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Comprehensive phytochemical profiling and antioxidant assessment of *Rhinanthus major* var *apterus* Fr. Using GC-MS and HPLC-DAD analysis of volatile and phenolic compounds in leaf and flower tissues

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Abstract

This study represents the first comprehensive phytochemical characterization of *Rhinanthus major* var. *apterus* Fr. **(sin.** *Rhinanthus serotinus* subsp. *Aestivalis*) an understudied hemiparasitic species from the Orobanchaceae family. Using GC-MS and HPLC-DAD analyses, volatile and phenolic compound profiles were determined in leaf and flower extracts, along with antioxidant capacity evaluation via FRAP and CUPRAC assays. GC-MS analysis identified 16 volatile compounds, with matsutake alcohol (1-octen-3-ol) as the predominant constituent at exceptionally high concentrations (68.42% in flowers, 38.34% in leaves), significantly exceeding levels reported in related species. HPLC analysis revealed distinct tissue-specific phenolic profiles, with leaves demonstrating superior total phenolic content (7.26±0.28 mg GAE/g) and antioxidant capacity compared to flowers. Notable compounds included rutin (828.69 ppm), rosmarinic acid (594.16 ppm), and ascorbic acid (289.89 ppm) in leaves, while flowers were enriched in trans-caffeic acid (459.07 ppm) and pyrogallol (1062.84 ppm). These findings establish *R. major var. apterus* as a promising source of bioactive compounds with potential applications in natural product development, functional foods, and pharmaceutical research, while providing fundamental data for future pharmacognostic studies.

Keywords: *Rhinanthus major* var. *apterus* Fr., phytochemical analysis, matsutake alcohol, phenolic compounds, antioxidant activity, Orobanchaceae

1. Introduction

Plant secondary metabolism produces an extensive range of phenolic compounds that have attracted significant scientific attention due to their broad biological activities and beneficial effects on human health [1–4]. These naturally occurring polyphenols are recognized as important bioactive molecules because of their multiple properties, including antioxidant activity, antimutagenic and anticarcinogenic effects, anti-inflammatory activity, and neuroprotective functions. The dual behavior of phenolic compounds represents an intriguing aspect of their biological activity, as research has shown that these molecules can act both as protective antioxidants against oxidative damage and as pro-oxidants that can damage biomolecules and induce cell death [5,6]. This dose-dependent behavior highlights the complexity of their action mechanisms and underscores the importance of understanding their specific functions in various biological contexts.

The therapeutic use of medicinal plants is closely linked to the presence of secondary metabolites, especially phenolic acids and flavonoids [7,8]. The increasing scientific interest in plant polyphenols arises from their demonstrated antioxidant effects, multiple health benefits, and various industrial applications. In addition to their therapeutic uses, medicinal plants serve important nutritional functions globally and are often registered and marketed as food products according to regulations in many countries [8–10]. Due to their significant therapeutic potential, the identification and characterization of phenolic compounds have become important research areas in analytical sciences [11,12]. Extensive studies have confirmed the presence of polyphenols in plant materials and have aimed to understand their therapeutic and nutraceutical applications, as well as their antioxidant activities [13].

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The development of analytical methods for identifying and characterizing phenolic compounds has become essential in phytotherapy research. Various analytical techniques have been developed for characterizing phenolic compounds in medicinal plants, including gas chromatography [14], high-performance liquid chromatography [7,15], and capillary electrophoresis [15,16]. However, the thermal characteristics of phenolic acids and flavonoids create specific analytical challenges. These compounds have high melting points and undergo thermal degradation when heated above 200 °C, making liquid chromatographic methods necessary for effective separation procedures. These liquid chromatographic techniques are usually combined with different detection systems including UV spectrometric, UV/Vis fluorimetric, mass spectrometric, or electrochemical detection methods. Electrochemical detection methods have become particularly important as highly sensitive analytical procedures that allow direct detection of electroactive phenolic compounds without requiring chemical derivatization procedures.

High-Performance Liquid Chromatography (HPLC) has become the most commonly used separation technique for detecting and quantifying phytochemicals in plants [17–20]. Different detection methods are used with HPLC systems, including on-line diode array detection (DAD) [21,22], ultraviolet detectors [23], and electrochemical detectors [24]. The use of advanced analytical techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) operating in negative or positive ion modes, combined with tandem mass spectrometry using triple quadrupole systems or ion trap mass analyzers for MSn experiments, provides definitive structural confirmation for peak identification. However, these sophisticated methods require significantly higher capital investment and technical expertise compared to conventional approaches [25–30]. In contrast, HPLC-DAD systems provide a cost-effective alternative that simplifies the analytical workflow while maintaining adequate sensitivity and selectivity for most applications, making them particularly suitable for routine phenolic compound analysis in laboratories with limited resources.

Solid-phase microextraction (SPME) is widely used for the isolation and pre-concentration of volatile organic compounds before gas chromatography-mass spectrometry (GC-MS) analysis [31–34]. Compared to conventional sample preparation techniques such as solid-phase extraction or liquid-liquid extraction, SPME offers several distinct operational advantages, including the elimination of organic solvents, compatibility with automated systems, and minimal sample volume requirements [35]. These methodological advantages make SPME particularly valuable for high-throughput studies requiring analysis of large sample sets, such as genetic mapping studies conducted by plant breeders investigating the molecular basis of specific agronomic traits [36,37]. The technique shows exceptional utility in applications where analytical efficiency, reproducibility, and cost-effectiveness are critical factors.

Genetic mapping studies have successfully identified genes and quantitative trait loci (QTLs) that regulate volatile compound biosynthesis, including those responsible for aroma production, across diverse plant species including tomato [32], melon [38,39], apple [40,41], and grape [42–44]. Plant volatile profiling studies typically employ GC-MS methodology for both targeted quantitative analysis of specific volatile metabolites [45,46] and untargeted metabolomics approaches that comprehensively profile the complete volatile organic compound repertoire [31,32,38,39,40,47]. The convergence of traditional analytical chemistry methods with contemporary genomics and metabolomics technologies continues to advance our understanding of phenolic compound biosynthesis, regulation, and their diverse applications in human nutrition, therapeutics, and industrial biotechnology.

R. major var. *apterus* is an annual herbaceous plant from the Orobanchaceae family, exhibiting hemiparasitic characteristics. This subspecies constitutes one of the characteristic elements of grassland and steppe ecosystems and possesses considerable ecological significance. The taxon is also documented in the literature under the synonym Rhinanthus major var. apterus.

Approximately 30 annual hemiparasitic species constitute the genus Rhinanthus, which are distributed predominantly throughout Europe [48]. Among the approximately 25-30 recognized species within the genus, only a few exhibit widespread distribution across Europe. The genus Rhinanthus possesses a complex taxonomy attributed to remarkable morphological diversity, with numerous species, subspecies, and varieties having been described.

Parasitism has evolved independently multiple times throughout the plant kingdom. Parasitic species are predominantly found within the Orobanchaceae family, as well as in the Santalaceae and Convolvulaceae families. The total number of known parasitic plant species approximates 4,500 across 28 families [49]. The classification of parasitic plants employs three groups of characteristics that define dependency on host interaction for life cycle completion, attachment site to the host plant, and photosynthetic capacity. In contrast to holoparasites, which lack chlorophyll and are incapable of CO_2 fixation, hemiparasites retain

photosynthetic function to at least some degree. Holoparasites invariably function as obligate parasites, whereas hemiparasites may exhibit either obligate or facultative parasitic behavior. All parasitic plant types sustain their existence by establishing connections to either the stem or root system of host plants [50–55].

Plants of the genus Rhinanthus represent typical constituents of the Rhinanthoid group, which comprises 10 hemiparasitic and one holoparasitic genera [56]. Unlike numerous parasitic plants that are considered economically significant weeds (Striga, Orobanche, Phelipanche), Rhinanthus species have been demonstrated to exert positive effects in enhancing grassland biodiversity. These species are also utilized in traditional medicine for the treatment of ocular disorders caused by specific bacterial pathogens [54]. Owing to these distinctive characteristics, Rhinanthus species have received considerably less research attention regarding the mechanisms of parasitic relationship establishment and host plant responses compared to other parasitic plants.

In this investigation, phenolic profiles of methanolic extracts obtained from leaf and floral specimens of *R. major* var. *apterus* were analyzed, volatile component analysis through solid-phase microextraction (SPME) methodology was performed, and antioxidant efficacies were comprehensively assessed. While isolated compositional analyses on analogous plant taxa exist within the literature, neither HPLC-DAD methodology for concurrent analysis of phytochemicals encompassing diverse chemical architectures such as phenolic acids and flavonoids, nor SPME-GC-MS techniques for volatile constituent characterization have been documented for the plant under investigation.

Despite the existence of studies on prevalent species within the Rhinanthus genus, comprehensive phytochemical characterization investigations specifically addressing *R. major* var. *apterus* remain absent from the literature. This deficiency results in the untapped potential bioactivity of the species remaining unexplored. Currently, insufficient data exists regarding the biochemical composition and therapeutic prospects of the foliar and floral components of this taxon.

The principal objective of this research endeavor is to elucidate the detailed phytochemical profile of *R. major* var. *apterus*, a species inadequately characterized within existing literature, to define its volatile metabolite composition, and to establish its antioxidant activity levels. This study aims to bridge the critical knowledge gap in the literature by unveiling the chemical potential of the species. The comprehensive phytochemical characterization findings obtained will establish a robust scientific foundation for evaluating the therapeutic potential of *R. major* var. *apterus* and will constitute a pioneering contribution to future pharmacognostic investigations. The outcomes of this study will serve as a reference framework for subsequent in vitro and in vivo bioactivity studies exploring the antibacterial, antiviral, anti-inflammatory, anti-allergic, and antithrombotic activities of the species.

2. Materials and methods

2.1. Plant collection and extract preparation of *R. major* var. *apterus*

The habitat characteristics and morphological features of *R. major* var. *apterus* are illustrated in Fig. 1. These specimens were collected on June 24, 2022, from grassland areas adjacent to forest edges and roadsides near Kılıçlı neighborhood, Taşlıca village, within Hatila National Park at an elevation of 1550 m (41°08'09.0" N, 41°42'40.0" E), and were taxonomically identified by Dr. Nurşen AKSU KALMUK. A portion of the collected material was dried under controlled ventilation conditions without direct sunlight exposure and deposited in the herbarium under accession number Aksu 385. Leaf and floral tissues designated for analyses were ground and preserved at -20 °C for subsequent determination of antioxidant potential, selected phenolic and flavonoid compound levels, and volatile metabolite profiles.

Figure 1. Habitat of *R. major* var. *apterus*

2.2. Sample processing protocol

The compound isolation procedure was developed by adapting previously established techniques [57,58]. In this study, pulverized dried leaf and flower specimens (20 g each) were individually treated with methanol (200 ml) to maximize recovery of bioactive substances. Cellular structure disruption and effective mass transfer were achieved by exposing samples to sonic wave treatment for 30 minutes. Subsequently, the mixtures were transferred to agitation equipment and incubated at ambient temperature under light-protected conditions for 24 hours to ensure optimal extraction yield.

Following the incubation phase, samples underwent a dual-stage purification procedure. In the first stage, coarse particulates were eliminated using standard filtration paper, while in the second stage, micron-sized residues were removed using 0.45 μm pore diameter membrane filters. This comprehensive extraction strategy, incorporating sonic wave pretreatment, extended contact periods, and sequential filtration steps, was specifically designed to target maximum migration of natural compounds from solid phase to liquid phase. The clarified final solutions were subsequently utilized for analytical procedures [57,58].

2.3. Evaluation of antioxidant properties in *R. major* var. *apterus* leaf and floral tissues

2.3.1. Chemical reagents and laboratory materials

The phytochemical analysis utilized high-purity analytical reagents obtained from established suppliers. Primary chemicals including methanol (HPLC grade), Trolox standard, 2,4,6-tripyridyl-s-triazine, and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Supporting reagents such as sodium carbonate, glacial acetic acid, neocuproine, aluminum nitrate nonahydrate, copper(II) chloride, iron(II) sulfate heptahydrate, and ammonium acetate were sourced from Merck KGaA (Darmstadt, Germany). All chemicals met analytical grade specifications with >99% purity.

2.3.2. Total phenolic content analysis

Total phenolic compound quantification was performed using a modified Folin-Ciocalteu colorimetric method following established protocols. Gallic acid calibration standards were prepared in a six-point series at concentrations of 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/mL in methanol. The analytical procedure consisted of combining 20 μL of sample extract (1 mg/mL in methanol) or standard with 400 μL of diluted Folin-Ciocalteu reagent (1:1 with distilled water) and 680 μL of deionized water. Following 3 minutes of reaction time, 400 μL of 10% sodium carbonate solution was added for mixture neutralization. Sample incubation proceeded for 120 minutes at room temperature under dark conditions prior to absorbance measurement at 760 nm using a UV-Vis spectrophotometer. Calculations were performed using the gallic acid standard curve with results expressed as mg gallic acid equivalents (GAE) per g dry plant material.

2.3.3. Total flavonoid content determination

Flavonoid content determination utilized aluminum chloride complexation methodology combined with spectrophotometric detection. This analytical approach utilizes aluminum ion coordination with flavonoid carbonyl and hydroxyl groups, specifically at the C-4 position and C-3/C-5 hydroxyl sites in flavones and flavonols. Quercetin was used as the reference standard, with concentration ranges from 0.03125 to 1.0 mg/mL prepared in methanol. Sample extracts (0.5 mL at 1 mg/mL) were combined with 1.5 mL methanol, 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 M potassium acetate, and 2.8 mL distilled water. After 30 minutes incubation at ambient temperature, absorbance measurement was performed at 415 nm. Flavonoid content calculation used the quercetin calibration curve with results reported as mg quercetin equivalents (QE) per g dry sample weight.

2.4. Metal reduction-based antioxidant capacity assessment

2.4.1. Ferric reducing antioxidant power (FRAP) analysis

FRAP methodology quantifies electron-donating capacity via reduction of ferric-tripyridyltriazine complex to its ferrous form under acidic conditions. Fresh FRAP working reagent preparation involved combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride in a 10:1:1 ratio. Assay mixtures contained 3.0 mL of FRAP reagent and 0.1 mL of sample extract or blank solvent. Reaction mixture incubation occurred at 37 °C, with absorbance change monitoring at 593 nm over 4 minutes. Calibration curves using iron(II) sulfate heptahydrate standards (100-1000 μmol/L) enabled reducing power quantification, with expression as μmol Fe^{2+} equivalents per g dry plant matter.

2.4.2. Cupric ion reducing antioxidant capacity (CUPRAC) evaluation

CUPRAC methodology measures copper(II) reduction to copper(I) in the presence of chromogenic chelator neocuproine at physiological pH. Reaction mixtures consisted of 1.0 mL each of copper(II) chloride solution (10 mM), neocuproine methanolic solution (7.5 mM), and ammonium acetate buffer (1 M, pH 7.0), combined with 0.2 mL sample extract and 0.9 mL distilled water for a total volume of 4.1 mL. Following thorough mixing,

Table 1. Phenolic standards analyzed by Method A (Acetonitrile-based gradient)

No	Compound
1	*L-Ascorbic acid*
2	*Gallic acid*
3	*3,4-Dihydroxy benzoic acid*
4	*Vanillic acid*
5	*p-Coumaric Acid*
6	*Trans-Caffeic acid*
7	*Ferulic acid*
8	*Rosmarinic acid*
9	*(+)-Catechin*
10	*(-)-Epicatechin*
11	*Rutin*
12	*Myricetin*
13	*Quercetin*
14	*Apigenin*

tubes underwent room temperature maintenance for 60 minutes to ensure complete reaction development. Absorbance recording occurred at 450 nm against appropriate blanks. Antioxidant capacity determination used a Trolox standard curve (50–500 μM) with results expressed as μmol Trolox equivalent antioxidant capacity (TEAC) per g dry plant material.

2.5. Chromatographic analysis of phenolic constituents using HPLC-DAD

2.5.1. Analytical reagents and solvents

High-performance liquid chromatography required gradient-grade organic solvents sourced from certified suppliers. Acetonitrile (HPLC-grade) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), while methanol (HPLC-grade) was obtained from Merck KGaA (Darmstadt, Germany). Authentic phenolic reference standards employed for compound identification and quantification were exclusively sourced from Sigma-Aldrich, ensuring consistent quality and analytical reliability for comparative studies.

2.5.2. Method A: Phenolic compound analysis

Chromatographic separation of target compounds (1-14 as listed in Table 1) was achieved using a reverse-phase ACE 5 C18 analytical column (dimensions: 250 × 4.6 mm, particle size: 5 μm). The mobile phase system employed a binary gradient consisting of solvent A (acetonitrile) and solvent B (aqueous acetic acid, 1.5% v/v). Gradient elution initiated at 15% A:85% B composition, progressing linearly to 40% A:60% B over 29 minutes. The analytical system comprised an Agilent 1260 series configuration including: diode array detector (DAD WR) with simultaneous monitoring at wavelengths 250, 270, and 320 nm; quaternary pump operating at 0.7 mL/min constant flow rate; autosampler with 10 μL injection volume; and thermostatted column compartment maintained at 35 °C.

Table 2. Phenolic standards analyzed by Method B (Methanol-based gradient)

No	Compound
1	*Syringic acid*
2	*Pyrogallol*
3	*Chlorogenic acid*
4	*Resveratrol*
5	*Oleuropein*
6	*Cyanidin chloride*
7	*Hesperidin*
8	*Kaempferol*
9	*Baicalein*
10	*Chrysin*

2.5.3. Method B: Extended phenolic profiling

Comprehensive analysis of additional phenolic compounds (1-10 as listed in Table 2) utilized identical column specifications (ACE 5 C18, 250 × 4.6 mm, 5 μm). The binary mobile phase comprised solvent A (methanol) and solvent B (1.5% aqueous acetic acid). The extended gradient program began at 10% A:90% B, increasing to 40% A:60% B at 29 minutes, further progressing to 60% A:40% B by 40 minutes, and concluding at 90% A:10% B from 40-53 minutes. Detection employed the same 1260 DAD system with expanded wavelength monitoring at 280, 290, 320, 370, and 535 nm. Instrumental parameters remained consistent: 0.7 mL/min flow rate, 10 μL injection volume, and 35 °C column temperature.

2.5.4. Standard calibration procedures

Quantitative analysis relied on external standard calibration methodology utilizing six-point concentration series for each reference compound. Standard solutions were prepared at precisely defined concentrations of 25, 50, 75, 100, 200, and 300 μg/mL in appropriate solvents. Each calibration standard underwent HPLC-DAD analysis using identical chromatographic conditions as sample extracts to ensure analytical consistency and reliability.

2.6. Volatile compound profiling via SPME-GC-MS

2.6.1. Solid-Phase microextraction protocol

Prior to analysis, SPME fibers underwent conditioning according to manufacturer specifications to ensure optimal performance. Plant material was mechanically processed using a laboratory mill to achieve uniform particle size distribution. Approximately one-third volume of powdered sample was transferred to 20 mL headspace vials equipped with PTFE/silicone septa (Supelco). Sample vials were equilibrated at 45 °C for 15 minutes to promote volatile compound release. SPME fiber extraction proceeded for 40 minutes at the same temperature, followed by thermal desorption in the GC injection port for 20 minutes to ensure complete compound transfer.

Table 3. Volatile compounds identified in *R. major* var. *apterus* flowers by SPME-GC-MS analysis

No	RT (min)	RI	RI*	Name of the compound	Cont. [%]
1	6.33	807	769-817	Hexanal	3.83
2	10.8	933	922-935	alpha.-Pinene	5.90
3	12.63	977	974-985	Matsuika alcohol	68.42
4	13.23	991	970-1002	2-Amylfuran	2.77
5	14.626	1022	1015-1050	o-Cymene	0.46
6	14.80	1026	1015-1040	1-Hexanol, 2-ethyl-	5.25
7	16.73	1068	1060-1094	3,5-Octadien-2-one, (E,E)-	0.47
8	17.82	1092	1090-1103	Benzoic acid, methyl ester	3.74
9	18.03	1096	1080-1112	Linalool	5.61
10	18.25	1101	1084-1126	Nonanal	1.31
11	22.62	1196	1106-1158	Chrysanthenone	0.450
12	22.868	1201	1187-1210	Decanal	0.390
13	32.19	1416	1415-1451	Caryophyllene	0.937

Rt -Retention times on an HP-5MS UI column
RI - Experimentally determined retention indices on an HP-5MS UI column
RI*- Values obtained from the NIST library.
Cont.-Content

2.6.2. Gas Chromatography-Mass spectrometry parameters

Volatile compound analysis employed an Agilent Technologies gas chromatograph (Santa Clara, CA, USA) equipped with an HP-5MS ultra inert fused silica capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness). Ultra-high purity helium (≥99.99%) served as carrier gas at 1.0 mL/min constant flow. The injection port was maintained at 250 °C for optimal compound desorption. The temperature program initiated at 50 °C (2-minute hold), ramped to 150 °C at 2.5 °C/min (5-minute isothermal period), then increased to 250 °C at 6.5 °C/min with a final 1-minute hold. Mass spectrometric detection utilized electron impact ionization at 70 eV energy, scanning mass-to-charge ratios from 35-500 m/z for comprehensive volatile compound characterization.

3. Results and discussion

3.1. GC-MS analysis and photochemical profile of *R. major* var. *Apterus*

The chemical profile obtained through GC-MS analysis of *R. major* var. *apterus* in this study exhibits remarkable characteristics when compared to similar studies in the literature. Due to the limited number of studies on this species in the literature, comparisons with species from the same family (Orobanchaceae) reveal the uniqueness of the chemical composition of this subspecies.

GC-MS analysis of the flower extract identified 13 different compounds (Table 3), which constitute 98.66% of the total volatile oil. The analytical chromatogram of the flower extract is presented in Fig. 2. According to the analysis results, the most dominant compound in the flower extract was determined to be matsutake alcohol (1-octen-3-ol), comprising 68.42% of the total composition. This high concentration contributes significantly to the characteristic aroma of the plant. The second highest concentration compound was α-pinene (5.90%), a monoterpene class compound. This was followed by 2-ethyl-1-hexanol (5.25%) and linalool (5.61%). The identification of all compounds was accomplished through comparison of experimentally calculated retention indices (RI) values with reference values (RI*) obtained from the NIST library.

Table 4. Volatile compounds identified in *R. major* var. *apterus* leaf by SPME-GC-MS analysis

No	RT (min)	RI	RI*	Name of the compound	Cont. [%]
1	3.405	683	649-697	Isovaleraldehyde	6.97
2	3.60	688	645-696	2-pentylfuran	4.51
3	4.06	700	700-712	Furan, 2-ethyl-	4.41
4	6.02	795	769-817	Hexanal	8.38
5	7.61	846	821-855	2-Hexenal, (E)-	3.120
6	7.68	847	844-857	3-Hexen-1-ol, (E)-	5.75
7	10.60	929	917-940	alpha.-Pinene	3.12
8	11.68	954	929-965	Benzaldehyde	2.25
9	12.48	973	974-986	Matsutake alcohol(1-Octen-3-ol)	38.34
10	12.84	982	965-992	Sulcatone	3.32
11	13.031	990	988-996	2-pentylfuran	2.61
12	14.68	1023	1015-1040	1-Hexanol, 2-ethyl-	3.24
13	17.95	1095	1080-1112	Linalool	1.90
14	18.17	1099	1084-1126	Nonanal	2.05
15	47.79	1917	1910-1928	Palmitic acid, methyl ester	2.12
17	49.82	2087	2081-2092	Linoleic acid, methyl ester	0.70

Rt -Retention times on an HP-5MS UI column
RI - Experimentally determined retention indices on an HP-5MS UI column
RI*- Values obtained from the NIST library
Cont.-Content

GC-MS analysis of the leaf extract identified 16 different compounds (Table 4), which constitute 98.83% of the total volatile oil. The analytical chromatogram of the leaf extract is presented in Fig. 3. Unlike the flower extract, matsutake alcohol was found at a lower concentration (38.34%) in the leaf extract, though it remains the dominant compound. The second highest concentration compound in the leaf was hexanal (8.38%), which is significantly higher than its concentration in the flower. Isovaleraldehyde (6.97%) was detected in substantial amounts in the leaf extract as an aldehyde compound, while it was not detected in the flower extract.

1-Octen-3-ol represents an alkenyl alcohol compound that is naturally produced by plants and fungi [59,60]. High concentrations of this compound are particularly detected in Japanese Matsutake mushrooms, and it has received approval as a food additive from the United States Food and Drug Administration [61]. Among the notable characteristics of 1-octen-3-ol is its strong antimicrobial activity. This molecule serves as a major component in volatile oils from different plant species and demonstrates antibacterial and antifungal

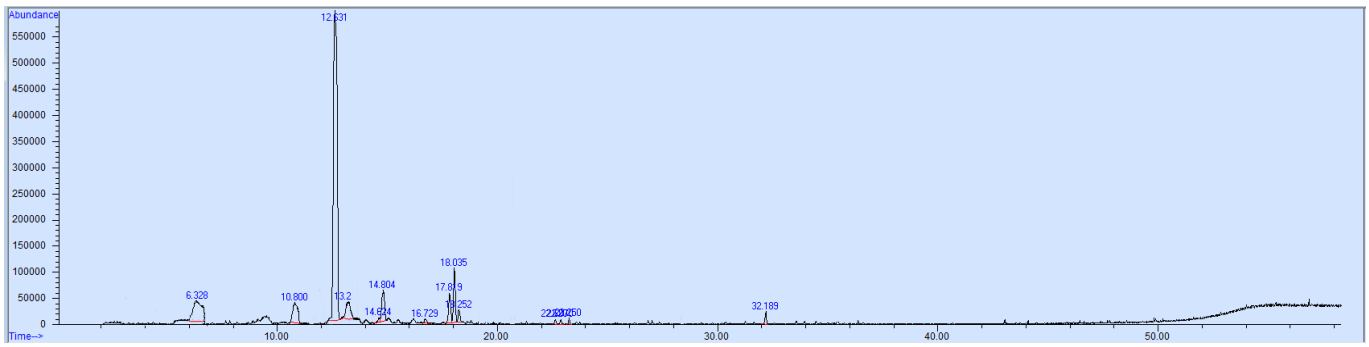

Figure 2. GC-MS chromatogram of volatile compounds from *R. major* var. *apterus* leaf extract analyzed by SPME

properties [62–65]. The compound acts as an effective antibacterial agent against gram-positive bacteria, showing minimum inhibitory concentration (MIC) values of 1.0 mg/mL and minimum bactericidal concentration (MBC) values of 4.0 mg/mL [66].

In the GC-MS analysis conducted by Khodaei and colleagues on *Pedicularis sibthorpii* from the same family, a total of 19 compounds were detected, with 1-octen-3-ol as the main compound at 55.2% concentration [67]. In this study, linalool (5.5%) was also reported as another important component. In the GC-MS analysis performed by Novy and colleagues on *Euphrasia rostkoviana* from the same family, more than 70 compounds were detected, with linalool (4.65%) among the main compounds [68]. In this study, 1-octen-3-ol was detected at only 1.82%, with matsutake alcohol concentration remaining at quite low levels.

In our current study, matsutake alcohol was detected at extraordinarily high concentrations in *R. major* var. *apterus*, at 68.42% in flowers and 38.34% in leaves. These values are approximately 1.2-1.4 times higher than the concentration reported in *P. sibthorpii* (55.2%) from the same family, and 38-40 times higher than that detected in *E. rostkoviana*. Significant superiorities are also observed in terms of other important compounds; α-pinene was found at concentrations of 5.90% in flowers and 3.12% in leaves, while this compound was not reported in the compared species. Linalool was detected at 5.61% in flowers and 1.90% in leaves, showing similar or slight superiority compared to 5.5% in *P. sibthorpii* and 21% higher concentration compared to 4.65% in *E. rostkoviana*.

The differences in compound profiles between leaf and flower extracts reveal the variability in secondary metabolite production in different organs of the plant. Although matsutake alcohol is the dominant compound in both extracts, concentration differences and the presence of organ-specific compounds demonstrate the chemical diversity of the plant. These results indicate that *R. major* var. *apterus* exhibits a superior profile in terms of matsutake alcohol among Orobanchaceae species compared in the current literature and possesses significant advantages in other important terpene compounds as well.

The fact that 1-octen-3-ol possesses important potential in pest control in addition to its antimicrobial properties [69–71] considering the trend toward biological-based solutions from synthetic pesticides in agricultural applications, makes this natural compound a versatile agent with great potential for developing new generation plant protection products [72]. In conclusion, the high matsutake alcohol content of *R. major* var. *apterus* makes this plant an extremely valuable resource for natural antimicrobial agents, food preservatives, perfumery raw materials, and biological pesticide development studies, presenting great potential for future biotechnological applications and industrial production.

3.2. Antioxidant capacity and phenolic content of *R. major* var. *apterus* by CUPRAC and FRAP methods

The evaluation of the antioxidant activity profile of *R. major* var. *apterus* revealed that both tissues of the plant possess significant antioxidant potential. These results

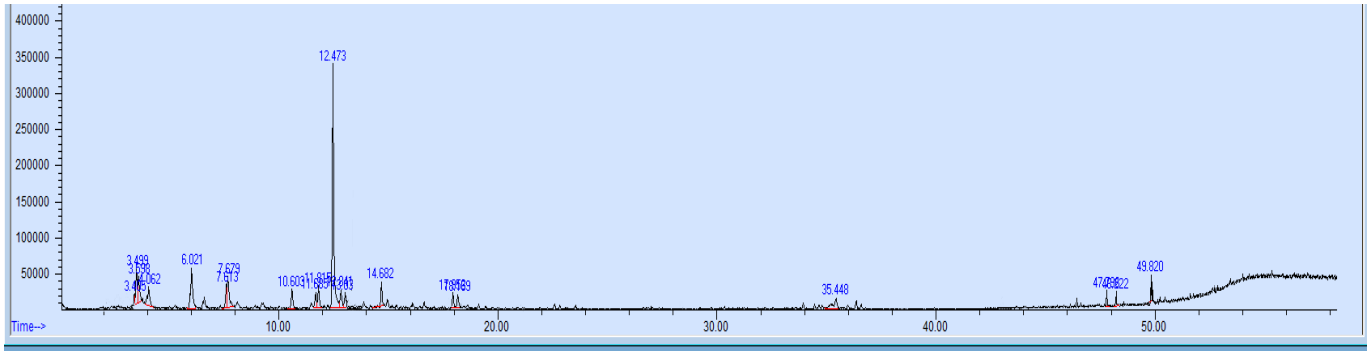

Figure 3. GC-MS chromatogram of volatile compounds from *R. major* var. *apterus* leaf extract analyzed by SPME

are shown in Table 5. In terms of total phenolic content, leaf extract exhibited 31% higher values with 7.26±0.28 mg GAE/g dry sample compared to flower extract (5.54±0.47 mg GAE/g dry sample). This result indicates that leaf tissue demonstrates more active metabolism in terms of phenolic compound accumulation.

When total flavonoid content was examined, minimal difference was observed between leaf and flower extracts, determined as 5.46±0.12 mg QE/g dry sample in leaves and 5.31±0.26 mg QE/g dry sample in flowers. These close values demonstrate that flavonoid metabolism is active at similar levels in both tissues.

The iron reducing capacity determined by FRAP (Ferric Reducing Antioxidant Power) method was found approximately 10% higher in leaf extract with 10.29±0.26 μmol $FeSO_4.7H_2O$/g sample compared to flower extract (9.36±0.17 μmol $FeSO_4.7H_2O$/g sample). This result demonstrates that leaf tissue possesses superior electron-donating capacity and therefore exhibits stronger reducing antioxidant activity.

CUPRAC (Cupric Ion Reducing Antioxidant Capacity) analysis results revealed that in terms of copper reducing capacity, leaf extract was detected at 0.08±0.00 mmol TEAC/g sample and flower extract at 0.06±0.00 mmol TEAC/g sample. The 33% higher CUPRAC activity exhibited by leaf tissue proves that the antioxidant defense mechanisms of this tissue are more effective. Although flower tissue contains potent antioxidant compounds such as pyrogallol (1062.84 ppm) and trans-caffeic acid (459.07 ppm) at high concentrations, the lower overall antioxidant activity compared to leaves indicates that the contribution of these two compounds to total antioxidant capacity is limited. The fundamental reason for this situation is that antioxidant activity depends not only on the high concentration of a few compounds, but on the balanced distribution of numerous antioxidant compounds. The presence of multiple antioxidant compounds such as ascorbic acid, rosmarinic acid, syringic acid, and rutin together at high concentrations in leaf tissue creates a synergistic effect, enhancing the total antioxidant capacity. Overall evaluation established that the leaf tissue of *R. major* var. *apterus* exhibits superior performance compared to flower tissue in terms of both total phenolic content and antioxidant activity parameters, and this situation is determined to be a natural consequence of the balanced distribution of multiple antioxidant compounds identified in HPLC analyses. This correlation supports at the molecular level that the plant, particularly the leaf extract, possesses strong antioxidant potential and may serve as a valuable natural antioxidant source for future pharmacochemical research.

3.3. HPLC-Based quantification of phenolic and flavonoid compounds in *R. major* var. *apterus* Extract

This investigation employed HPLC analysis for L-ascorbic acid determination alongside a comprehensive range of phenolic and flavonoid compounds in leaf and flower methanolic extracts of *R. major* var. *apterus*. Phenolic compound profile determination targeted 12 phenolic acid standards: gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, trans-caffeic acid, ferulic acid, rosmarinic acid, pyrogallol, chlorogenic acid, resveratrol, and oleuropein. Flavonoid composition characterization utilized 11 reference standards: (+)-catechin, (-)-epicatechin, rutin, myricetin, quercetin, apigenin, cyanidin chloride, hesperidin, kaempferol, baicalein, and chrysin. Classification of these flavonoid compounds as bioactive compounds resulted from their demonstrated bioactive potentials. Methanolic extract characterization required optimization of two distinct HPLC methods. Method A enabled quantitative determination of phenolic compounds specified in Table 1, whereas Method B facilitated quantitative determination of bioactive compounds listed in Table 2. Table 6 presents detailed HPLC analysis results and compound composition data for the methanolic extracts. Chromatographic data evaluation included HPLC chromatograms of flower methanolic extracts shown in Fig. 4 and HPLC chromatograms of leaf methanolic extracts presented in Fig. 5. Comparative HPLC analysis demonstrated that both methanolic extract types exhibit distinct phenolic compositions and revealed tissue-specific compound distributions. The optimized HPLC methods delivered high separation resolution and selectivity for targeted phenolic compounds in both plant methanolic extracts. Chromatographic system performance facilitated successful separation and identification of complex compound compositions within the methanolic extract matrices.

The phytochemical analysis results conducted on the flower and leaf portions of *R. major* var. *apterus* revealed that the plant exhibits a rich profile in terms of significant bioactive compounds. As indicated in Table 6, the analyzed compounds include vitamins, phenolic acids, and flavonoids.

Table 5. Phenolic content, flavonoid content, and antioxidant activity results (FRAP and CUPRAC) for *R. major* var. *apterus* extracts

Taxon	Used part	Total phenolic content (mg GAE/g dry sample)*	Total flavonoid content (mg QE/g dry sample)*	FRAP (μmol $FeSO_4.7H_2O$/g sample)*	CUPRAC (mmol TEAC/g sample)*
R.serotinus subsp. aestivalis	Leaf	7.26±0.28	5.46±0.12	10.29±0.26	0.08±0.00
	Flower	5.54± 0.47	5.31±0.26	9.36±0.17	0.06±0.00

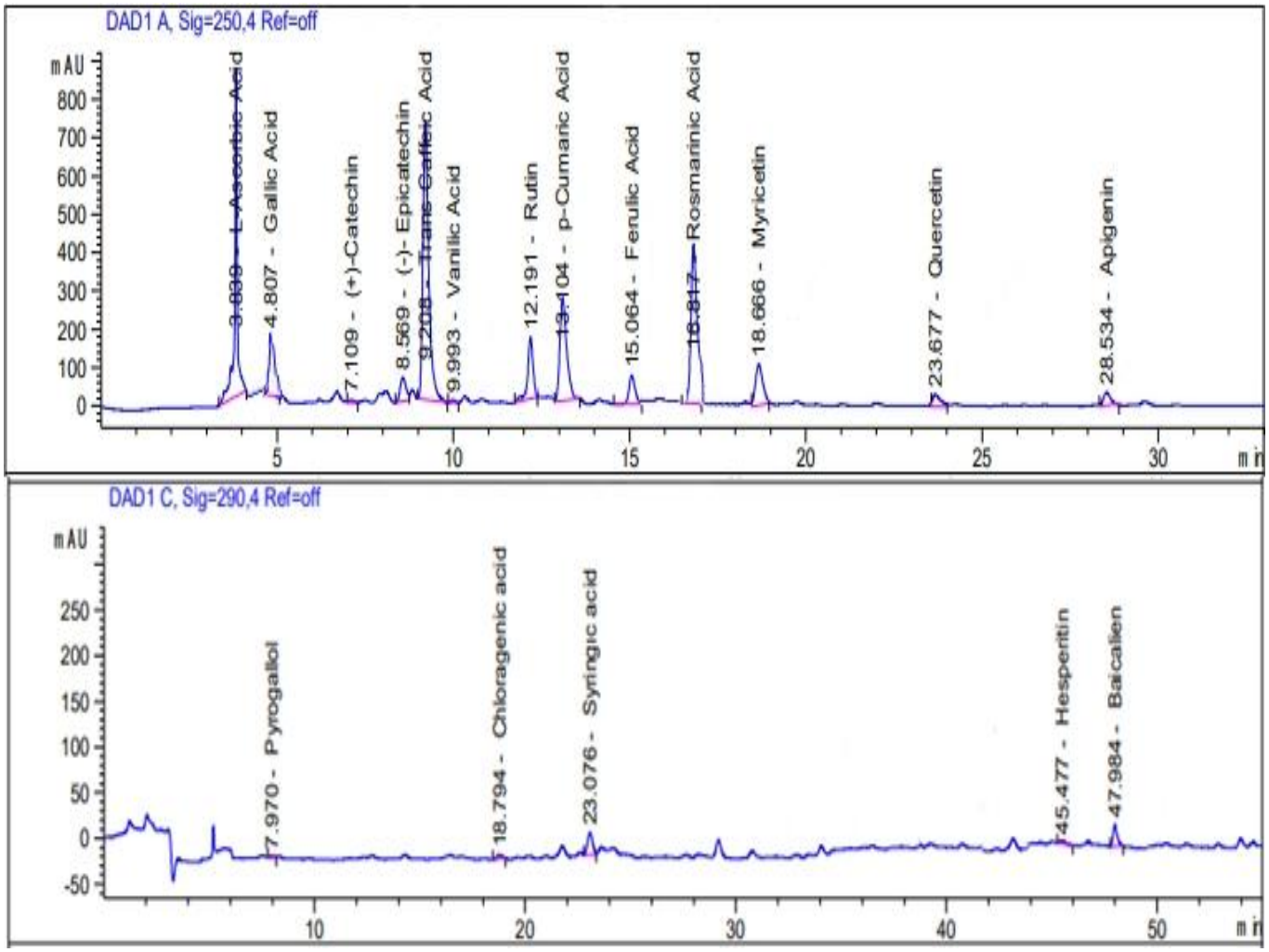

Figure 5. Chromatographic analysis results of *R. major* var. *apterus* flower samples

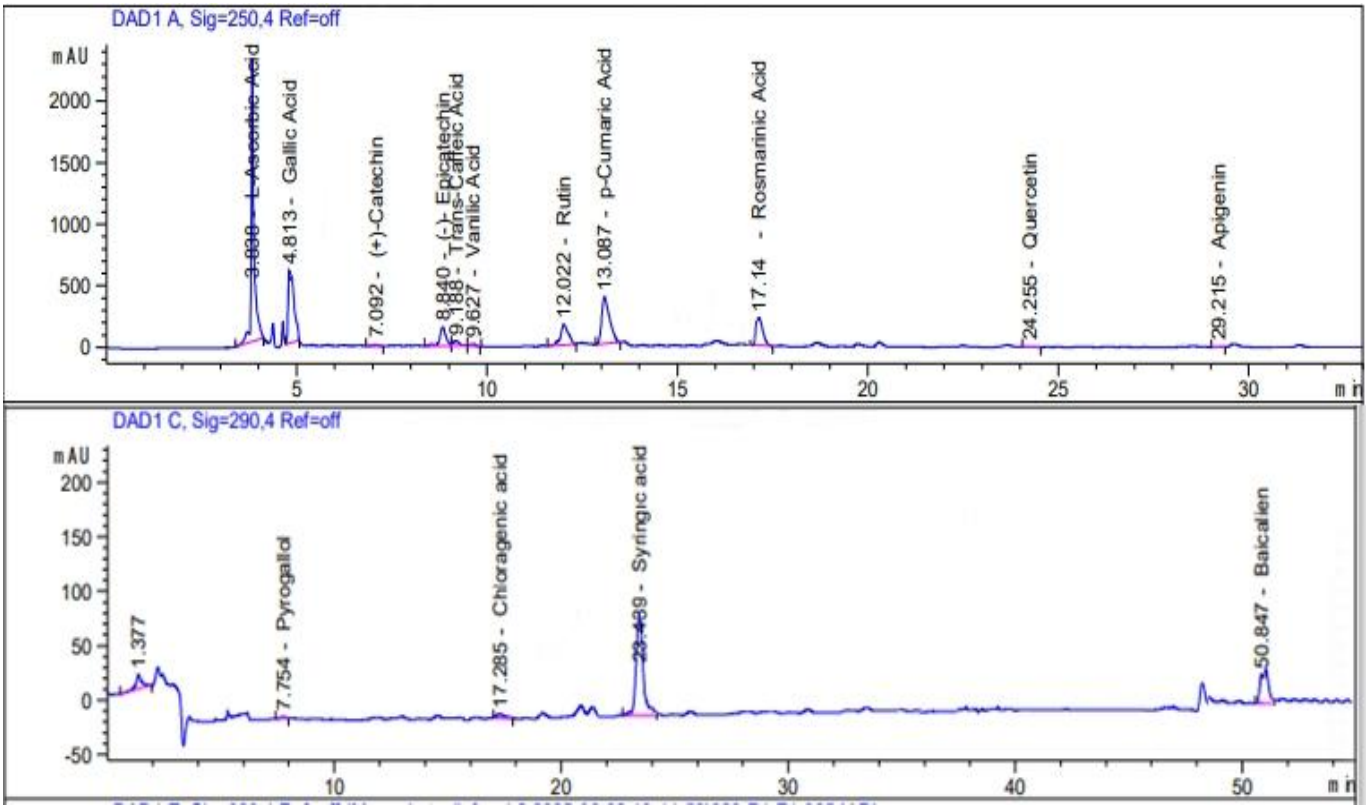

Figure 4. Chromatographic analysis results of *R. major* var. *apterus* leaf samples

Table 6. Phenolic compound analysis results for *R. major* var. *apterus* leaf and flower extracts

No	Compounds	*R. major* var. *apterus* flower *(ppm)*	*R. major* var. *apterus* leaf *(ppm)*
	Vitamin		
1	L-Ascorbic acid	109.5	289.89
	Phenolics		
2	*Gallic acid*	41.49	45.43
3	*3,4-Dihydroxy benzoic acid*	N/D	N/D
4	*Vanillic acid*	N/D	40.25
5	*Syringic acid*	2.79	141.24
6	*p-Coumarıc Acid*	182.44	321.35
7	*Trans-Caffeic acid*	459.07	29.04
8	*Ferulic acid*	1.65	N/D
9	*Rosmarinic acid*	424.81	594.16
10	*Pyrogallol*	1062.84	398.34
11	*Chloragenic acid*	90.02	91.75
12	*Resveratrol*	N/D	N/D
13	*Oleuropein*	N/D	N/D
	Flavonoids		
14	*(+)-Catechin*	76.02	142.44
15	*(-)-Epicatechin*	666.75	130.89
16	*Rutin*	570.83	828.69
17	*Myricetin*	283.96	N/D
18	*Qercetin*	126.59	47.13
19	*Apigenin*	90.87	12.94
20	*Cyanidin cloride*	N/D	N/D
21	*Hesperitin*	N/D	N/D
22	*Kaempferol*	N/D	N/D
23	*Baicalien*	16.72	78.33
24	*Chrysin*	N/D	N/D

N/D: Not detected

L-ascorbic acid (vitamin C) detected in this study is acknowledged as a significant nutritional antioxidant. Research has demonstrated that vitamin C substantially decreases oxidative damage to macromolecules including lipids, DNA, and proteins resulting from reactive oxygen and nitrogen species linked to chronic conditions such as cardiovascular disease, stroke, cancer, neurodegenerative diseases, and cataractogenesis [73]. Literature studies demonstrate that vitamin C possesses numerous health benefits including high levels of antioxidant, anti-atherogenic, anti-carcinogenic, and immunomodulatory properties. In *R. major* var. *apterus*, L-ascorbic acid was detected at 109.5 ppm in flowers and 289.89 ppm in leaves, establishing that the leaf portion is significantly enriched in terms of vitamin C content.

Plant phenolics are generally products of the phenylpropanoid metabolic pathway and consist of a diverse array of compounds. These compounds possess potent antioxidant properties and have been reported to exhibit protective effects against oxidative damage to biomolecules such as DNA, lipids, and proteins involved in chronic disorders including cancer and cardiovascular diseases [74]. In the analyses, gallic acid was detected at 41.49 ppm in flowers and 45.43 ppm in leaves. Vanillic acid was found only in leaves at 40.25 ppm levels, while it could not be detected in flower portions. Ferulic acid was determined only in flowers at 1.65 ppm and was not detected in leaf portions. p-Coumaric acid was detected at high concentrations in both flower and leaf portions, found at 182.44 ppm in flowers and 321.35 ppm in leaves. These results demonstrate that the leaf portion is more enriched in terms of p-coumaric acid content.

When syringic acid concentrations were examined, it was detected at low levels of 2.79 ppm in flowers, while it was found at approximately 51-fold higher concentration of 141.24 ppm in leaves. This situation demonstrates that the leaf is considerably enriched in terms of syringic acid accumulation compared to the flower. Literature studies indicate that caffeic acid possesses significant therapeutic potential in cancer treatment [75,76] and is recognized as a potent natural antioxidant [77]. Studies have reported that caffeic acid induces apoptosis in cancer cells by increasing reactive oxygen species (ROS) levels and disrupting mitochondrial function, while also suppressing metastasis and reducing the aggressive behavior of tumors . In this study, trans-caffeic acid was detected at high levels of 459.07 ppm in flowers, while it was determined at 29.04 ppm in leaves. This result demonstrates that the flower is approximately 16-fold more enriched in terms of caffeic acid compared to the leaf.

Rosmarinic acid was detected at 424.81 ppm in flowers and 594.16 ppm in leaves, establishing that the rosmarinic acid content of the leaf portion is approximately 1.4-fold higher compared to the flower. Pyrogallol is a phenolic compound naturally present in oak, eucalyptus, and other hardwood plants as a decomposition product of hydrolysable tannins, possessing potent antioxidant, anti-fungal, and anti-psoriatic properties [78–85]. This compound was one of the compounds detected at the highest concentration in the flower portion at 1062.84 ppm. In the leaf portion, it was found at 398.34 ppm, establishing that the flower is approximately 2.7-fold more enriched in terms of pyrogallol compared to the leaf. Chlorogenic acid was detected at similar levels in both tissues, found at 90.02 ppm in flowers and 91.75 ppm in leaves.

Flavonoids are polyphenolic compounds produced by plants with potent antioxidant properties, and are reported in the literature to possess anti-inflammatory, antiviral, free radical scavenging, and anticarcinogenic effects [86]. In the analyses conducted on the methanolic extract of *R. major* var. *apterus*, among the examined flavonoids, (+)-catechin, (-)-epicatechin, rutin, quercetin, and apigenin were found at detectable levels. (-)-Epicatechin, a flavonoid type belonging to a subclass of flavanols, was detected at high concentrations of 666.75 ppm in flowers, while it was found at 130.89 ppm in leaves. This compound possesses potent antioxidant

properties and has been reported in literature studies to demonstrate free radical neutralization, inflammation reduction, cardiovascular health support, and potential neuroprotective effects.

Rutin, one of the major flavonoids, has been reported in the literature to exhibit significant antioxidant activity by demonstrating reducing effects on different oxidizing species such as superoxide, peroxyl, and hydroxyl radicals [87]. Additionally, studies have shown that rutin possesses various pharmacological properties including anticancer, antimicrobial, and anti-inflammatory effects [88]. Rutin, which serves as a natural protective mechanism against harmful organisms and UV radiation, was detected at 828.69 ppm in the leaf portion and 570.83 ppm in the flower portion, establishing that the leaf possesses approximately 1.5-fold higher rutin content compared to the flower. This result suggests that the leaf portion may have higher potential for anticancer, antimicrobial, and anti-inflammatory effects.

Myricetin was detected only in the flower portion at a concentration of 283.96 ppm, while it could not be found in the leaf portion. This situation demonstrates that myricetin specifically accumulates in flower tissues. Among other detected flavonoids, (+)-catechin was found at 76.02 ppm in flowers and 142.44 ppm in leaves; quercetin at 126.59 ppm in flowers and 47.13 ppm in leaves; apigenin at 90.87 ppm in flowers and 12.94 ppm in leaves. Baicalein was detected at 16.72 ppm in flowers and 78.33 ppm in leaves, establishing that the leaf portion is approximately 4.7-fold more enriched in terms of this flavonoid compared to the flower. Other flavonoids such as cyanidin chloride, hesperidin, kaempferol, and chrysin could not be detected in either tissue. Compounds such as resveratrol and oleuropein were also not detected in the analyzed samples.

Comprehensive phytochemical studies on *R. major* var. *apterus* are limited in the literature, and the detailed elucidation of this species' metabolic profile for the first time emphasizes the scientific originality and importance of our study. Comparative analyses conducted among members of the Orobanchaceae family reveal the extraordinary phytochemical potential of *R. major* var. *apterus*. While Mihailović et al. (2016) reported in their comprehensive study on *Verbascum nigrum, V. phlomoides*, and *V. thapsus* species that caffeic acid concentration reached a maximum value of 3.93 mg/g after hydrolysis, the finding of trans-caffeic acid in its natural form at remarkably high levels of 459.07 ppm in the flower tissues of *R. major* var. *apterus* in our current study dramatically reveals the bioactive potential of this species [89]. This concentration difference is approximately 117-fold, proving the superiority of the Rhinanthus species in terms of caffeic acid accumulation within the family.

Similar superiority is observed in terms of flavonoid composition, and while the rutin compound could not be detected in Verbascum species, it was determined that this potent antioxidant flavonoid accumulates at levels of 828.69 ppm in *R. serotinus*. The comparative analysis conducted by Benedec et al. (2024) with *Euphrasia officinalis subsp*. pratensis and *E. stricta* species from the same family also supports the superiority of *R. major* var. *apterus* [90]. While chlorogenic acid was detected at 353.86 μg/mL in E. stricta and rutin concentration was detected in the range of 57-61 μg/mL in both Euphrasia species, the rutin levels in *R. serotinus* exceed these values by 13-14 fold. These findings scientifically demonstrate that *R. major* var. *apterus* occupies a unique position within the Orobanchaceae family in terms of phenolic compound richness and should be evaluated as a highly valuable natural resource from a pharmacochemical perspective. It is anticipated that these bioactive compounds contained in the plant may serve as an important resource for future pharmacological studies.

Comparative HPLC analyses conducted across species from distinct taxonomic families reveal that *R. major* var. *apterus* exhibits a remarkable phenolic compound profile in terms of compositional richness. These inter-taxon comparisons demonstrate pronounced metabolomic variations and underscore the unique bioactive potential inherent to each species. Among these comparative investigations, the phytochemical analysis of *Cakile maritima* from the Brassicaceae family presents particularly noteworthy parallels and contrasts. While both species demonstrate substantial phenolic compound content, the quantitative distributions reveal significant disparities [91]. In *C. maritima*, pyrogallol was detected at 579.9 mg/L and rutin at 219.6 mg/L concentrations. Conversely, the present investigation determined that pyrogallol concentrations in *R. major* var. *apterus* floral tissues reached 1062.84 ppm, representing approximately a two-fold elevation compared to the Brassicaceae species, while foliar rutin concentrations (828.69 ppm) substantially exceeded those documented in *C. maritima*. Rosmarinic acid concentrations further exemplify this pronounced differential pattern, with *C. maritima* exhibiting levels of 7.67 mg/L while *R. major var. apterus* demonstrated concentrations of 594.16 ppm, representing an approximately 77-fold enhancement. This comparative analysis provides compelling evidence that *R. major* var. *apterus* occupies a notable position within the Orobanchaceae family regarding phenolic compound abundance and merits scientific recognition from a pharmacochemical perspective. Collectively, these findings establish that *R. major* var. *apterus* possesses an enriched phenolic composition when compared to both

congeneric species within the same family and taxa from different taxonomic families, demonstrating valuable resource potential for future pharmacological investigations.

4. Conclusions

This comprehensive investigation elucidated the detailed phytochemical profile of *R. major* var. *apterus* for the first time, thereby addressing a significant lacuna in the existing literature. Gas chromatography-mass spectrometry analyses revealed the presence of 16 distinct volatile compounds across floral and foliar tissues, with matsutake alcohol (1-octen-3-ol) exhibiting extraordinarily elevated concentrations of 68.42% in flowers and 38.34% in leaves, thereby underscoring the remarkable antimicrobial potential of this species. High-performance liquid chromatography with diode array detection elucidated the phenolic compound profile, demonstrating the superiority of leaf tissue compared to floral tissue in terms of total phenolic content (31% higher) and antioxidant capacity (10% and 33% higher in FRAP and CUPRAC assays, respectively). The detection of elevated concentrations of rutin (828.69 ppm), rosmarinic acid (594.16 ppm), and ascorbic acid (289.89 ppm) in leaf tissues, contrasted with the predominance of trans-caffeic acid (459.07 ppm) and pyrogallol (1062.84 ppm) in floral tissues, substantiates the occurrence of tissue-specific metabolite accumulation patterns within the plant. These findings illuminate the potential of *R. major* var. *apterus* as a natural antioxidant source, antimicrobial agent, and functional food constituent, while establishing a robust scientific foundation for future pharmacognostic investigations and biotechnological applications.

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