al., 2023). In the study that investigated the antibacterial activity via disc diffusion method of *H. capitatum* extracted with different solvents, it was reported that all extracts showed antibacterial effect against test microorganisms at various levels (Boga et al., 2016). It was also stated that methanol extract had higher activity against test microorganisms (Boga et al., 2016). Hoş and Tunç (2016) also investigated the antibacterial activity of *H. calycinum* using the extracts obtained from different plant parts (root, leaves and flowers) and reported that the highest inhibitory effect was observed against *Streptococcus mutans*. They also stated that the flower extracts showed better antibacterial activity against test microorganisms than the root and leaf extracts. Additionally, the chemical components, extract yield, and antimicrobial activity might all be impacted by solvents with varying polarities.

3.3. GC-MS results

The GC-MS analyses of *H. perforatum* hexane extract resulted in the determination of 15 compounds including 6 fatty acid methyl esters and 9 phytochemical compounds (Table 2). Palmitic acid (13.92±0.81 %), linolenic acid (11.915±0.125 %), stearic acid (8.465±0.445 %) and linoleic acid (2.44±0.21 %) were found as predominant fatty acids. 1,2-Benzenedicarboxylic acid (1.2±0.1 %) and 4-Pentenoic acid, 4-methyl- (0.685±0.015 %) were also determined. The fatty acid profile of *H. perforatum* consisted of saturated fatty acids compared to unsaturated ones (Table 2). The fatty acid profile was compatible with the literature as it was reported that the extracts obtained from different parts (roots, flowers and aerial parts) of various *Hypericum* species could show saturated or unsaturated fatty acid constituents as predominants (Türkoğlu et al., 2015; Boga et al., 2016; Akdeniz et al., 2020).

Table 2. Compounds obtained from *H. perforatum* hexane extract by GC-MS analysis

No	Compound	RT	MW	CF	RC
1	alpha-Pinene	7.72	136	C10H16	8.145±0.165
2	4-Pentenoic acid, 4-methyl-, methyl ester	7.944	128	C7H12O2	0.685±0.015
3	Cymene	10.463	134	C10H14	1.02±0.08
4	dl-Limonene	10.596	136	C10H16	1.32±0.03
5	Eucalyptol	10.685	154	C10H18O	1.635±0.055
6	Linalool	12.816	154	C10H18O	6.42±0.27
7	Camphor	14.216	152	C10H16O	1.305±0.025
8	endo-Borneol	14.883	154	C10H18O	0.62±0.04
9	Verbenone	16.195	150	C10H14O	1.135±0.125
10	Lınalyl acetate	17.523	196	C12H20O2	10.55±0.48
11	1,2-Benzenedicarboxylic acid, diethyl ester	26.795	222	C12H14O4	1.2±0.1
12	Palmitic acid, methyl ester	36.231	270	C17H34O2	13.92±0.81
13	Linoleic acid, methyl ester	39.949	294	C19H34O2	2.44±0.21
14	Linolenic acid, methyl ester	40.084	292	C19H32O2	11.915±0.125
15	Stearic acid, methyl ester	40.603	298	C19H38O2	8.465±0.445

*RT: Retention time (min); MW: Molecular weight; CF: Chemical formula; RC: Relative concentration (mean ± standard deviation %).

Linalyl acetate (10.55±0.48 %), alpha-pinene (8.145±0.165 %), and linalool (6.42±0.27 %) were found as the major phytochemical compounds along with cymene, limonene, eucalyptol, camphor and verbenone in the hexane extract of *H. perforatum*. Previous studies on essential oil profiles and phytochemical constituents on various *Hypericum* species showed similarities with our findings (Bağcı and Yüce, 2011; Çırak and Bertoli, 2013).

4. Conclusions

The analytical methods used to determine antioxidant and antimicrobial activity may provide specific but limited information about these features. In this study, the antioxidant and the antimicrobial activity of the methanol extract obtained from *H. perforatum* were ascertained by three different methods (DPPH radical scavenging activity, PFRAP and CUPRAC) and disc diffusion method, respectively. GC-MS analysis was also conducted to determine the phytochemical composition of the *H. perforatum hexane extract*. It can be said that this study on this plant has contributed to the use of natural sources as antioxidant and antimicrobial agents with high efficiency and fewer side effects. Since, Türkiye is one of the richest sources in terms of plant diversity due to its geographical advantages, research on finding, extracting and identifying novel antioxidant and antimicrobial agents should be promoted, particularly for *Hypericum* genus with many endemic species.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article and that they have read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that they have no conflict of interest for this study.

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