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Original Research Article

HPLC-DAD and HPLC-MS/MS Analyses of Phenolic Compounds, Antioxidant and Antibacterial Activities of the Methanol Extract of *Atractylis caespitosa* Desf

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ABSTRACT

The Asteraceae family are known for their diverse pharmacological activities, such as hepatoprotective, antioxidant, antibacterial, and anti-inflammatory effects. This study aimed to investigate the phenolic compounds composition, antioxidant, and antibacterial activities of the aqueous-methanol extract of the aerial parts of *Atractylis caespitosa* Desf., a plant species from the Asteraceae family. This species is used in conventional medicine to treat various ailments across different regions of Algeria. The phenolic compounds were analyzed using a newly developed High-Performance Liquid Chromatography coupled with Diode Array Detection (HPLC-DAD) and High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) techniques. The antioxidant activity was assessed using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging and the Ferric Reducing Antioxidant Power (FRAP) assays. Antibacterial activity was evaluated against notable pathogenic bacterial strains using the disk diffusion method. Through area normalization, 13 phenolic compounds were identified from the HPLC-DAD analysis of *Atractylis caespitosa* aerial parts with rosmarinic acid being the predominant compound at a concentration of 36.662 ppm. Additionally, 5 phenolic compounds were detected through the HPLC-MS/MS analysis, with gentisic acid being the most abundant at a concentration of 8.492 µg/g. The extract exhibited significant antioxidant activity with IC_{50} of 366.65 ± 1.25 µg/mL in the DPPH assay. The extract showed notable antibacterial activity, particularly against *Pseudomonas aeruginosa* and *Escherichia coli*, with the extract showing the lowest Minimum Inhibitory Concentration (MIC) of 2.5 mg/mL against both bacterial strains. The present findings pave the way for further exploration of *Atractylis caespitosa*.

Keywords: *Atractylis caespitosa* Desf., Phenolic compounds, Antioxidant activity, Antibacterial activity.

Introduction

Throughout history, plants have played a significant role in medicine due to their capacity to generate secondary metabolites with remarkable biological properties. Plants have been an integral part of traditional healing methods, they are used to treat various disease conditions. According to the World Health Organization (WHO), over four-fifths of the world's population still relies on native and botanical treatments, many of which are based on plants. Remedies derived from medicinal flora in customary practices are often more affordable, easily obtainable, and associated with fewer side effects compared to synthetic drugs.

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Recently, advanced research techniques have been employed to investigate various traditional medicinal plants, leading to the discovery of numerous potential compounds. These naturally sourced substances may act as a foundation for developing new drugs or enhancing existing ones.[1,2] The investigation of natural antioxidants, particularly plant-derived phenolic compounds, has highlighted their numerous benefits, including their ability to reduce oxidative damage and exhibit anti-inflammatory, anti-cancer, and antibacterial properties.[3] Owing to its geographical location in the southern Mediterranean basin, Algeria is characterized by a diverse array of climate zones and plant life. The country is home to over 3,139 species of spontaneous and naturalized plants, which contribute significantly to the identity of its communities. The Algerian population utilizes many of these species for various purposes, as demonstrated by the extensive use of medicinal plants among herbalists, alternative medicine practitioners, and individuals seeking remedies for various diseases.[4] The majority of species in the Asteraceae family possess healing properties and have a longstanding history in traditional medicine, with several having been cultivated for food and medicinal purposes for over 3,000 years. While they are recognized globally, they are particularly abundant in dry and semi-arid regions of the subtropics. Members of the Asteraceae family are known for their diverse beneficial characteristics, such as hepatoprotective, antioxidant, antibacterial, and anti-inflammatory effects.[5]

Following the identification of the species *Atractylis cancellata* L,[6] the genus *Atractylis* is classified as a new genus of the Asteraceae family. This classification serves as a basis for analyzing future classifications of the genus as a representative example. However, the definition of the *Atractylis* genus remains unclear, and there is considerable disagreement among authors regarding the number of species that belong to it.[7] In 1889, Batandier and Trabut classified the Algerian species of this genus under the rank *Carlinodia*.[8] The species included *Atractylis caespitosa* Desf., *A. humilis* L., *A. polystachya* Coss., *A. faeolipes* Pom., *A. echinata* Pom., *A. ceratoidolidis* (Ceh. ex Cass.), and *A. carduus* (Forssk.). Although the *Carlinoideae* family was not specifically mentioned in the genus classification at that time, the name *Carlinoideae* is no longer applicable, particularly after January 1, 1953, as it designates a subgroup and cannot serve as the primary name for the genus.[9] The genus *Atractylis* is known for its diuretic effects and various medicinal properties. Plants under this genus are used to treat conditions such as cholelithiasis, tumors, circulatory system disorders, intestinal parasites, ulcers, snakebite envenomation, and hepatitis. Additionally, the biological activities of *Atractylis* are broad and diverse, demonstrating effectiveness in treating inflammatory and infectious diseases, as well as conditions related to oxidative stress.[10-12] The flowers of *A. caespitosa* are utilized in southern Europe to coagulate milk.[13] In Algeria's Aurès area, the herb, locally recognized as "Degaa," is applied as an ointment to treat adolescent acne. According to Cheriti *et al*.[14] *A. caespitosa*, referred to as "Knoud" in the El-Bayadh region, is recommended for its anti-gastralgic effects when its roots are prepared as a decoction.[14] This species is a recently discovered medicinal plant that has not been previously documented in Algeria. Known by its native name "Kanouda," it is used to treat rheumatism, obesity, bloating, constipation, and hepatitis.[15] *Atractylis caespitosa Desf* grows in dense tufts and produces heads of globose flowers, which can sometimes feature striking ray blooms. The leaves are small, lanceolate-linear, and may be either pubescent or glabrous, with regularly toothed-spiny edges and thicker margins along the marginal vein. The plant typically has an average of 15 to 20 flower heads, although it can sometimes produce more. The involucre has additional bracts that resemble leaves or are more spiny. The dorsal vein of the involucre continues into a subulate spine, and the bracts are truncated. Occasionally, the bracts may display a black stain on their upper surfaces. This species is commonly found in steppes, rocky pastures, and wooded areas.[16] This study is the first to conduct both qualitative and quantitative analyses of phenolic compounds in the *Atractylis caespitosa Desf.* plant extract using High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) and High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD). This study aimed to highlight the phenolic compounds composition, the antioxidant and antibacterial potentials of *Atractylis caespitosa* Desf. aerial parts due to its traditional medicinal uses in the region where it was collected. In addition, the dearth of scientific studies on this species, drew our attention to investigate the phenolic profile of this plants and its potential biological activities.

Material and Methods

Reagents and Equipment

The solvents and reagents used for the study included methanol, acetic acid, acetonitrile, formic acid, hexane, Folin-Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3), gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), butylated hydroxyanisole (BHA), potassium dihydrogen phosphate (KH_2PO_4), dimethyl sulfoxide (DMSO), and ascorbic acid (Vitamin C). All reagents were of analytical grade. The equipment used were Rotary Evaporator (Heidolph instruments), UV-Vis spectrophotometer (Agilent BioTek Epoch 2 Microplate Spectrophotometer), and LC-MS/MS (HP-Agilent 1200 Infinity Series 1290 Infinity HPLC system hyphenated with Triple Quadrupole MS/MS system).

Collection and identification of plant sample

The aerial parts of *Atractylis caespitosa* Desf. (synonym: *Atractylis humilis* subsp. *caespitosa* (Desf.) Maire) were collected in May 2023 from the Hammam Dalaa Province of M'sila, located in northeastern Algeria (35°58'57"N, 04°23'59"E) at an altitude of 994 meters. The plant material was identified by Pr. K. Rebbas, Department of Natural and Life Sciences, M'Sila University. A herbarium specimen with voucher number N° KR0091 was deposited at the herbarium of the same institution.

Preparation of extract

The aerial parts of the plant were cleaned and dried in a shaded area before being ground using a laboratory mill. The powdered plant sample was macerated in methanol:water (30:70) at room temperature for 24 hours, with gentle agitation. The extraction process was repeated three times. The combined extract was filtered using Whatman filter paper (125 mm), and the resulting filtrate was concentrated using a Rotary Evaporator at 45°C at a speed of 40 rpm. The residual solvent was removed by drying the concentrated extract in a laboratory drying chamber. The dried extract was stored in a refrigerator at 4°C until used for the experiment.[17]

Determination of total phenolic content

The total phenolic content of the extract was determined spectrophotometrically using the Folin-Ciocalteu reagent according to previuosly described method.[18] In this procedure, 200 µL of the diluted extract (1 mg/mL) was mixed with 1 mL of Folin-Ciocalteu reagent. After 4 minutes, 800 µL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The mixture was incubated at room temperature for 2 h protected from light. The absorbance of the reaction mixture was therafter measured at 765 nm. Gallic acid (200 µg/mL) was used to create the standard calibration curve. The total phenolic content was reported as microgram gallic acid equivalent per milligram of crude extract (µg GAE/mg extract).[19]

High-performance liquid chromatography analysis with diode-array detection

The phenolic compounds composition of the extract was determined using the method outlined by Caponio *et al*. (2007)[20] with minor modification. The HP-Agilent 1200 Infinity Series 1290 Infinity HPLC system, fitted with a C18 column and a diode array detector (DAD), was used for the analysis. The mobile phase was composed of 3% acetic acid (v/v) in water (A) and methanol (B). Injection volume was set at 10 µL, with extract concentrations at 20 mg/mL in methanol. The eluates were observed at 278 nm. A gradient elution with a flow rate of 0.8 mL/min was applied as follows: 93% A - 7% B for 0.1 min; 72% A - 28% B for 20 min; 75% A - 25% B for 8 min; 70% A - 30% B for 7 min; 67% A - 33% B for 15 min; 58% A - 42% B for 2 min; 50% A - 50% B for 8 min; 30% A - 70% B for 3 min; 20% A - 80% B for 2 min; and 100% B for 5 min until completion. The standards used for the analysis were 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, benzoic acid, catechin hydrate, chlorogenic acid, caffeic acid, epicatechin, gallic acid, hesperidin, p-coumaric acid, quercetin, rosmarinic acid, sinapic acid, syringic acid, t-cinnamic acid, and t-ferulic acid. The phenolic compounds in the extract was identified by comparison with established reference standards. The phenolic compounds were quantified using external calibration curves tailored to each standard, the concentration of each phenolic compound was calculated and reported as milligrams per kilogram of extract.

High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

A 50 mg of the sample contained in a 2 mL Eppendorf tube was dissolved with 1 mL of a mixed solution consisting of acetonitrile, methanol, and water (1:1:1). The sample was vortexed until fully dissolved. If the sample was insoluble, it was treated in an ultrasonic bath. Extraction was performed by the addition of 0.8 mL of hexane, and the mixture was centrifuged at 7000 rpm for 5 minutes. The subphase was collected and diluted at a ratio of 1:4, and then filtered through a 0.25 µm filter. Finally, LC-MS/MS analysis was performed using the Agilent 6460 Triple Quad LC-MS system with a reverse-

phase C18 Poroshell 120 column (50 mm × 4.6 mm I.D., 2.7 µm). The injection volume was 4 µL, and the analysis was conducted at 30°C for 40 minutes at a flow rate of 0.4 mL/min. The mobile phase consisted of water (0.1% formic acid, 5 mM ammonium formate) (A) and acetonitrile (0.1% formic acid) using the gradient outlined below (B) : Zero min, 85% A and 15% B; 5 min, 75% A and 25% B; 15 min, 25% A and 75% B; 16 min, 0% A and 100% B; 20 min, 0% A and 100% B; 22 min, 85% A and 15% B; 40 min, 85% A and 15% B.

Determination of total antioxidant activity
DPPH radical scavenging assay
The antioxidant capacity of the extract of *Atractylis caespitosa* Desf. aerial parts was evaluated using the DPPH free radical scavenging assay following the method outlined by Topcu *et al*.[21] with minor modification. This method is based on reducing the violet DPPH (2,2-diphenyl-1-picrylhydrazyl) to the yellow 2,2-diphenyl-1-picrylhydrazine. In this assay, 1 mL of the extract at various concentrations (4 - 1000 µg/mL) was mixed with 1 mL of a fresh methanol solution of DPPH at a concentration of 0.025 mg/mL. The mixture was incubated at room temperature for 30 minutes, protected from light. The absorbance of the reaction mixture was measured at 517 nm using Agilent BioTek Epoch 2 Microplate Spectrophotometer. A lower absorbance value indicates greater ability in scavenging DPPH free radicals. Three trials of the experiment were conducted, and the average values ± standard deviation (SD) were reported. Butylated hydroxyanisole (BHA) was used as a reference standard. The percentage DPPH radical scavemging activity was calculated using the formula below.

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{Ac + As}{Ac}\right) \text{X } 100$$

Where; As is the absorbance of the extract sample with DPPH, and Ac is the absorbance of the methanol blank (Ac) with DPPH.
The 50% inhibitory concentration (IC_{50}) measured in µg/mL was determined from the plot of percentage inhibition versus concentration.

Ferric reducing antioxidant power (FRAP) assay
The FRAP assay was performed using the method described by Oyaizu (1986)[22] with minor modification. Briefly, a mixture of 10 µL of different concentrations of the extract (4 - 1000 µg/mL), 50 µL of 1% potassium ferricyanide ($K_3Fe(CN)_6$) and 40 µL of phosphate buffer (pH 6.6) was prepared. After a 20-minute incubation period at 50°C, 10 µL of 0.1% ferric chloride ($FeCl_3$), 40 µL of distilled water, and 50 µL of 10% trichloroacetic acid (TCA) were introduced. The absorbance of the resulting solution was then measured at 700 nm. The concentration at which the absorbance reached 0.50 was noted and represented as A0.5 (in µg/mL). Ascorbic acid was used as the reference standard.

Determination of antibacterial activity
Test organisms
Typed culture of a Gram-positive bacterium, *Staphylococcus aureus* (ATCC 25923) and two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27253) were used in the study. These strains were sourced from the Pasteur Institute in M'Sila, Algeria, where the antibacterial activity assays were also performed.

Antibacterial susceptibility testing
The antibacterial activity of *A. caespitosa* aerial parts extract was evaluated against the test bacterial strains using the disk diffusion method.[23] First, the bacterial strains were incubated at 37°C for 24 h in BHA medium. Following this, they were isolated into colonies using the dilution method and cultured for an additional 24 h in the same medium at 37°C. Five milliliters of nutrient broth were then added to one or more colonies from each pure culture. After homogenizing the bacterial suspension, it was cultured at 37°C for 10 to 24 h. After incubation, the optical density (OD) of 1 mL of the inoculum was measured using a spectrophotometer set at 625 nm, ensuring that the opacity was equal to 0.5 McFarland. Every culture sample was evenly spread onto plates with Mueller-Hinton agar using a sterile swab. Sterile Whatman paper disks (6 mm diameter) were saturated with 10 µL of the extract at different concentrations and carefully placed on the treated agar surface using sterile tweezers. Negative control disks were infused with DMSO. The Petri dishes were incubated at 37°C for 24 h. The tests were performed in triplicates. The results were presented as the diameters in millimeters of the growth inhibition zones surrounding the disks.

Statistical analysis
The data were presented as the mean ± standard deviation (Mean ± SD) of triplicate determination. Data were analyzed using one-way analysis of variance using Microsoft Excel 2010 (Microsoft Corp., USA, 2010).

Results and Discussion

Extraction yield and total polyphenolic content
The primary sources of intrinsic antioxidants are phenolic compounds, which include phenols, flavonoids, tannins, phenolic acids and lignans. Antioxidants derived from phenolic groups act as efficient nucleophiles and lipid peroxidation inhibitors, binding free radicals and preventing oxidation reactions.[24] Additionally, phenolic compounds function as metal ion chelating agents, further inhibiting oxidation. These valuable natural antioxidants are commonly present in significant concentrations within wild plants. For the extract of *Atractylis caespitosa* Desf., both the extraction yield and total polyphenolic content (TPC) were assessed. The TPC values were expressed as micrograms of gallic acid equivalent (GAE) per milligram (mg) of extract. The applied extraction method resulted in an overall extraction yield of 15.26%, with a maximum TPC content of 125.81 ± 0.95 µg GAE/mg of plant extract. The TPC of *A. caespitosa* extract obtained in the present study is in congruence with the values obtained for ethyl acetate extract (140.97 ± 0.53 µg GAE/mg of plant extract) and the n-butanol extract (113.77 ± 0.62 µg GAE/mg) of the plant as reported by Sifouane. [25]

In vitro antioxidant activity
The antioxidant activity of *Atractylis caespitosa* Desf extract was evaluated using the ferric reducing antioxidant power assay and the free-radical scavenging assay, both of which are important indicators of antioxidant potential. The DPPH test was used to measure the free radical scavenging capacity, while the potassium ferricyanide reduction assay was used to assess the ferric reducing antioxidant power. A lower IC_{50} value and a lower A0.5 value for the DPPH assay and FRAP assay, respectively indicate greater antioxidant activity of the extract. High antioxidative capacity of the *A. caespitosa* extract was consistently exhibited across all the concentrations tested. However, it was notably lower than that of the standard antioxidants used in each assay. Table 1 shows the IC_{50} and A0.5 values of the aqueous methanol extract of *A. caespitosa* in comparison with the standard antioxidants butylated hydroxyanisole (BHA) and ascorbic acid used in each of the assays.

Table 1: Antioxidant activity of aqueous-methanol extract of *Atractylis caespitosa* aerial parts

Extract and standard	DPPH IC_{50} (µg/mL)	**FRAP A0.5**
A. caespitosa	366.65 ± 1.25	-
BHA	32.71±1.46	**NT**
Ascorbic Acid	**NT**	218.18 ± 1.50

NT: Not Tested, IC_{50}: 50% Inhibitory Concentration 50%, BHA: Butylated hydroxyanisole

Atractylis caespitosa Desf. extract exhibited an IC_{50} value of 366.65 ± 1.25 µg/mL in its capacity to neutralize free radicals, as determined by the DPPH assay (Figure 1). This value was lower than that of BHA, which exhibited an IC_{50} value of 32.71 ± 1.46 µg/mL. This result is somewhat similar to the findings of the study by Sifouane .[25] Regarding

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the ferric reducing antioxidant power (FRAP) activity, the extract demonstrated the capacity to reduce ferric cyanide (Fe^{3+}) to ferrous cyanide (Fe^{2+}), with an A0.5 value for the extract lower compared to that reported for the n-butanol and ethyl acetate extracts from the aerial parts of *A. humilis*.[26] The extract also showed a lower A0.5 value compared to ascorbic acid (Figure 2).

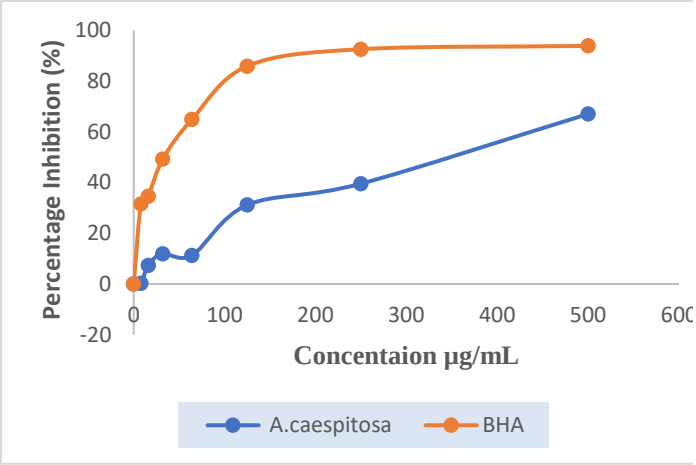

Figure 1: DPPH free radical scavenging activity of *Atractylis caespitosa* aerial parts extract and butylated hydroxyanisole (BHA)

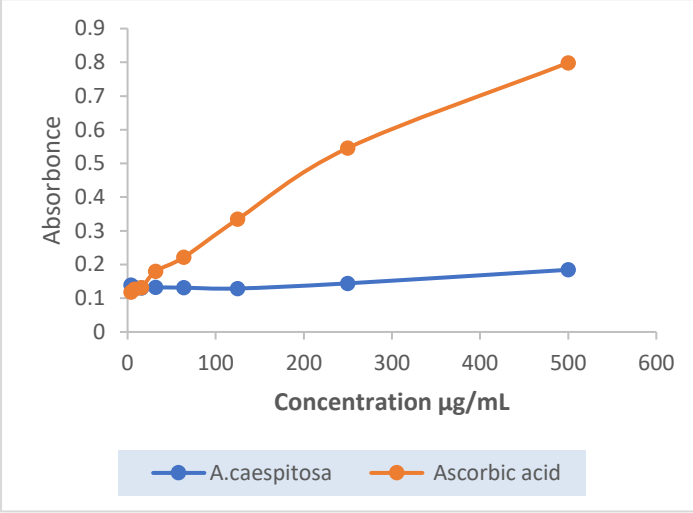

Figure 2: Ferric reducing antioxidant power (FRAP) of *Atractylis caespitosa* aerial parts extract and ascorbic acid

To identify the phenolic compounds present in the methanol extract of *A. caespitosa* and understand their roles in reducing and scavenging free radicals, the current study employed HPLC-DAD and HPLC-MS/MS techniques. Previous research has shown that phenolic compounds possess antioxidant properties, which can be attributed to three primary mechanisms: neutralizing reactive oxygen species *via* hydrogen donation, electron transfer, and metal ion chelation.[26] The antioxidant properties of phenolic compounds are largely attributed to their distinctive structural features and functional groups. Their hydroxyl groups and aromatic rings enable them to effectively neutralize free radicals and mitigate oxidative damage by donating electrons and hydrogen atoms.[27] The results of the present study revealed that the plant species under investigation has antioxidant properties. This finding is supported by the findings from the study of Zakaria *et al*.[28] on the antioxidant activity of Asteraceae family, to which *A. caespitosa* belongs, where a significant role of flavonoids in the actioxidant activity of this plant family was shown.[28]

Phenolic compounds identified from HPLC-DAD analysis of Atractylis caespitosa extract

The phenolic compounds composition of the extract from *Atractylis caespitosa* extract was determined using HPLC-DAD technique. This technique enables a comprehensive analysis of the phytochemical profile of the extract and facilitates the detection and quantification of various phenolic compounds. The phenolic composition of the aqueous methanol extract of *A. caespitosa*, as identified through HPLC-DAD analysis is shown in Figure 3.

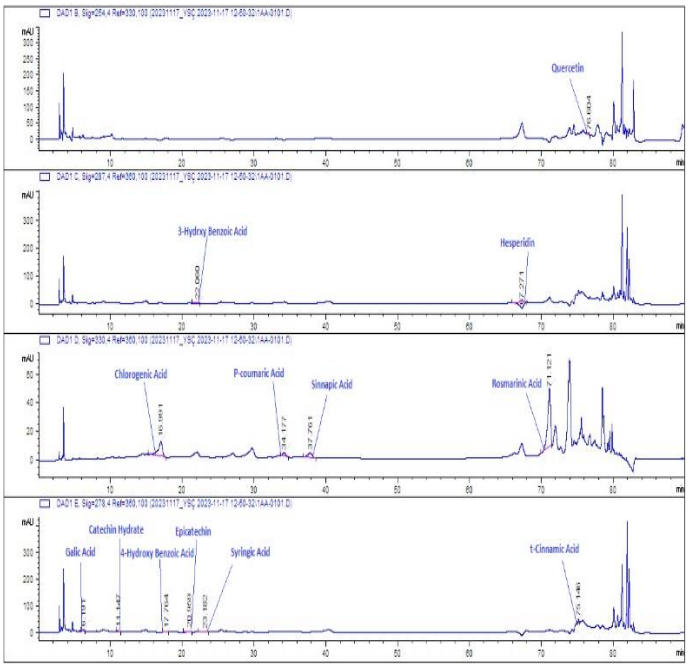

Figure 3: HPLC-DAD chromatogram of phenolic compounds from *Atractylis caespitosa* aerial parts extract

Thirteen (13) phenolic compounds were accurately identified in the aqueous-methanol extract of *A. caespitosa* aerial parts using HPLC-DAD technique, with the UV wavelength of the DAD detector set at 254, 278, 287, and 330 nm, the compounds were identified based on their absorption characteristics in the UV spectrum. Quercetin was identified at 254 nm, while 4-hydroxy benzoic acid, benzoic acid, catechin hydrate, epicatechin, gallic acid, syringic acid, and t-cinnamic acid were identified at 278 nm. 3-Hydroxy benzoic acid and hesperidin were analyzed at 287 nm, and chlorogenic acid, caffeic acid, rosmarinic acid, *p*-coumaric acid, sinapic acid, and *t*-ferulic acid were identified at 330 nm. Each compound's identity was verified by contrasting its retention times and UV spectra with those of reliable standards. The retention times for the peaks of gallic acid, catechin hydrate, chlorogenic acid, 4-hydroxybenzoic acid, epicatechin, caffeic acid, 3-hydroxy benzoic acid, syringic acid, *p*-coumaric acid, *t*-ferulic acid, sinapic acid, benzoic acid, hesperidin, rosmarinic acid, *t*-cinnamic Acid, and quercetin were found to be 5.912, 11.499, 16.239, 17.647, 20.169, 21.476, 22.545, 22.628, 33.597, 37.202, 37.264, 47.629, 65.989, 70.655, and 75.207 min, respectively (Table 2).

Rosmarinic acid was identified as one of the predominant compounds in the extract of *A.caespitosa* aerial parts. Rosmarinic acid has been shown to exhibit various biological activities, including astringent, antimutagenic, antioxidative, antibacterial, anti-inflammatory, and antiviral properties.[29] Notably, rosmarinic acid has been used in preparations of *Melissa officinalis* to treat Herpes simplex infections. Its anti-inflammatory effects are believed to stem from its ability to interfere with the complement cascade and inhibit lipoxygenases and

cyclooxygenases.[29] Furthermore, phenolic compounds like rosmarinic acid have been shown to possess cancer-preventive properties. Additionally, it enhances the antioxidant qualities of plants used in cosmetics.[30] Bnzoic acid, caffeic acid, and sinapic acid were absent in the extract, while chlorogenic acid, apigenin, and hesperidin were present in significant amounts. Apigenin is noted for its anti-diabetic effects, as it can boost insulin secretion and inhibit the activity of *β*-glucosidase,[31] and it may help counteract reactive oxygen species (ROS).[32] Due to its plant origin, apigenin is recognized as one of the bioactive compounds that may reduce cancer risk.

Table 2: Phenolic compounds identified from the HPLC-DAD analysis of aqueous-methanol extract of *Atractylis caespitosa* aerial parts.

N°	**Phenolic Compound**	Correlation Coefficient (r^2)	λ (nm)	RT (min)	Concentration (ppm)
1	3-Hydroxy Benzoic acid	0.99928	287	22.545	3,41
2	, 4-Hydroxy Benzoic acid	0.99994	278	17.647	0.804
3	Benzoic acid	0,99986	278	47.629	**N.D**
4	Catechin Hydrate	0.99906	278	11.499	2.378
5	Chlorogenic Acid	0.99970	330	16.239	12,696
6	Caffeic Acid	0.99892	330	21.476	**N.D**
7	Epicatechin	0.99879	278	20.169	10.148
8	Gallic Acid	0.99966	278	5.912	2.145
9	Hesperidin	0.99705	287	65.989	26.101
10	*p*-Comaric acid	0.99982	330	33.597	1.516
11	Quercetin	0.99962	254	76.313	5.267
12	Rosmarinic acid	0.99907	330	70.655	36.662
13	Sinnapic acid	0.99925	330	37.264	**N.D**
14	Syringic acid	0.99839	278	22.628	1.306
15	*t*-Cinnamic acid	0.99998	278	75.207	0.899
16	*t*-Ferruc acid	0.99993	330	37.202	1.73

RT: Retention Time, ppm: parts per million, ND: Not Detected.

A high dietary intake of flavonoids from fresh produce has been linked with a lower risk of cancer. Notably, a study that investigated the relationship between the consumption of flavonoids such as kaempferol, quercetin, apigenin, luteolin, and myricetin and the risk of lung cancer found an inverse correlation.[33] Hesperidin, a flavonoid found in citrus fruits, is recognized for its antibacterial, anticancer, antioxidant, and anti-inflammatory activities.[34] Chlorogenic acid, another phenolic compound, is known for its anti-inflammatory and antioxidant effects.[35] The remaining polyphenols were detected at modest concentrations, ranging from 0.804 to 10.148 ppm. A review by De *et al*. (2011)[36] provided a comprehensive overview of recent publications concerning the cytotoxic and anticancer properties of cinnamic acid derivatives. Additionally, both syringic acid and gallic acid have demonstrated anti-inflammatory properties.[35,37,38] Moreover, catechin, a flavonoid abundant in tea leaves, is well-known for its antioxidant qualities and potential antiviral, anticancer, antibacterial, antiallergenic, and anti-inflammatory effects.[35]

The preliminary phytochemical study and HPLC analysis of *A. caespitosa* aerial parts extract has demonstrated the diversity of bioactive secondary metabolites in the plant, highlighting its potential as a valuable natural resource with antioxidant properties. Additionally, this study confirms the significant role of the secondary metabolites found *A. caespitosa*, highlighting their importance in health and pharmaceutical applications.

Compounds identified from HPLC-MS/MS analysis of Atractylis caespitosa extract

The HPLC-MS/MS analysis of the methanol extract of *A. caespitosa* identified five phenolic compounds. The extract showed the highest content of gentisic acid (8.492358 µg/g), followed by *p*-coumaric acid (4.578683 µg/g), vanillin (2.593676 µg/g), and chlorogenic acid (1.085519 µg/g), while, the compound rutin was present at a lower concentration (0.042877 µg/g) (Table 3). The HPLC chromatogram is displayed in Figure 4 The compounds were eluted as follows: 3-Chlorogenic acid, 2.76 min; 9- Rutin, 3.93 min; 5-Gentisic acid, 3.41 min; 13- *p*-coumaric acid, 4.30 min; 15- Vanillin, 4.85 min. Gentisic acid (GA) a well known phenolic compound has been demonstrated to possess antioxidant properties. Due to the presence of two hydroxyl (OH) groups oriented perpendicularly to each other, GA exhibits stronger antioxidant properties than monohydroxy phenolic acids.[39] The antioxidant activity of gentisic acid has also been shown to contribute to its anticarcinogenic effects. One study investigated the potential of gentisic acid to inhibit tumor growth in Swiss albino mice, and the results indicated that topical application of gentisic acid (2 and 4 µg/0.2 mL acetone for three days) markedly enhanced antioxidant enzyme concentrations and effectively inhibited tumor growth.[40] Additionally,

gentisic acid (GA) has been used in the management of rheumatoid arthritis.[41]

Antibacterial activity

The extract of *Atractylis caespitosa* aerial part exhibited antibacterial activity against all tested bacterial strains. *Pseudomonas aeruginosa* (ATCC 27253) was the most sensitive to the extract at a minimum inhibitory concentration (MIC) of 2.5 mg/mL (Table 4**)**. Previous studies have indicated that *P. aeruginosa* is a major cause of septicaemia, lung infections, urinary tract infections and hospital-acquired infections, particularly in patients with weakened immune systems.[42] This bacterium has developed resistance to several classes of antibiotics through various resistance mechanisms.[43] *Staphylococcus aureus* (ATCC 25923) was the next most sensitive organism after *Pseudomonas aeruginosa*, and the MIC of the extract against *S. aureus* was 10 mg/mL (Table 4, Figure 5). *S. aureus* strains are known for their resistance to many antibiotics, presenting a significant challenge for treatment.

Table 3: Phenolic compounds identified from HPLC-MS/MS analysis of of aqueous-methanol extract of *Atractylis caespitosa* aerial parts

N°	Compound	Retention time	Formula	Final Conc (µg/g)	M.I. (m/z)	F.I. (m/z)
1	Gallic acid	2.37	/	ND	/	/
2	Epigallocatechin	2.33	/	ND	/	/
3	Chlorogenic acid	2.76	$C_{16}H_{18}O_9$	1.085519	350.0	353.0
4	Catechin	2.85	/	ND	/	/
5	Gentisic acid	3.41	$C_7H_6O_4$	8.492358	152.8	152.8
6	Caffeic Acid	3.30	/	ND	/	/
7	Syringic acid	3.47	/	ND	/	/
8	Vanillic acid	3.52	/	ND	/	/
9	Rutin	2.93	$C_{27}H_{30}O_{16}$	0.042877	610.56	609.10
10	Isoquercitrin	4.49	/	ND	/	/
11	Polydatin	4.45	/	ND	/	/
12	Hydroxybenzaldehyde	4.52	/	ND	/	/
13	p-coumaric acid	4.30	$C_9H_{10}O_3$	4.578683	163.1	163.10
14	Sinapic acid	5.27	/	ND	/	/
15	Vanillin	4.85	$C_8H_8O_3$	2.593676	152.9	152.9
16	trans-ferulic acid	5.65	/	ND	/	/
17	Taxifolin	5.77	/	ND	/	/
18	Salicylic Acid	7.87	/	ND	/	/
19	*o*-coumaric acid	7.77	/	ND	/	/
20	Baicalin	7.67	/	ND	/	/
21	Protocatechuic ethyl ester	8.48	/	ND	/	/
22	Protocatechuic acid	8.40	/	ND	/	/
23	Kaempferol	10.05	/	ND	/	/
24	Trans-cinnamic acid	10.65	/	ND	/	/
25	Naringenin	11.26	/	ND	/	/
26	Morin	11.92	/	ND	/	/
27	Quercetin	11.97	/	ND	/	/
28	7-Hydroxyflavone	12.02	/	ND	/	/
29	Chrysin	13.59	/	ND	/	/
30	Luteolin	14.08	/	ND	/	/
31	Biochanin A	14.70	/	ND	/	/
32	5-Hydroxyflavone	15.74	/	ND	/	/
33	Diosgenin	20.63	/	ND	/	/

FI : Fragment ions**; MI :** Molecular ions of the standard analytes(m/z ratio); **R.T:** Retention Time; **ND:** Not Detected **(/) :** Not determined.

Table 4: Antibacterial activity of aqueous-methanol extract of *Atractylis caespitosa* aerial parts

Test bacteria	Extract concentration (mg/mL)			
	20	**10**	**5**	**2.5**
Pseudomonas aeuginosa	12	10	7	-
Escerichia coli	9	8	7	-
Staphylococcus aureus	10	7	-	-

Values represent inhibition zone diameter in millimeters

The manifestations of *S. aureus* bacteremia can range from mild skin infections to severe conditions like sepsis. This bacterium is notorious for causing infections in various sites, including the skin, bones, joints, lungs, and vascular locations, often leading to septicemia, pneumonia, endocarditis, and ocular infections. It frequently colonizes surfaces, particularly surgical wounds and on implanted medical devices.[44] Furthermore, *Escherichia coli* (ATCC 25922) strain also showed sensitivity to the methanol extract of *Atractylis caespitosa* aerial parts, and the extract gave an MIC of 2.5 mg/mL. Although *Atractylis* species are generally thought to have weak inhibitory effects on this strain, the present results showed substantial efficacy. These findings corroborated a previous study where *Atractylis caespitosa* extract exhibited antibacterial activity against the three test strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*).[25]

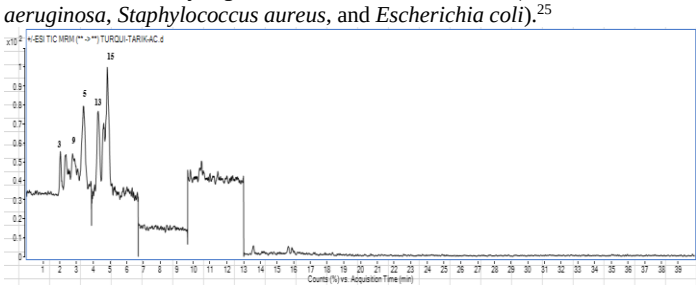

Figure 4: LC-MS/MS chromatogram showing the phenolics compounds identified in *Atractylis caespitosa* aerial parts extract

Figure 5: Antibacterial activity of *Atractylis caespitosa* aerial parts extract with Petri dishes showing the zones of inhibition

Conclusion

This study examined the total polyphenol content, phenolic composition, antioxidant, and antimicrobial activities of the wild plant extract *Atractylis caespitosa* Desf. The phenolic compounds were identified using a combination of HPLC-DAD and HPLC-MS/MS analysis for the first time. The extract exhibited significant antioxidant activity by scavenging DPPH free radical and reducing ferric ions to ferrous ions. Additionally, the extract demonstrated strong antibacterial activity, especially against *Pseudomonas aeruginosa*, indicating the effectiveness of the extract in combating infections caused by this bacterium. HPLC-DAD and HPLC-MS/MS analysis identified numerous phenolic compounds in *Atractylis caespitosa* aerial parts, which gave shown potential antibacterial, anti-inflammatory, anticancer, and antioxidant properties. Furthermore, substantial amounts of rosmarinic acid along with a high concentration of gentisic acid were detected in the aerial parts of *A. caespitosa* through HPLC-DAD and HPLC-MS/MS analyses, respectively. These two compounds are widely recognized for their therapeutic benefits, making *Atractylis caespitosa* an important natural source of these compounds. This discovery opens up new avanues foe the potential pharmaceutical and medical applications of this plant. However, further investigation into the biological activities of the plant will deepen the understanding of its potential applications in the medical and pharmaceutical feilds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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