

Chemical Profiling and Antimicrobial Activity of Essential Oils from *Hypericum adenotrichum* Spach. An Endemic Species

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Received: 09 November 2022 / Revised: 09 February 2023 / Accepted: 09 February 2023

ABSTRACT: The purpose of this study was to determine the content of essential oils isolated from the herba and roots of *Hypericum adenotrichum* Spach., an endemic, wild-growing species of Turkish flora. Gas Chromatography-Mass Spectroscopy (GC-MS) and Gas Chromatography-Flame Ionization Detector (GC-FID) were used to examine the essential oils (EO). In the essential oils of the root of *H. adenotrichum*, the compounds were found, with undecane (64.4%), hexadecanoic acid (5.3%), and α -copaene (3.9%) as the primary components. Three significant herba compounds, germacrene D (13.3%), hexadecanoic acid (11.3%), and cadinene (6%) were found. Moreover, in this study antimicrobial effects of EO of endemic *H. adenotrichum* against *Escherichia coli* NRRL B-3008, *Staphylococcus aureus* ATCC 6538, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 700699, *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6258. *H. adenotrichum* root and herba extracts were made, and LC-MS/MS analysis was used to determine the primary constituents of the extracts. The chemicals found in the herb and root, respectively, include 3-caffeoylquinic acid, 5-caffeoylquinic acid, quercetin hexoside, quinic acid, quercetin, and catechine.

KEYWORDS: Antimicrobial activity; GC-MS; GC-FID; *Hypericum adenotrichum*, LC-MS/MS

1. INTRODUCTION

There are 400 species in the *Hypericum* L. genus, which is primarily found in temperate regions of the world and is a member of the Hypericaceae family. Furthermore, for hundreds of years, plants in the genus have been used as a traditional folk treatment [1,2]. In Turkey, there are 96 different *Hypericum* species, 46 of which are endemic, making Turkey a significant geographic distribution hub for these species [3]. Various *Hypericum* species are generally known as “Kantaron, binbirdelik otu, kan otu, koyunkıran, kuzukıran, kılıçotu, mayasilotu, püren, sarı kantaron, and yara otu” in Turkey [3,4]. Secondary metabolites from *Hypericum* species have been classified into at least 11 different kinds, including naphrodiantrons, flurogonol derivatives, flavonoids, organic acids, essential oils, amino acids, xanthones, tannins, proxyanidins, and other water-soluble components. Because of its antispasmodic, antiseptic, anti-depressive, and therapeutic properties for skin conditions like wounds and burns, it is one of the frequently employed genus in pharmacological research [2, 5, 6]. Results from recent studies have shown that hyperforin is the main chemical responsible for the antidepressant effects of *Hypericum* extracts [7]. In addition, when the pharmacological activities of *Hypericum* extracts were evaluated, it was reported that naphthodiantrones, hypericin, and pseudohyperin were mainly responsible for their antidepressive and antiviral activities.

Hypericum adenotrichum Spach is an endemic herbaceous perennial herb that grows naturally in dry grassland and stony areas of Northwestern Turkey. It is known that the stem of the plant is erect or oblique, sometimes prostrate, its leaves are oblong or oblanceolate to linear, its flowers are yellow and numerous, and

How to cite this article: Saltan N, Köse YBK, Kırıcı D, Göger F., Demirci B. Chemical Profiling and Antimicrobial Activities of Essential Oils from *Hypericum adenotrichum* Spach. An Endemic Species. J Res Pharm. 2023; 27(4): 1645-1657.

it is slightly black-dotted [8]. Folk medicine employs *H. adenotrichum* for its antispasmodic, antibacterial, and wound-healing properties, particularly for burns [9]. Since all of the compounds are found in the aerial parts of the plant while it is fully flowering in the wild, this species has a great deal of potential as a medicinal agent [10]. In a study involving 74 species of *Hypericum*, it was shown that *H. adenotrichum* had amentoflavone but no rutin, hyperoside, pseudohypericin, or hyperforin [11]. In the study by Sarimahmut et al., besides the chemical composition analyses of *H. adenotrichum* methanol extract, its genotoxic/antigenotoxic effects were investigated in human lymphocyte culture, and the anti-growth effect of the extract was evaluated in human breast cancer cell lines using MTT and ATP viability assays, and it has been reported that it has cytotoxic and genotoxic effects in a cell type-dependent manner [12]. In another study, the bioactivity of plant extracts prepared from *H. adenotrichum*, proliferation test, cell death detection, determination of specific protein expression profiles for cell cycle arrest and apoptosis, as well as their composition were investigated by HPLC analysis. A powerful p53-independent antineoplastic effect of *H. adenotrichum* has also been demonstrated and it has been reported to be used in wound healing and as an antiseptic in Turkey [13]. Extracts of 10 edible plants, including *H. orientale* L., grown in Ordu province by Kurt et al., were prepared with different solvents (hexane, ethanol, and water separately), and their antioxidant, anticholinesterase, and antiaflatoxic activities were evaluated. It has been reported that *H. orientale* extract shows acetylcholinesterase and butyrylcholinesterase inhibitory activity with IC₅₀ values of 165.20 ± 0.62 and 50.57 ± 0.32 µg/mL, respectively [14]. Although many studies on the phytochemistry, biological activity, and essential oil composition of *Hypericum* species have been conducted [6, 15-20], there is still limited research on *H. adenotrichum*. However, the knowledge of the essential oil compositions of the *H. adenotrichum* species is limited [21]. Also, to the best of our knowledge, no study has been found on the antimicrobial activity of the essential oil obtained from *H. adenotrichum*.

The aim of the present study was (a) to examine the chemical composition of essential oil isolated from the root and herba of endemic *H. adenotrichum* by GC-MS and GC-FID; (b) to evaluate the antimicrobial activity of the essential oil; (c) to determine the compounds, thus adding for the database to phytochemical knowledge for the species. Further investigations on *Hypericum* species are necessary to provide additional knowledge about these plants. In addition, unlike other members of the *Hypericum* genus, the lack of neither ethnomedical nor pharmacological reports on this endemic species in the current literature makes the study meaningful.

2. RESULTS AND DISCUSSION

Thirty-six constituents corresponding to 91.4% of the oil from the root of *H. adenotrichum*, forty-seven constituents corresponding to 91.9% of the oil from herba were identified. The analyses were performed and the results were given in Table 1. Germacrene D (13.3%), hexadecanoic acid (11.3 %), δ -cadinene (6 %), and α -copaene (5.9 %) were identified as major compounds of herba. The main constituents of the volatile oils were given in Table 2.

Table 1. The volatile composition of *H. adenotrichum*

RRI	Compound	Root %	Herba %	Identification Method
1000	Decane	tr	-	RRI, MS
1032	α -Pinene	tr	3.0	RRI, MS
1100	Undecane	64.4	5.3	RRI, MS
1244	2-Pentyl furan	0.3	-	MS
1300	Tridecane	1.2	-	RRI, MS
1466	α -Cubebene	0.2	-	MS
1481	α -Amorphene	0.2	-	MS
1492	Cyclosativene	1.9	0.5	RRI, MS
1497	α -Copaene	3.9	5.9	MS
1544	α -Gurjunene	0.3	-	MS
1553	Linalool	-	0.7	RRI, MS
1597	β -Copaene	-	0.5	MS

1612	β -Caryophyllene	0.2	1.5	RRI, MS
1655	(<i>E</i>)-2-Decenal	-	tr	MS
1661	Alloaromadendrene	0.4	2.4	MS
1677	<i>epi</i> -Zonarene	-	0.3	MS
1687	α -Humulene	-	1.2	RRI, MS
1704	γ -Muuroolene	0.7	2.7	MS
1709	α -Terpinyl acetate	0.2	0.7	RRI, MS
1722	2-Undecanol	0.2	-	MS
1722	Bicyclosquiphellandrene	-	0.3	MS
1726	Germacrene D	0.5	13.3	MS
1729	β - Himachalene	0.5	-	RRI, MS
1740	α -Muuroolene	0.4	1.1	MS
1751	Carvone	-	tr	RRI, MS
1755	Bicyclogermacrene	-	1.5	MS
1758	(<i>E,E</i>)- α -Farnesene	-	0.4	MS
1765	(<i>E</i>)-2-Undecenal	0.3	-	MS
1773	δ -Cadinene	1.8	6.0	MS
1776	γ - Cadinene	-	1.2	MS
1779	(<i>E,Z</i>)-2,4-Decadienal	0.1	-	MS
1799	Cadina-1,4-diene	-	0.3	MS
1807	α -Cadinene	-	0.3	MS
1827	(<i>E,E</i>)-2,4-Decadienal	0.5	-	MS
1849	Cuparene	0.5	-	MS
1849	Calamenene	0.3	0.5	MS
1893	Dodecyl acetate	0.2	-	MS
1941	α -Calacorene	1.4	-	MS
1945	1,5-Epoxy-salvial-4(14)-ene	-	0.4	MS
2008	Caryophyllene oxide	-	0.9	RRI, MS
2071	Humulen epoxide II	-	0.5	MS
2080	Junenol	-	0.7	MS
2080	Cubenol	-	0.9	MS
2088	1- <i>epi</i> -Cubenol	-	1.1	MS
2104	Viridifurolol	-	0.9	MS
2131	Hexahydrofarnesyl acetone	0.7	0.7	MS
2144	Spathulenol	0.4	3.6	MS
2179	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	-	tr	MS
2187	<i>T</i> -Cadinol	-	0.1	MS
2192	Nonanoic acid	-	0.1	RRI, MS
2209	<i>T</i> -Muurolol	0.4	0.1	MS
2210	Copaborneol	1.1	-	MS
2219	δ -Cadinol	0.3	0.1	MS
2255	α -Cadinol	0.7	5.2	MS
2256	Cadalene	0.7	-	MS
2289	4- <i>oxo</i> - α -Ylangene	0.5	1.8	MS
2298	Decanoic acid	-	0.1	RRI, MS
2503	Dodecanoic acid	-	1.3	RRI, MS
2622	Phytol	-	2.8	MS
2670	Tetradecanoic acid	0.7	3.2	RRI, MS
2822	Pentadecanoic acid	-	0.8	RRI, MS
2900	Nonacosane	-	5.7	RRI, MS
2931	Hexadecanoic acid	5.3	11.3	RRI, MS

Total **91.4** **91.9**

Yield **0.05** **tr**

RRI: Relative retention indices calculated against n-alkanes %: calculated from FID data

Tr: Trace (< 0.1 %)

IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column;

MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

Table 2. The main components of essential oils of *H. adenotrichum*

RRI	Compound	Root %	Herba %
1100	Undecane	64.4	5.3
1497	<i>α</i> -Copaene	3.9	5.9
1773	δ -Cadinene	1.8	6.0
2255	<i>α</i> -Cadinol	-	5.2
2900	Nonacosane	-	5.7
2931	Hexadecanoic acid	5.3	11.3
1726	Germacrene D	0.5	13.3

As a result of this research, undecane (64.4%), hexadecanoic acid (5.3 %), and *α*-copaene (3.9%) were identified as major volatile constituents of the root. Although there are many species in *Hypericum* genus, the composition of volatile chemicals is known in only a few species except *H. perforatum* L. . Previous research has demonstrated that the essential oil composition of *Hypericum* species varies depending on where the plants are grown [2, 6, 21-23]. The most often identified volatile chemicals in *Hypericum* flowers and leaves are the aliphatic hydrocarbons *n*-nonane and *n*-undecane, the monoterpenes *α*- and β -pinene, the sesquiterpene β -caryophyllene, and caryophyllene oxide. There have also been reports of *Hypericum* species with essential oil components that are similar to *H. adenotrichum* (undecane, germacrene D, hexadecanoic acid). We also observed that the main components were similar to previous studies related to *H. adenotrichum*. In the study conducted in 2001, it was noted that the volatiles of endemic *H. adenotrichum* comprised undecane (16.5%) and germacrene D (38%) as the primary components, and that 45 compounds, or 93% of the total compounds, had been discovered. Hexadecanoic acid was not detected in any of these compounds [21]. In a taxonomic point of view, *H. adenotrichum* belongs to the section *Crossophyllum* Spach. Many differences are evident when comparing the chemical makeup of the essential oils within the same section. Mathis and Ourisson [24] considered predominant the hydrocarbon sesquiterpene *α*-humulene for *H. orientale* L. while Bertoli et al. detected β -selinene in huge amounts (37.1%) as a major component [25]. The highest level of hydrocarbon sesquiterpene distinguishes *H. orientale* from *H. adenotrichum*, which is in the same section. There are differences in terms of major components when studies on the essential oil composition of various endemic *Hypericum* species are reviewed. By using GC and GC-MS, Yuce and Bagci determined the *H. uniglandulosum* Hausskn. ex Bornm. essential oil concentration of aerial parts growing naturally in Turkey. Twenty-six compounds were identified in the essential oils of *H. uniglandulosum* with *α*-pinene (35.1%) and undecane (19.2%) as main constituents [20]. In another study, *H. aviculariifolium* Jaub. & Spach is analyzed for essential oil composition [26]. Germacrene D (8.5%) and *α*-pinene (52.1%), which were identified using GC and GC-MS analysis of aerial parts, were the predominant components [26]. According to another GC and GC-MS analysis, the main constituents of the essential oils extracted from the aerial parts of *H. capitatum* Choisy var. *capitatum* Choisy plant were undecane (3.8%), β -caryophyllene (6.5%), hexadecanoic acid (8.9%), caryophyllene oxide (11.8%), and *α*-pinene (20.3%) [27]. In a study employing the hydrodistillation process, the compositions of essential oils extracted from the aerial parts of five endemic species of Turkey were determined. Hexadecanoic acid (17.7%) and spathulenol (5.3%) were characterized as the main components of the aerial parts of the endemic *H. scabroides* N. Robson&Poulter. In the same study, the main components in *H. kotschyianum* Boiss were α -pinene (14.4%), nonacosane (11.1%), hexadecanoic acid (9.2%), β -pinene (8.7%), spathulenol (6.3%) and limonene (5.1%) has been noted [28]. Studies have shown that hexadecanoic acid has an anti-inflammatory effect due to its enzyme inhibitory activity. It has also been reported that indirectly validates the rigorous use of medicated oils rich in hexadecanoic acid for the treatment of rheumatic symptoms in Ayurveda, the traditional medicine system of India [29]. Germacrene D, which we identified as one of the primary components of the essential oil extracted from the *H. adenotrichum* herba, has been shown in a prior study to have anticancer effects on various types of cancer cell lines [30]. Also, it is known that germacrene D possess strong antimicrobial activity [31]. It has also been claimed to have insecticidal activity against mosquitoes, insect repellent activity against aphids, and repellent activity against ticks [32-34]. In addition, undecane, one of the primary ingredients in the essential oil extracted from the root, was found to suppress mast cell production of inflammatory mediators like histamine in the prior study [35]. As drugs used to treat multidrug-

resistant microorganisms become less effective, they become more difficult to treat. Therefore, new methods to control microorganisms need to be evaluated. These data indicate that the chemotaxonomic significance of the compounds contained in *H. adenotrichum* and the phytochemical evaluation of the plant are important. In previous studies, essential oil compositions of aerial parts of *Hypericum* species were frequently investigated. According to the results of this research, root and herba volatile components are similar. Our findings suggest that variations in the volatile content of plant material may be dependent on a variety of environmental and climatic circumstances, seasonal periods, and geographic origins when compared to earlier research on the chemical composition of *Hypericum* species.

The essential oils were tested against several bacteria and two fungi (Table 3). The MICs of the essential oils were within concentration ranges from 19.5 µg/mL to >10 mg/mL. The results of antimicrobial activity indicated that the essential oils were active against *Staphylococcus aureus*, *Candida albicans*, and *C. krusei*. The essential oil from the root had higher inhibitory activity against some bacteria than the essential oil from the herba while the results were the contrary for antifungal activity. The antibacterial activity results of *H. adenotrichum* root (0.156 mg/mL) essential oil against *S. aureus* is close to the standard compounds (Ampicillin and Clarithromycin). In a prior study, Gudzcic et al. reported that the essential oil from *H. maculatum* Crantz in Serbia exhibited a wide range of antibacterial effects (12-16 µL/mL), particularly against *S. aureus*, *E. coli*, *P. aeruginosa*, *S. enteritidis*, *K. pneumoniae*, and *B. subtilis* [36]. Kizil et al. investigated the antimicrobial activity of the essential oils of *H. scabrum* L., *H. scabroides*, and *H. triquetrifolium* Turra against the test organisms and a yeast [37]. All the essential oils exhibited large spectrum antibacterial activity, at a concentration of 80 µg/ml. In a different paper, the results of antimicrobial activity of *H. hircinum* L. indicated that the essential oils were active against *S. aureus*, *S. mutans*, *B. subtilis*, *E. coli*, and *C. albicans* by Maggi et al. [38]. The range of 155–625 µg/mL was found to be the MIC value for the bacteria and yeast species that are sensitive to the essential oils [38]. The interaction between the main ingredients and additional constituents of the oils could be used to explain the antibacterial properties of essential oils from the *Hypericum* genus. Many different methods such as LC/UV [39,40], LC/MS [41, 42], LC/SPE/ NMR [43], TLC screening [44] and HPLC [9, 19, 45-47] have been reported to determine the content of major bioactive substances such as naphthodianthrones, phloroglucinols and different phenolics in *Hypericum* species.

Table 3. Antimicrobial activities of *H. adenotrichum* essential oil (MIC, mg/mL)

	Root	Herba	Ampicillin	Clarithromycin	Ketoconazole	Itraconazole	Fluconazole
<i>Escherichia coli</i> NRRL B-3008	>10	>10	0.01	0.02	-	-	-
<i>Staphylococcus aureus</i> ATCC 6538	0.0195	0.078	0.00063	0.00063	-	-	-
<i>Salmonella typhimurium</i> ATCC 14028	>10	>10	0.0013	0.04	-	-	-
<i>Staphylococcus aureus</i> ATCC 700699	0.156	0.3125	0.02	0.16	-	-	-
<i>Candida albicans</i> ATCC 90028	>10	1.25	-	-	0.01	0.04	-
<i>Candida krusei</i> ATCC 6258	-	0.156	-	-	-	0.01	0.04

Dash means no activity

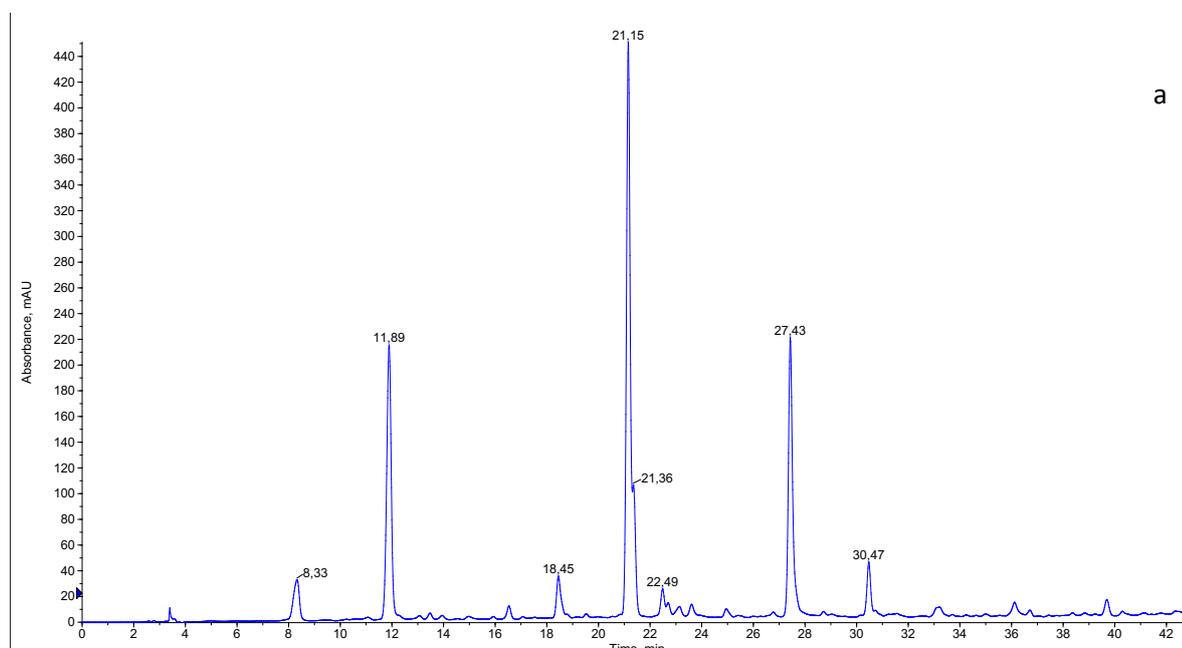
Flavonoids are a group of bioactive compounds found in *Hypericum* species and have been reported to play an important role in the prevention of cardiovascular diseases and various types of cancer [48]. In this study, the LC-MS/MS method recommended as one of the most reliable tools in the analysis of complex plant

extracts was used. According to the results (Fig 1-4) it has been determined that 5-caffeoylquinic acid, quercetin glucoside, and quercetin are the main components, as well as quinic acid, 3-caffeoylquinic acid, catechine, and quercetin hexoside in both the herba and root (Table 4). In addition, myricetin derivative, quercetin pentoside, and luteolin hexoside were detected only in the herba of *H. adenotrichum* (Table 4).

Table 4. Compounds determined with LC-MS/MS in *H. adenotrichum*

Compound No	Rt	M-H	MS2	Identification	Extract	Reference
1	3,4	191	173, 127, 108	Quinic acid	H, R	[47]
2	8,3	353	191, 179, 135	3-Caffeoylquinic acid	H,R	[47]
3	11,9	353	191, 179, 135	5-Caffeoylquinic acid	H,R	[47]
4	12,9	289	271, 245	Catechine	H, R	[47]
5	18,5	479	315, 287, 271	Myricetin derivative	H	[47]
6	21,2	463	301, 271, 255	Quercetin hexoside	H, R	[47]
7	22,5	433	300,271, 255	Quercetin pentoside	H	[47]
8	23,3	447	284, 255, 227	Luteolin hexoside	H	[47]
9	27,4	301	271, 151, 121	Quercetin	H, R	[47]

H: herba; R: root



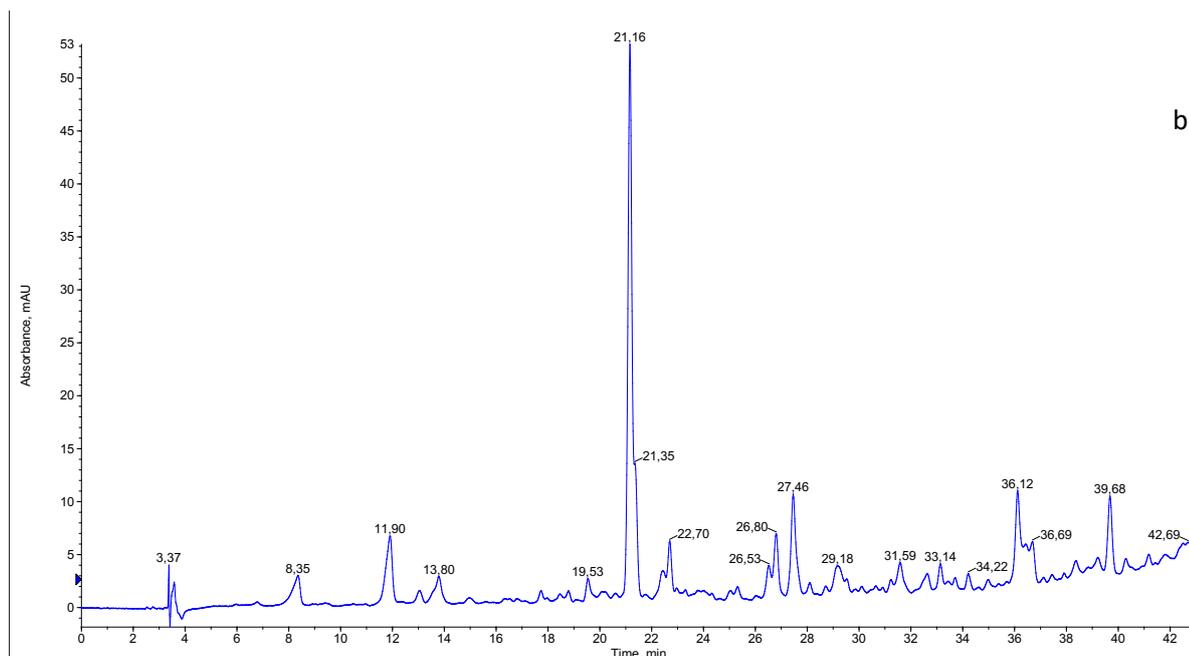


Figure 1. Typical chromatograms (a-herba), (b-root), of *H. adenotrichum* plant material extracts were obtained by LC-MS/MS.

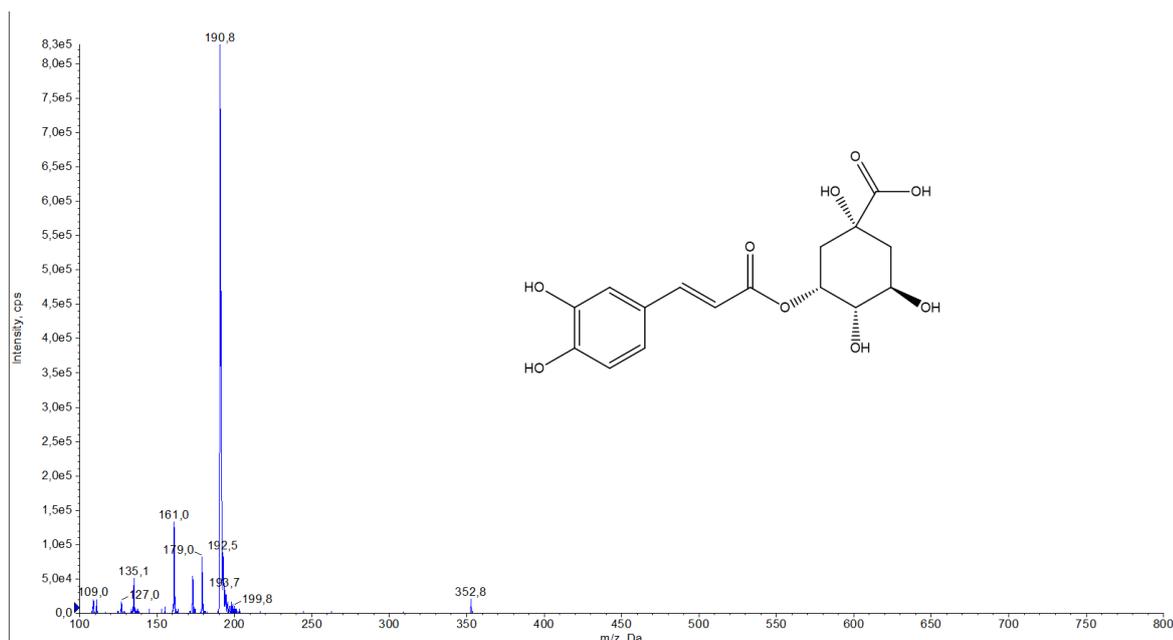


Figure 2. Mass spectrum of compound: 5-Caffeoylquinic acid

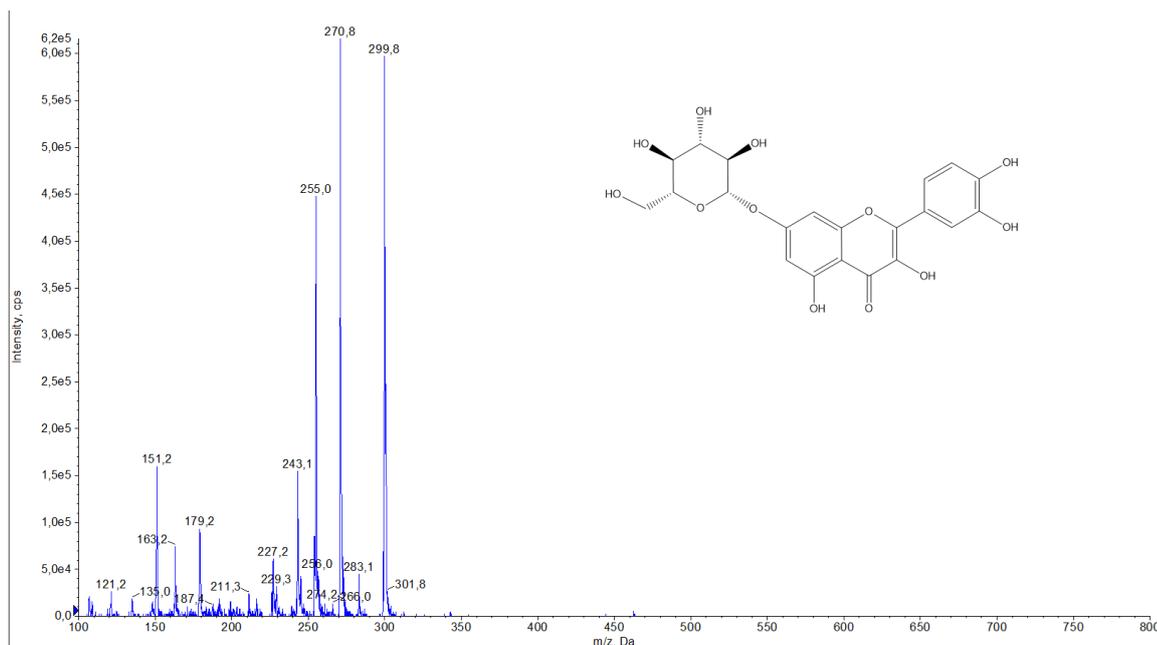


Figure 3. Mass spectrum of compound: Quercetin glucoside

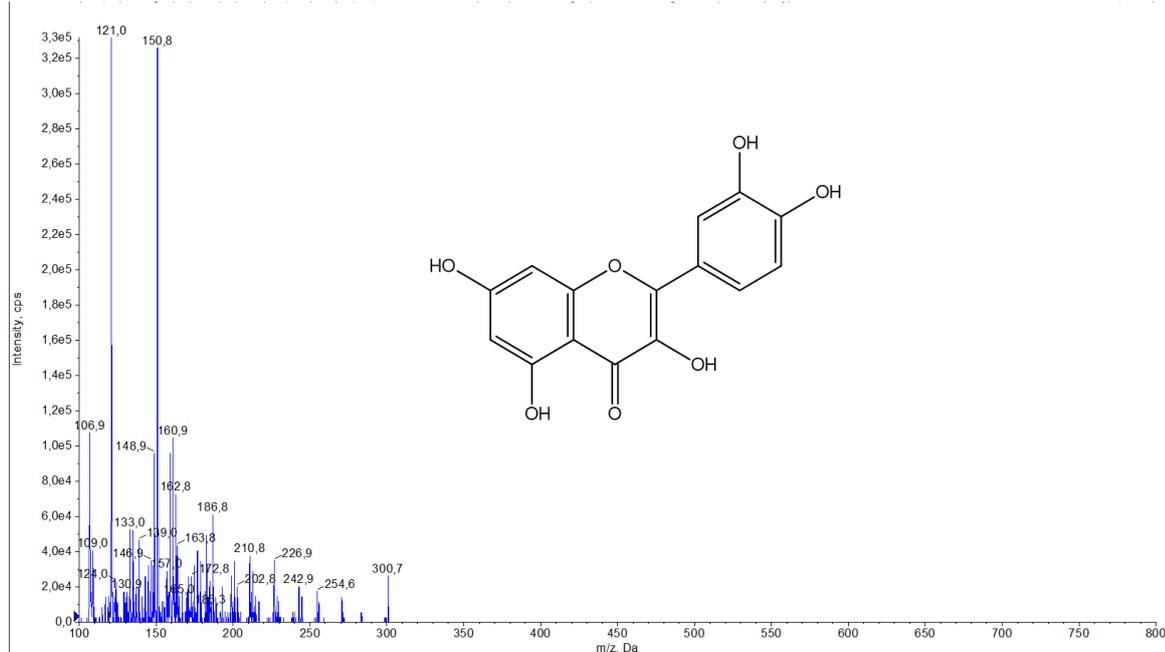


Figure 4. Mass spectrum of compound: Quercetin

The aerial parts of *H. adenotrichum* contain hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, kaempferol, apigenin-7-O-glucoside, and amentoflavone, according to a study [10]. The findings show that when compared to *H. perforatum*, a well-known and commercial source of the secondary metabolites under study, *H. adenotrichum* accumulates lower concentrations of chlorogenic acid, hyperoside, quercetin, and rutin; comparable amounts of hypericin, quercitrin, and amentoflavone; and higher concentrations of pseudohypericin and apigenin-7-O-glucoside [10]. When we compared our findings to those of this earlier study, we observed that there were some differences in the main components (5-caffeoylquinic acid, 3-caffeoylquinic acid, catechin, myricetin derivative, and luteolin hexoside). Cirak et al. showed that the flowers were rich in chlorogenic acid, hyperoside, apigenin-7-O glycoside, kaempferol, quercetin, and amentoflavone, and rutin and quercitrin were found more in the leaf parts in the comparison

between different *H. triquetrifolium* parts [48]. Hyperforin, hypericin, pseudohypericin, rutin, adhyperforin, hyperocyte, quercetin, quercetin, caffeic acid, and chlorogenic acid have all been found in *H. orientale* belonging to the *Crossophyllum* section in recent studies [49-54]. In a study in which HPLC analyses of individual plant parts (stems, leaves, and reproductive tissues) as well as herbs of two *Hypericum* species (*H. aviculariifolium* subsp. *depilatum* (Frey and Bornm.) Robson var. *depilatum* and *H. orientale*) were performed, no accumulation of kaempferol was observed in *H. aviculariifolium* Jaub. et Spach and the highest hypericin, pseudohypericin, and quercitrin levels were observed. It was reported that the plants harvested during the flower budding period produced the highest amount of rutin, hyperoside, and isoquercitrin. In addition, it was reported that hypericin, pseudohypericin, or kaempferol production was not observed in *H. orientale*, rutin, hyperoside, and isocercetin levels were highest in flower development, and the highest amount of quercetin and quercetin were produced in plants harvested in fresh fruits [55]. The comparison of our results with the previously published report revealed that the two species in the same section have different sequences of components. The distribution of *Hypericum* species in different regions has also been demonstrated to be a major source of chemical variation in previous studies. Similarly, in this study, some differences were found in chemical accumulations in *H. adenotrichum*. The chemical variability in the content of chemicals between populations can be attributed to the different environmental conditions of the habitat where the samples were collected. Hence, the detection of the compounds in *H. adenotrichum* in the present study supports the taxonomic position of the section *Crossophyllum* within the genus *Hypericum*. The presence of bioactive compounds in *H. adenotrichum* is promising as it is thought to be a source of medicine, and is the basis of its effectiveness as a medicinal plant.

3. CONCLUSION

Staphylococcus aureus is a major bacterial individual pathogen that causes a wide type of clinical manifestations. Infections are usually found including bacteremia, chemical burns, infective endocarditis, soft tissue, and skin infections. Drug application of *S. aureus* diseases depends mainly on the variety of infections as well as the lack or presence of drug-resistant strain. The period and type of therapy are largely related to the infection type and other factors when antimicrobial treatment is required. Generally, penicillin and vancomycin are the preferred drug therapy for methicillin-sensitive *S. aureus* strains. In some events, an alternative therapy is needed in addition to antibacterial treatment [56]. The results of the study indicate that the antimicrobial effect of the essential oil of *H. adenotrichum*, which is used for alternative treatment, can be benefited from. In addition, since the extraction and characterization of various active phytochemicals from plants has resulted in the emergence of some highly active drugs, in this study, the secondary metabolite content of *H. adenotrichum* root and herb was determined using a highly selective LC-MS/MS method. Considering that secondary metabolites in plants play an important role in human health and nutrition, it is thought that the results of phytochemical studies may contribute to pharmacological activity studies as a preliminary study.

4. MATERIALS AND METHODS

4.1. Plant Material

The plant was collected from Aydın province of Turkey during the flowering time. Herbarium specimens prepared by pressing and drying of the plant were stored in the Herbarium of the Faculty of Pharmacy of Anadolu University, in Eskisehir, Turkey (Fig. 5 ESSE 15493).



Figure 5. *H. adenotrichum*

4.2. Isolation of the essential oils and Plant extraction

Air-dried herba and root were crushed just before hydrodistillation by Clevenger-type apparatus for 3 h. Essential oils (EO) were stored at +4°C until analyses.

H. adenotrichum dried herbal components were macerated with methanol 70% at 25°C for 24 hours. The aqueous portion was freeze-dried once the methanol had evaporated, and the dry extract was employed in all tests.

4.3. GC-FID and GC/MS analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. For GC-FID analyses an Agilent* 6890N GC system was used. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. Other essential oil analysis criteria were carried out as in previous reports [57,58]. By comparing the relative retention times of the essential oil constituents to those of real samples or by comparing the relative retention index (RRI) of the constituents to a group of n-alkanes, the components of essential oils were identified.

For the identification, computer matching against publicly available databases (Wiley GC/MS Library, MassFinder 3 Library) [59,60] and an internal database called the "Baser Library of Essential Oil Constituents," which was created by real compounds and parts of known oils, as well as MS and RI from the literature [22,28], were used. The analysis results are given in Table 1.

4.4. Antimicrobial Activity Assay

The EO obtained from the root and herba of endemic *H. adenotrichum* were examined for antimicrobial activity by the microdilution broth susceptibility assay against *E. coli* NRRL B-3008, *S. aureus* ATCC 6538, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 700699, *C. albicans* ATCC 90028 and *C. krusei* ATCC 6258 using the

microdilution method according to modified CLSI protocols M7-A7 and M27-A2 [61,62]. Ampicillin and clarithromycin were used as standard antibacterial agents, while ketoconazole, itraconazole, and fluconazole were used as standard anticandidal agents. Standard antifungals and EOs dissolved in sterile 50% DMSO (CLSI document M27-A2). Serial two-fold dilutions of the samples were achieved by using sterile 96 U-shaped multi-well plates (Brand). The microplates were incubated at 35°C for 24 h. The MIC (minimal inhibitory concentration) is defined as the lowest concentration in which an optically clear well is observed. The experiments were repeated in duplicates, and MIC values were reported as the mean.

4.5. LC-MS/MS analysis

Using a previously described procedure, the results of the LC-MS/MS analysis of the methanolic extract of *H. adenotrichum* were evaluated [63]. LC-MS/MS analysis was performed with the Shimadzu 20A HPLC system connected to the Applied Biosystems 3200 Q-Trap MS/MS detector. The ionization mode was realized in negative mode with Electro Spray Ionization (ESI). A 150 × 4.6 mm, 3 μm ODS column was used for chromatographic separation. Analyzes were carried out at 40 °C and with a PDA detector. The mobile phase was selected as (A) Methanol: water: formic acid (10:89:1, v/v/v) and (B) Methanol: water: formic acid (89: 10:1, v/v/v). The concentration of B was increased from 10% to 100% in 40 minutes. The flow was set at 0.5 mL/min. Software called Analyst 1.6 was used to collect and process LC-ESI-MS/MS data.

Author contributions: Author contributions: Concept – Y.B.K. N.S.; Design – N.S., Y.B.K., D.K. F.G B.D.; Supervision – Y.B.K B.D.; Resources – N.S., Y.B.K., D.K. F.G.; Materials – N.S., Y.B.K., D.K. F.G., B.D; Data Collection and/or Processing – N.S., Y.B.K., D.K., F.G.; Analysis and/or Interpretation – N.S., D.K. F.G., B.D.; Literature Search – N.S., F.G.; Writing – N.S., Y.B.K.; Critical Reviews – N.S., Y.B.K., F.G., B.D.

The authors declared no conflict of interest.

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