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Determination of phenolic content of *Hypericum aucheri* Jaub. & Spach by LC-HRMS and its antioxidant capacity

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Abstract: The study aims to identify the secondary metabolites of *Hypericum aucheri* Jaub. & Spach extracts using liquid chromatography-high resolution mass spectrometry (LC-HRMS) and to evaluate the plant's antioxidant activity. Antioxidant activity was assessed using DPPH free radical scavenging and CUPRAC assays. The primary secondary metabolite detected was hyperoside, with concentrations of 1059.53 mg/L in acetone (Ac) extract and 389.73 mg/L in methanol (MeOH) extract. Syringic acid was the predominant compound in the chloroform (C) extract, with a concentration of 6646.53 mg/L. In addition, Ac extract showed the highest DPPH free radical scavenging activity (85.90±0.26 - 61.19±0.47%) of all the extracts tested. The MeOH extract exhibited the highest value in the CUPRAC assay (2.53±0.05 mmol TR g^{-1}). The study highlights a numerical relationship between phenolic content and antioxidant activity, underscoring the importance of phenolics in their antioxidant functions.

Keywords: Hypericaceae; *Hypericum aucheri;* antioxidant; LC-HRMS; phenolic compounds.

1. Introduction

The genus *Hypericum* L., belonging to the Hypericaceae (Cluciaceae=Guttiferae) family, comprises more than 500 species worldwide, distributed across various geographic regions, particularly in warm, temperate, subtropical, and tropical areas. Türkiye is a significant region for *Hypericum* species, with by 112 taxa in 20 sections, of which 55 are endemic [1]. These species are known in Türkiye by various regional names, including "kantaron, kan otu, mayosıl otu, binbirdelik otu, kılıç otu, koyun kıran, yara otu" [2].

Hypericum species, known for their wide range of biological properties and rich phytochemical content, have recently gained significant attention. These plants are valued for their diverse medicinal properties and are commonly utilized in both traditional and modern medicine [3]. In folk medicine, *Hypericum* species are used as infusions, decoctions and oleates [1,4]. For many years, *Hypericum* species have been used in traditional Turkish medicine for their sedative, anthelmintic, and antiseptic properties. Additionally, the oil derived from these plants is commonly used to treat conditions such as depression, burns, wounds, bruises, skin inflammation, and nerve pain [2].

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The genus *Hypericum* L. is a taxon of significant economic and medicinal importance, as it serves as a source of various naturally occurring active pharmaceutical compounds [3].

The extraction and evaluation of pure, active substances from extracts and essential oils derived from medicinal and aromatic plants hold significant scientific and economic value [4]. Research indicates that these extracts and essential oils exhibit strong antioxidant activity [5]. It is crucial to understand how antioxidant phenolic compounds affect living organisms for their applications in the pharmaceutical, cosmetic, and food industries [6-10]. Therefore, investigating the active substances and antioxidant properties of plants abundant in phenolic compounds is of considerable importance.

Numerous studies have investigated the phytochemical content and biological activity of *Hypericum* species [11]. The antioxidant activities of ethanol extracts of *H. perforatum* were linked to phenolic compounds [12-14]. Another study investigated the antioxidant activities of ethanol extracts of *H. triquetrifolium* and *H. scabroides* species growing in Türkiye, concluding that their antioxidant activities were strong and that they could serve as natural antioxidants [15]. Similarly, the antioxidant effects of ethanol extracts from *Hypericum* species (*H. scabrum, H. lysimachioides* var. *lysimachioides*, and *H. retusum*) growing in Türkiye were examined using various methods, and all three species were found to exhibit high antioxidant activity [16]. In another study, on *H. perforatum* and *H. calycinum*, the % inhibition value of *H. calycinum* exceeded that of *H. perforatum* using the DPPH method, which is attributed to *H. calycinum*'s higher total phenolic content [17]. In another investigation, the antioxidant activities of 11 different *Hypericum* species growing in Sicily were assessed [18]. In a study by, the antioxidant activities and phenolic contents of *H. neurocalycinum* and *H. malatyanum* species were examined. *H. neurocalycinum* showed higher antioxidant activity than *H. malatyanum*, which is explained by the greater amount of flavonoid compounds in *H. neurocalycinum* [19].

Only a few phytochemical studies have been conducted on the composition of the title plant. Mangiferin, maclurin-6-*O*-*β*-D-glucopyranoside, 1-*O*-galloyl-*β*-D-glucose, vanillic acid, and 5-hydroxy-2-isopropylchromone-7-*O*-*β*-glucoside were isolated from the MeOH extract of the title plant [20]. Three new prenyloxy chromanone derivatives were identified from the dichloromethane extract of *H. aucheri* [21]. Furthermore, polar phenolic metabolites were identified and quantified in the ethanol extract from the title plant [22]. In addition, total tannins and flavonoid contents were determined of methanol extract of *H. aucheri* [23]. To our knowledge, no report on the phenolic profile of *H. aucheri* is available in the literature.

A previous study reported the isolation of three new acylated benzophenone *O*-glycosides and five known compounds from *H. aucheri* [20]. Many factors influence the chemical composition and biological activity of medicinal and aromatic plants, including geographical location, environmental conditions, prevailing climate, soil characteristics, and cultivation practices.

Phenolic compounds, whose quantities may vary among plant species, constitute the most abundant group of secondary metabolites in plants. Plants produce phenolics for defense under intense stress. Phenolics are also named "bioflavonoids" due to their positive influence in terms of nutritional physiology. Also, phenolics contribute to the diverse health benefits of plant extracts [24-26].

Because of the limited number of studies on the phytochemical and biological properties of *H. aucheri*, this study aims to present an analytical method for identifying differences in bioactive secondary metabolites and antioxidant activity among chloroform, acetone, and methanol extracts of *H. aucheri*. The method was validated, and uncertainties were calculated using a bottom-up approach. The antioxidant activities of the extracts were assessed using DPPH and CUPRAC assays. The data collected highlight the phytochemical profiles of less-studied species and underscore the potential of other *Hypericum* species as sources of biologically active compounds.

2. Experimental

2.1. Chemicals

Detailed information regarding the purity and origin of the reference materials and chemicals used in the study is provided in the Supporting Information section.

2.2. Plant Material

The aerial parts of *H. aucheri* (1 kg) were collected during the full-flowering period in June 2023 from Balıkesir (Edremit, Mount Ida, Sarıkız area, 489155X, 4393598Y, 1700 m) and were identified by Dr Selami Selvi. Voucher specimens were deposited at the Herbarium of Altınoluk Vocational School, Balıkesir University, Balıkesir, Türkiye (ES 102).

2.3. Preparation of the Extract

The air-dried aerial parts of *H. aucheri* were powdered, then weighed separately (100 g), and macerated in chloroform (C) (1 L) for 15 days at room temperature. The macerate was filtered, and this process was repeated for acetone (Ac) and methanol (MeOH) extracts. After filtration, the solvents were evaporated under reduced pressure at 40 °C using a rotary evaporator to yield extracts of chloroform (6.73 g), acetone (5.16 g), and methanol (22.59 g). All the extracts were stored at -20 °C until used for experimental studies.

2.4. LC-HRMS Analysis

2.4.1. Sample and Standard Solutions Preparation for LC-HRMS

The extract was dissolved in the mobile phase (2.5 mL; A: B; 50:50; v/v). An internal standard (100 mg/L curcumin, 97%) was then added to obtain a final concentration of 3 mg/L, and the volume was adjusted to 5 mL with the mobile phase mixture. The solution in the flask was filtered through a 0.45 µm filter, 2 µL and was injected into the instrument.

2.4.2. LC-HRMS (Liquid Chromatography-High-Resolution Mass Spectrometry) Conditions

LC-HRMS utilizing a Thermo ORBİTRAP Q-EXACTIVE mass spectrometer coupled with a Troyasil C18 HS – 150 x 3 mm 3.5 µ particle size was used to determine the secondary metabolites of *H. aucheri*. A mobile phase consisting of (A) 1% formic acid in water and (B) 1% formic acid in methanol was used for analysis under electrospray ionisation (ESI) mode. The laboratory temperature was maintained at 22.0 ± 5.0 °C, and the relative humidity was 50 ± 15% rh. The gradient programs were composed of 50% A and 50% B for the initial 60 seconds, 100% B for the subsequent 180 minutes, 6.00–7.00 min: The mobile phase B ratio was reduced back to 50% and 7.00–15.00 min: Mobile phase was kept constant at 50% B. The flow rate was kept constant at 0.35 mL/min throughout the analysis. This gradient program is suitable for the effective separation of compounds with a wide range of polarities and for ensuring analytical reproducibility [27-30].f

Mass spectra were acquired over an m/z range of 100–900. Ionization conditions were set as follows: sheath gas flow rate, 45; auxiliary gas flow rate, 10. The spray voltage was set to 3.80 kV, the capillary temperature to 320 °C, and the auxiliary gas heater temperature to 320 °C. To increase ion transmission efficiency, the S-lens RF level was set to 50.0. The resulting mass spectra were characterized using the Bezmialem Vakif University, Drug Application and Research Center Library (ILMER library).

2.4.3. Method Validation

Specificity, accuracy, precision, LOD, and LOQ were used as the method validation parameters in this study. The evaluation of sources of uncertainty and quantification procedures was carried out in accordance with the EURACHEM/CITAC guide and based on previous studies [29-35].

2.4.3.1. Specificity

Specificity describes the ability to uniquely identify target-molecule peaks at a given retention time, differentiating them from contaminants, secondary metabolites, matrix components, and degradation products. The LC-HRMS technique was selected for its high specificity, required selectivity, and sensitivity within the matrix, while also effectively reducing the positive effects of treatments. The specificity of the improved LC-ESIHRMS method was assessed by directly analyzing all the solvents produced, the plant extract, and the added target analytes [30,34,35].

2.4.3.2. Recovery, Repeatability, and Intermediate Precision

Repeatability and intermediate precision of the developed LC-HRMS method were evaluated, along with the quantification of secondary metabolites, using C, Ac, and MeOH extracts for preliminary screening. Subsequently, we adjusted the extracts by adding compounds to achieve final concentrations of 0.1 mg/L, 0.5 mg/L, and 1 mg/L in 5-mL volumetric flasks, based on the initial detected levels. Unspiked plant extracts were also analyzed to determine the concentrations of target compounds in the blank samples. The recovery of each component at each fortification level was calculated using the following formula [35].

$$Recovery\ \% = Recovered\ concentration\ /injected\ concentration\ x\ 100$$

2.4.3.3. Linearity

A calibration curve was generated using standard solutions to analyze secondary metabolites with LC ESI HRMS. The curve, based on analyte responses in six replicates at different concentrations, includes the regression coefficient (R^2) and linear regression equation in Table S1 of the Supporting Information [27,28,35].

2.5. Antioxidant Capacity

The antioxidant capacities of aqueous extracts were evaluated through DPPH free radical scavenging [36-39], and the CUPRAC assay for cupric (Cu^{2+}) ion-reducing power [25]. The specific procedures for these activity measurements are detailed in the supplementary material.

3. Results and Discussion

3.1. LC-HRMS Analysis

In this study, chemical compositions of chloroform (C), acetone (Ac), and methanol (MeOH) extracts of aerial parts of *H. aucheri* collected from Edremit, Balıkesir were investigated by LC-HRMS, and the results are given in Table 1. The main component of *the H. aucheri C* extract was identified as syringic acid (6646.53 mg/L), a naturally occurring hydroxycinnamic acid. Other compounds detected in C were *p*-coumaric acid (77.11 mg/L), hesperidin (36.09 mg/L), (+)-t*rans* taxifolin (9.78 mg/L), penduletin (8.29 mg/L) and dihydrokaempferol (3.00 mg/L). As shown in Table 1, the C extract (6873.57 mg/L) has the highest phenolic compound content.

In the Ac extract, 18 compounds were available. Hyperoside (1059.53 mg/L), a flavonoid glycosides, is the most abundant phenolic compound in the Ac extract. It was determined that the Ac extract was richer in hyperoside compared to the MeOH extract. In contrast, hyperoside was not detected in the C extract. Additionally, Ac extract was found to be richer than the other analyzed extracts in terms of, *p*-coumaric acid (253.87 mg/L), caffeic acid (3.99 mg/L), quercetin (27.58 mg/L), (+)-t*rans* taxifolin (18.63 mg/L), myricetin (17.30 mg/L), penduletin (45.38 mg/L), quercitrin (43.93 mg/L) and luteolin-7-rutinoside (3.74 mg/L).

In the MeOH extract of *H. aucheri*, 18 metabolites were determined. The primary compound of the extract was hyperoside with 389.73 mg/L extract. Following this compound, *p*-coumaric acid (176.61 mg/L), vanilic acid (168.53 mg/L) and syringic acid (108.32 mg/L) were determined as the most abundant components.

Hypericum species are rich in secondary metabolites such as hypericin, hyperforin, pseudohypericin, hyperoside, rutin, quercetin, isoquercitrin, quercitrin, amentoflavone and chlorogenic acid. Smelcerovic et al. reported that hyperoside was the most common component in extracts of *H. nummularioides* [40]. Baroni Fornasiero et al. have previously determined that hyperoside was the most dominant constituent of *H. richeri* [41]. Several studies have examined the chemical components of *H. aucheri* found in Bulgaria. [20-23]. In a previous study, biflavones, flavonol glycosides, and xanthone glycosides were identified in the aerial parts of *H. aucheri* [42]. According to the results of the study,

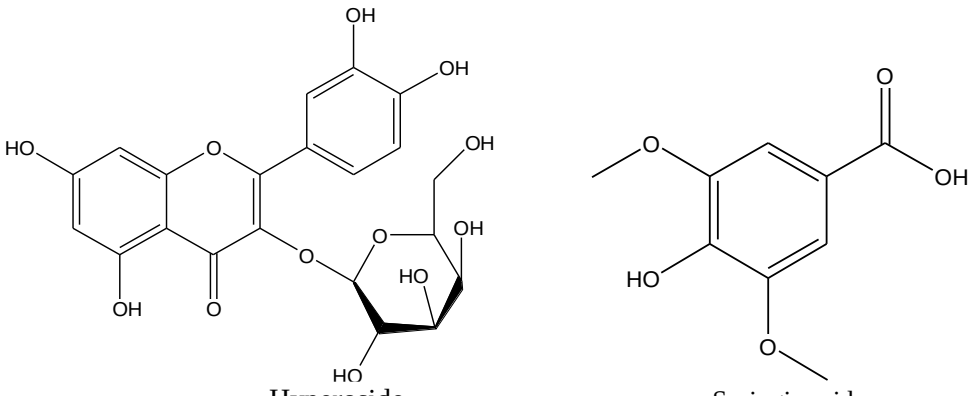
kaempferol, quercetin, myricetin and isoquercitrin were isolated [42]. Environmental factors also have a significant impact on the biosynthesis of secondary metabolites in medicinal and aromatic plants.

Within this context, the findings of this study are consistent with the existing literature; moreover, many phytochemical compounds from *H. aucheri* collected in Türkiye were identified for the first time in the present analysis.

Table 1. Phenolic compounds of extracts of *H. aucheri* quantified by LC- HRMS (mg/L)

No	Compounds	C	Ac	MeOH	U (%)
1	*p*-Coumaric acid	77.11	253.87	176.61	3.31
2	Vanilic acid	-	142.72	168.53	3.49
3	Caffeic acid	0.52	3.99	1.93	3.74
4	Syringic acid	**6646.53**	898.60	108.32	3.57
5	Apigenin	0.03	0.61	0.10	2.87
6	Naringenin	0.63	0.66	0.01	4.20
7	Dihydrokaempferol	3.00	0.92	4.79	2.86
8	Quercetin	-	27.58	6.25	2.95
9	(+)-t*rans* taxifolin	9.78	18.63	2.88	3.35
10	Myricetin	-	17.30	2.44	4.18
11	Penduletin	8.29	45.38	9.50	3.20
12	Apigenin 7-glucoside	-	0.1	0.39	3.59
13	Quercitrin	-	43.93	8.34	3.78
14	Hyperoside	-	**1059.53**	**389.73**	3.46
15	Luteolin-7-rutinoside	-	3.74	1.72	3.06
16	Hesperidin	36.09	4.57	3.31	3.79
	Total (mg/L)	**6873.57**	**2583.19**	**896.05**	

C: chloroform extract, Ac: acetone extract, MeOH: methanol extract



Hyperoside Syringic acid

Figure 1. The chemical structures of the three most abundant phenolic compounds of *H. aucheri* extracts

3.2. Antioxidant Capacit Evaluation

The antioxidant potential of the C, Ac and MeOH extracts of *H. aucheri* was evaluated using DPPH free radical scavenging and CUPRAC assays in vivo. DPPH free radical scavenging effects were assessed at concentrations of 10, 25, 50, and 100 µg/mL, and the results are presented in Table 2. BHA and BHT were employed as standard antioxidant reference compounds in the DPPH assay.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable radical frequently used to assess antioxidant activity. It shows a distinctive absorption at 517 nm, which diminishes significantly when it reacts with radical scavengers, such as hydrogen atoms from antioxidants. A decrease in absorbance at 517 nm indicates greater radical-scavenging capacity of the extract. In this assay, the Ac extract showed the highest activity among all extracts and surpassed the standards BHA and BHT, with inhibition rates from 85.90% to 61.19%. The DPPH assay results indicated that the MeOH extract exhibited moderate antioxidant activity, with inhibition values ranging from 41.05% to 78.99%. A positive correlation was observed between DPPH values and the phenolic content of *H. aucheri*, with the most active extract being rich in flavonoid glycosides (hyperoside). These findings align with earlier results [43].

The antioxidant capacities of the C, Ac and MeOH extracts of *H. aucheri* were defined using Trolox Equivalent Antioxidant Capacity (TEAC) amount, as evaluated by the CUPRAC assay, which employs Cu^{2+}- neocuproine reagent as a chromogenic oxidizing agent. In the CUPRAC assay, a higher value demonstrates strong antioxidant activity. In this case, the Cu^{2+} reducing capacity of the MeOH extract of *H. aucheri* (2,53±0,05 mmol TR g^{-1}) was found to be higher than that of curcumin (2,47±0,01 mmol TR g^{-1}).

Table 2. DPPH free radical scavenging activity of the extracts, BHA, and BHT

	DPPH Assay (Inhibition%)			
H. aucheri	**10 µg/mL**	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**
C	19.32±0.41	33.94±0.97	19.23±0.55	27.32±0.82
Ac	61.19±0.47	83.72±0.48	74.96±0.14	85.90±0.26
MeOH	49.52±0.80	45.76±0.83	50.29±0.14	66.95±0.13
BHA	51.85±1.58	78.69±0.71	81.39±0.38	82.76±0.20
BHT	47.58±0.63	76.26±0.84	83.24±0.40	84.01±0.14

Dimitrov et al. documented the acute toxicity, antidepressant effects, and MAO-inhibitory activity of mangiferin derived from *H. aucheri* in Bulgaria [43]. Many studies have evaluated the antioxidant potential of various *Hypericum* species. Overall, the evidence collected from these studies clearly indicates that all *Hypericum* species possess significant antioxidant activity [14, 43-46]. Our results align with earlier reports. While variability in antioxidant potency assessment is evident, it stems from factors such as the extraction method, the assay type, the way data are expressed, and the extract's chemical constituents.

4. Conclusions

This study offers the first detailed analysis of the phenolic content and antioxidant activity of various extracts from the aerial parts of *H. aucheri* collected from Mount Ida in Türkiye. To provide new insights into the antioxidant properties of this ethnobotanically significant plant, the phytochemicals identified by LC-HRMS in the C, Ac, and MeOH extracts of *H. aucheri* from Türkiye were examined. The Ac extract was the richest, containing 18 different constituents and the highest hyperoside content. As expected, the Ac extract demonstrated the strongest antioxidant activity in the DPPH assay, likely due to hyperoside. This flavonoid glycoside has been shown in various studies to possess significant anticancer, anti-inflammatory, antibacterial, antiviral, antioxidant, neuroprotective, antidepressant, and organ-protective activities. *H. aucheri* could serve as a new source of rich hyperoside and antioxidant compounds for our daily diets.

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Supporting Information

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