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Antioxidant, Antibacterial Activity, Ethnopharmacology, Phytochemical in Different Extracts of *Melilotus officinalis* L. as an Anti-infection and Anti-diabetic in Traditional Uses of Two Northern Provinces From Iran

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Abstract

Objectives: The current research delves into ethnopharmacology, specifically focusing on the influence of extraction solvent on parameters such as total phenol, flavonoid, coumarin, tannin, antioxidant, and antibacterial activity. This investigation spans different parts of *Melilotus officinalis* L. within two northern provinces of Iran. It is a holistic approach, exploring both traditional applications and the scientific impact of extraction methods on the plant's bioactive properties.

Materials and Methods: In various observational studies across different fields, ethnopharmacological data were acquired by collecting various parts of the *M. officinalis* plant (flowers, stem, and root) at different blooming stages from Charbagh Mountain (2340 msl) and Gorgan region (200 msl) between June and July 2016. "Ethanol and water extracts" of these plant parts were obtained through the maceration method. The quantification of "total phenol" (TP), "flavonoid" (TF), coumarin (CO), and tannin (TA) content in the extracts was conducted using spectrophotometry. The in vitro "antioxidant capacity" of the extracts was assessed through the DPPH free radical scavenging assay, the total antioxidant capacity (TAC) assay, and the reducing power assay. Additionally, the antibacterial activity of the extracts was examined against nine gram-positive and gram-negative bacteria using disc diffusion and minimum inhibitory concentration (MIC) assays.

Results: Based on the results, the ethanol extract obtained from the flowers of *M. officinalis* collected at a high altitude (2340 meters) in Semnan province demonstrated the highest concentrations of total phenols (38.08 ± 0.13 mgGA/g), flavonoids (62.04 ± 0.01 mgQU/g), coumarins (19.32 ± 0.08 mL/g), and tannins (33.89 mg/g). Notably, coumarin and tannins were absent in the water extract. The ethanol extract from the flowers in Semnan province exhibited superior antioxidant activity, particularly in the DPPH method (IC50=10.61 ± 0.81 µg/mL), surpassing the efficacy of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Moreover, the ethanol extract from high-altitude flowers displayed potent antimicrobial activity against gram-positive bacteria, specifically *Staphylococcus aureus, S. epidermidis,* and *Bacillus cereus,* with inhibition zone (IZ) values of 19.1 ± 0.01 mm, 15.8 ± 0.2 mm, and 14.4 ± 1.12 mm, respectively. The MIC values against these bacteria were 24.5 µg/mL, respectively. In summary, the ethanol extract exhibited greater effectiveness against gram-positive bacteria compared to gram-negative bacteria.

Conclusions: Our results indicate a robust association among the extraction solvent, plant part, phytochemical composition, antibacterial efficacy, and antioxidant potential of *M. officinalis* L. Specifically, the ethanol extract derived from the flowers displayed the most significant antioxidant and antibacterial characteristics. This implies that the ethanol extract from the flowers of *M. officinalis* L. holds promise as a valuable reservoir of natural antioxidants, aligning with its traditional utilization in addressing various infections.

Keywords: Antioxidant, Antibacterial, Ethnopharmacology, Gorgan and Semnan province, *Melilotus officinalis* L., Phytochemical

Introduction

Reactive oxygen species (ROS) constitute a diverse category of molecules, encompassing radicals like the hydroxyl radical (OH) and superoxide anion (O_2^-) alongside nonradicals such as hydrogen peroxide (H2O2) and inglet oxygen ($^1O_2$). Recent research underscores that oxidative stress can prompt various degrees of cell dysfunction, emerging as a significant contributor to cellular impairment due to the heightened generation of ROS. These ROS interfere with the body's ability to neutralize free radicals, affecting enzymatic and non-enzymatic antioxidant mechanisms. Furthermore, ROS can potentially compromise the structural integrity of the cell membrane's biochemical components, particularly impacting polyunsaturated fatty acids (1-3). ROS-induced DNA damage can lead to the inhibition of cell functions, catralyzing the development of numerous disorders, including cancer, inflammatory conditions, and infectious diseases (4,5).

Indeed, there has been a keen interest in exploring new natural antioxidants derived from various sources, including secondary metabolites found in wild plants. Compounds such as flavonoids, tannins, coumarins, phenols, and terpenoids have garnered attention for their potential antioxidant properties. These secondary metabolites can neutralize free radicals, making them

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valuable as antioxidants. Additionally, they exhibit properties that make them effective as anti-fungal and antibacterial agents, further highlighting their potential therapeutic benefits (2,6,7). Incorporating these secondary metabolites, including flavonoids, tannins, coumarins, phenols, and terpenoids, into antioxidant supplementation is crucial for promoting a healthy lifestyle. By harnessing their antioxidant properties, these natural compounds enhance free radical protection in the body. Including them in antioxidant supplements can be a valuable strategy to support overall well-being and counteract oxidative stress, ultimately contributing to a healthier lifestyle (8). While synthetic antioxidant drugs can effectively combat oxidative damage, it is important to note that some may come with significant side effects. These side effects can contribute to the development of various modern illnesses. This emphasizes the need to explore and develop natural antioxidants, such as those found in secondary metabolites of wild plants, as safer alternatives with potentially fewer adverse effects in the pursuit of maintaining health and well-being (5). *Melilotus officinalis* (L.) Pall. (yellow sweet clover) belongs to the Fabaceae family, and it is wild and widespread beside the sunny position of the roads and fields in many Mediterranean and Eurasian regions. In some traditional uses, the aerial parts of this plant in bloom have been used as a natural ointment to treat headaches and stomach pains. It is also used to compress swelling, diabetic ulcers, and skin infections (9). In another literature review, they reported that the medicinal activity of *M. officinalis* is attributable to the presence of bioactive secondary metabolites such as coumarin, dihydrocoumarin, melilotin, tannins, flavonoids, and polyphenols, which can be used as an anticoagulant, antioxidant, anti-inflammatory, and antibacterial activity in many pharmaceutical industries as a natural effective drug (9). Coumarins and flavonoids extracted from *Melilotus* species have found application in the pharmaceutical industry, where they are utilized as key ingredients in formulating antioxidant and anti-inflammatory medications. These natural compounds play a significant role in combating free radicals, contributing to the effectiveness of medications designed to address oxidative stress and inflammation. Using such bioactive compounds from *Melilotus* species underscores the potential of natural sources in developing therapeutic agents for maintaining health and managing various conditions (8,10-12).

The data suggests that coumarins and flavonoids extracted from both water and ethanol extracts of *M. officinalis* possess strong antioxidant, antibacterial, and anti-inflammatory activities. This underscores the therapeutic potential of these compounds derived from *M. officinalis* in addressing oxidative stress, combating bacterial infections, and mitigating inflammatory processes. The multi-faceted properties of coumarins and flavonoids highlight their promising role in pharmaceutical and medicinal applications (8). A study in male rabbits provides evidence that an extract from *M. officinalis*, containing 0.25% coumarin, demonstrated a reduction in acute inflammation. This finding supports the anti-inflammatory potential of coumarin derived from *M. officinalis*, suggesting its efficacy in mitigating inflammatory responses in vivo. It adds to the growing body of research supporting the medicinal benefits of natural compounds found in plants, particularly in addressing inflammatory conditions (13). The results from another study suggest that breast cancer patients treated with coumarin isolated from *M. officinalis* extracts experienced improved recovery. This finding highlights the potential therapeutic effects of coumarin in breast cancer treatment and recovery. It is fascinating to see how natural plant compounds from plants, such as *M. officinalis*, can positively impact health outcomes in specific medical conditions (13,14). It has been reported that the different extracts of *M. officinalis* had great antibacterial and anti-fungal activity using the disk diffusion method (14). The available evidence suggests that antioxidant activity in plants arises from the presence of secondary metabolites, whose production is influenced by environmental and extraction factors (3,14). This study investigated the ethnopharmacology, phytochemical composition, antibacterial properties, and antioxidant activity of various extracts from different parts of *M. officinalis* (L.). The research focused on two provinces in northern Iran, where the plant is widely used and has traditional significance.

Materials and Methods

Chemicals and Reagents

The study utilized analytical reagent-grade chemicals and reagents of the utmost purity obtained from Merck without additional purification. This ensures the reliability and precision of the experimental procedures, emphasizing the commitment to using high-quality materials in scientific investigations.

Materials and Extraction of Plant

In the course of field surveys, researchers gathered information on the traditional medicinal uses of this plant by consulting with rural healers. Then, the distinct parts of the plant (flowers, stems, and roots) were collected from two distinct growing places: Chaharbagh Mountain (2340 m above sea level) in Semnan province and Gorgan province (250 m above sea level) in the north of Iran, from early June to July 2015. Local botanists identified the plant initially, and the specimen was deposited in the RCMP Herbarium (Medicinal Plants Research Center, "Islamic Azad University, Gorgan Branch, Golestan Province", Iran; Herbarium No. HRCMP:96). Next, plant samples were finely powdered, and then 30 g of dried

plant material were extracted using a solution of 500 mL of ethanol (80%) and water, using the soaking method for 12 hours with stirring. Then, the obtained extract was filtered and stored at 4 °C. Subsequently, the samples were allowed to return to room temperature and stored for further analysis, including evaluation of phytochemicals, total phenols, flavonoids, coumarins, and tannins, as well as antioxidant and antibacterial activities.

Determination of Total Phenolic Content

Following the Folin-Ciocalteu method, Eghdami et al (15) determined the total phenolic content (TPC) in the extract of *M. officinalis* L. The reaction mixture consisted of 200 µL of diluted plant extract, 800 µL of freshly prepared and diluted Folin-Ciocalteu reagent, and 2 mL of 7.5% sodium carbonate solution. This method provides a quantitative measure of the TPC in the plant extract, contributing to the understanding of its chemical composition. In the experimental procedure, the volume of the reaction mixture was precisely adjusted to 7 mL with deionized water. This step ensures the standardization of the reaction mixture, maintaining a consistent volume for accurate measurements and reliable results in the analytical process. Following the preparation of the reaction mixture, it was incubated in the dark at ambient temperature for 2 hours to facilitate the completion of the reaction. After the incubation period, the absorbance of the reaction mixture was measured at a wavelength of 765 nm. Gallic acid served as the standard for calibration, and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of plant extract. This quantification provides a standardized measure of the TPC in terms of gallic acid equivalents, aiding in comparative analyses and understanding the phenolic composition of the plant extract.

Determination of Total Flavonoid Content

The otal flavonoid content (TFC) in the *M. officinalis* L. extract was determined using a reliable method based on aluminum chloride (AlCl3), with quercetin serving as the standard, following the procedure outlined by Chong et al (16). In this method, 0.1 mL of the plant extract was combined with 0.3 mL of distilled water and 0.03 mL of 5% w/v NaNO2. After the initial 5-minute incubation at 25°C, 0.03 mL of 10% AlCl3 was introduced to the mixture. Following an additional 5-minute interval, the reaction mixture was combined with 0.2 mL of 1 mM NaOH. Subsequent to this step, the reaction mixture was diluted to a final volume of 1 mL with water, and the absorbance was measured at 510 nm. The results were then quantified and expressed in milligrams of quercetin equivalent (QE) per gram of *M. officinalis* L. extract. This quantification provides valuable information about the TFC in the extract, using quercetin as a standard for reference.

Determination of Total Extractable Tannin Content

The determination of the total extractable tannin (TET) content utilized an indirect spectrophotometric method relying on the precipitation of tannins with polyvinyl polypyrrolidone, following the protocol detailed by Nawaz et al (17). The TET content was quantified and expressed as mg GAE/g extract.

Determination of Total Extractable Coumarin Content

The chromatography method was used to evaluate the coumarin content in ground cinnamon samples based on the protocol proposed in the study by Sproll et al (18).

The extract's coumpounds were analyzed using high-performance liquid chromatography (HPLC) with UV detection. The separation process was carried out using a Kinetex 2.6 µm, C18, 10 nm, 150 × 4.6 mm column provided by Phenomenex (Torrance, CA, USA). An isocratic mobile phase consisting of 5 mmol/L ammonium acetate buffer and acetonitrile/methanol (1:2) was employed, maintaining a flow rate of 0.6 mL/min. Under these specific conditions, coumarin displayed a retention time of 3.6 minutes. The injection volume for the analysis was set at 5 µL, and the column temperature was consistently maintained at 35 °C throughout the analysis. These parameters ensure the effective separation and identification of coumarin in the sample.

Validation Parameters

The quantification of coumarin was accomplished through external calibration. Initially, a standard stock solution of coumarin (1000 mg/L) was prepared in methanol/water (1:1) and stored at 4 °C. Subsequently, nine working standard solutions were created in methanol/water (1:1). Each working standard solution underwent five injections into the HPLC, and the average peak areas obtained were utilized to construct the standard calibration curve. The calibration curve exhibited linearity over the concentration range of 0.5 to 200 mg/L. Linear regression analysis, facilitated by Empower Chromatography software (Waters), produced the equation used for quantification. This rigorous calibration process accurately determines coumarin levels in the sample: $y = 30{,}315x + 13{,}944$ ($R2 = 0.9996$). This equation facilitated the calculation of coumarin amounts in cinnamon samples, with a detection limit (3σ) of 0.1 mg/L. The analysis's reproducibility was assessed by conducting ten measurements of the same cinnamon sample under identical HPLC conditions, resulting in a relative standard deviation of 0.93%. Injection reproducibility was gauged by injecting the same standard solution ten times, yielding a relative standard deviation of less than 0.4%. A recovery test was performed to validate the method's reliability and suitability. In this experiment, 1 mL of a standard solution containing 500 mg/L coumarin was added to a 50 mL cinnamon sample solution, and the recovery rate was determined to be 96%.

Evaluating Antioxidant Activity Using the DPPH Assay

The antioxidant activity of *Coreopsis grandiflora* L. extract samples was assessed through the DPPH assay. An 80 µL portion of the extract was diluted 15-fold with distilled water. the assessment of antioxidant activity was conducted using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. For each extract, a 1 M DPPH solution was prepared. The absorbance of the resulting solution, comprising 1 mL of the extract and 1 mL of the DPPH solution, was measured at a wavelength of 517 nm. This method provides valuable insights into the ability of the extracts to scavenge free radicals, contributing to the overall understanding of their antioxidant potential. The radical scavenging activity was calculated using the following equation:

$$\text{Percent radical scavenging activity}=\left(\frac{\text{Abs}_{\text{control}}-\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right)\times 100$$

where $\text{Abs}_{\text{control}}$ represents the absorbance of the DPPH solution without extract, and $\text{Abs}_{\text{sample}}$ represents the absorbance of the solution containing extract and DPPH.

Reducing Power

Evaluating the reducing power (RP) of the ethanolic extract of *C. grandiflora* L. adhered to the method delineated by Salmanipour et al (19). In this process, 1 mL of the extract was combined with 2.5 mL of 200 mM phosphate buffer (pH 6.6) and 2.5 mL of 30 mM potassium ferricyanide. The resulting solution underwent incubation at 50°C for 20 minutes. Post-incubation, 2.5 mL of 600 mM trichloroacetic acid was introduced, and the mixture was centrifuged at 3000 rpm for 10 minutes. The upper layer (2.5 mL) was then mixed with 2.5 mL of distilled water and 0.5 mL of 6.0 mM FeCl3. The absorbance of the resulting solution was measured at 700 nm. Ascorbic acid was the positive control in this assessment of reducing power.

Total Antioxidant Capacity

To evaluate the antioxidant capacity of all extracts, the method proposed by Arabshahi-Delouee and Urooj (20) was used. The sampling method includes the following steps, described sequentially: The plant extract's total antioxidant activity was determined employing the phosphomolybdenum method. A 0.1 mL aliquot of the extract was mixed with 1 mL of a reagent solution comprising 0.6 M sulfuric acid, 28 M sodium phosphate, and 4 M ammonium molybdate. The reaction mixture underwent incubation at 95 °C for 90 minutes. Following cooling to room temperature, the absorbance of each solution was measured at 695 nm against a methanol blank using a UV-VIS spectrophotometer (UVmini-1240). The total antioxidant activity was quantified and expressed as gram equivalents of ascorbic acid (GAE) utilizing a calibration curve prepared with various concentrations of ascorbic acid (1000, 500, 250, 125, 62.5, and 31.25 µg/mL) in ethanol (80%).

Bacterial Strains

The bacterial strains utilized in this study were sourced from the microbiology laboratory at Golestan University of Medical Sciences. The ethanol extracts derived from various plant parts were individually assessed against nine strains of bacteria, encompassing both gram-positive and gram-negative types. The specific bacterial strains included *Shigella dysenteriae* (PTCC1188), *Pseudomonas aeruginosa* (PTCC1430), *Escherichia coli* (PTCC1399), *Staphylococcus aureus* (PTCC1431), *Bacillus cereus* (PTCC1015), *Salmonella typhimurium* (ATCC1596), *Staphylococcus epidermidis* (PTCC1114), *Enterococcus faecalis* (PTCC1393), and *Klebsiella pneumoniae* (PTCC1291).

Antimicrobial activity

In the initial investigation, the minimum inhibitory concentrations (MICs) of the plant extract and essential oil against the specified bacteria were determined by employing the agar serial dilution method. Two-fold serial dilutions of the extract and oil were prepared in Mueller-Hinton agar containing DMSO. Each well was inoculated with a standardized bacterial suspension, and the plates were then incubated for 24 hours at 37 °C. The MIC was defined as the lowest concentration of extract or oil at which no visible bacterial growth occurred. Muller Hinton agar containing DMSO without extract or oil was used as a negative control, while gentamicin served as the positive control.

Statistical Analysis

The statistical analysis results were expressed as the mean ± standard error of the mean. A one-way analysis of variance (ANOVA) was conducted using the SPSS package to assess the difference between means in different groups. Significance was established at a *P* value less than 0.05 in all cases.

Results

Ethnopharmacological Properties

The ethnopharmacological survey reveals that *M. officinalis* L. is a wild, annual herb deeply rooted in traditional practices. Its historical use spans various applications, serving as a tonic—independently or in conjunction with other medicinal herbs—and showcasing roles as an aphrodisiac, emollient, carminative, anti-flatulent, anti-inflammatory, and anti-aging agent. The herb has been traditionally employed for addressing conditions such as diabetes, infections, and pain relief. Additionally, its external application as a poultice has been recognized for alleviating pains and aches, as well as exhibiting anti-malarial properties. This diverse range of traditional uses underscores the multi-faceted significance of *M. officinalis* L. in folk medicine.

Phytochemical Evaluations

Table 1 indicates a robust positive correlation between solvent extraction, plant parts, and their respective TPC, TFC, coumarin (CO), and tannins (TA), as well as their antioxidant and antibacterial activities. The results highlight that the highest concentrations of total phenols (38.08 ± 0.13 mgGA/g), flavonoids (62.04 ± 0.01 mgQU/g), coumarin (19.32 ± 0.08 mL/g), and tannins (33.89 mg/g), along with the most potent antioxidant activity, especially in the DPPH method (10.61 ± 0.81), were observed in the ethanol extract of plant flowers at a high altitude in Semnan province (2340 m).

It is noteworthy that coumarin and tannins were absent in the water extract, and both the water and root extracts exhibited the lowest concentrations. The ethanol extract of flowers at 2340 m demonstrated the highest antimicrobial activity, particularly against *Staphylococcus aureus, S. epidermidis,* and *Bacillus cereus*, with inhibition zone values of 19.1 ± 0.01 mm, 15.8 ± 0.2 mm, and 14.4 ± 1.12 mm, respectively. The MIC values against these bacteria were 24.5 µg/mL, respectively. These findings underscore the potential therapeutic benefits of the ethanol extract, particularly in the context of antimicrobial activity against gram-positive bacteria.

Table 2 presents the inhibition zones (IZ) and MIC values of the ethanol extract of *M. officinalis* L., allowing for a comparison with the standard antibiotic (gentamycin), as detailed in Table 3. The results indicate that the ethanol extract displayed notable antibacterial activity against the tested bacteria, with IZ values ranging from 9.5 ± 0.2 to 19.1 ± 0.01 mm, except for *Salmonella typhimurium* and *S. dysenteriae*, which are gram-negative resistant bacteria.

It is noteworthy that *S. aureus, S. epidermidis*, *B. cereus,* and *Enterococcus faecalis* exhibited heightened sensitivity to the ethanol extract of flowers, with the highest MIC value recorded at 24.5 µg/mL against *S. aureus*. This suggests that the ethanol extract, particularly from plant flowers, holds promising antibacterial efficacy, especially against gram-positive bacteria.

Discussion

The choice of solvent for extraction and the specific plant parts utilized significantly influence both the quality and quantity of phytochemical compounds within the extract. Parameters such as TPC, TFC, total coumarin, and total tannin content, as well as the antibacterial and antioxidant activities, are intricately linked to these extraction variables. This underscores a direct correlation between the phytochemical composition of plant extracts and their biological activities. *Melissa officinalis* L. (Fabaceae), being an annual wild species, exhibits a wide distribution across regions, including the Mediterranean, Central Asia, North Africa, and America. The variability in its natural habitat could contribute to differences in its phytochemical

Table 1. Comparison of total phenolic, flavonoids, coumarin and tannin contents in different parts and extracts of *Melilotus officinalis* L. in Two Provinces (North of Iran)

Region	Plant Sample	Total Phenol (mgEGA/g)		Total Flavonoid (mgEQU/g)		Coumarin (mL/g)	Tanin (mg/g)
		Ethanol Extract	Water Extract	Ethanol Extract	Water Extract	Ethanol Extract	Ethanol Extract
Gorgan -250 m	Flower	24.32±0.09	18.12±0.1	48.21±0.01	21.42±0.1	4.13±0.03	11.01±0.1
	Stem	14.45±0.01	11.52±0.00	17.45±0.11	13.55±0.1	1.12±0.01	0.95±0.09
	Root	8.45±0.06	1.15±0.00	6.11±0.12	5.23±0.07	0.8±0.00	0.45±0.1
Charbagh-2340 m	Flower	38.08±0.13	21.16±0.09	62.4±0.01	31.14±0.09	19.32±0.08	33.89±0.05
	Stem	25.32±0.12	16.82±0.01	49.09±0.01	23.13±0.02	12.02±0.05	13.18±0.19
	Root	12.11±0.13	9.13±0.03	35.25±0.01	18.12±0.09	1.41±0.04	1.38±0.23

Table 2. In Vitro Antibacterial Activity and the MIC Values in the Ethanol Extract of *Melilotus officinalis* L. Flowers From the Charbagh Region (2340m) (North of Iran)

Microorganisms	Inhibition Zone (mm) ±SD	MIC (µg/mL)	Gentamycin
Staphylococcus aureus	19.1±0.01	24.5	16.7
Staphylococcus epidermidis	15.8±0.2	31.3	14.7
Bacillus cereus	14.4 ± 1.12	63.2	16.5
Enterococcus faecalis	12.1±0.76	78.1	9.6
Escherichia coli	11.1 ± 0.5	102.9	11
Pseudomonas aeruginosa	11.8±1.3	106.1	9
Klebsiella pneumoniae	12.7±0.2	119.7	-
Salmonella typhimurium	10.5 ± 0.17	258.6	11
Shigella dysenteriae	9.5±0.1	260.2	11

Table 3. Investigation of Antioxidant Activity in Different Parts and Extracts of *Melilotus officinalis* L. in Two Provinces (North of Iran)

Region	Plant Sample	DPPH (IC50 µg/mL)	Reduce Power (µg/mL)	TAC(µg/mL)
Gorgan -250m	Flower	15.59±0.11	37.28±0.6	57.15±1.90
	Stem	39.6±0.43	67.28±0.47	72.02±0.13
	Root	45.1±0.14	71.25±0.26	93.59±0.43
Charbagh-2340m	Flower	10.61±0.81	38.61±0.12	41.99±0.12
	Stem	11.32±0.41	73.08±0.74	93.4±0.34
	Root	3.61±0.07	91.83±1.02	118.31±0.37

composition, further emphasizing the importance of considering extraction parameters for obtaining extracts with specific bioactive properties (21). *M. officinalis* has a rich historical tradition of being employed for various purposes in traditional medicine. It has served as a tonic, aphrodisiac, emollient, carminative, and anti-flatulence agent. Additionally, its applications extend to being an anti-aging remedy. Externally, it has been utilized as a poultice to alleviate pains and aches. Notably, *M. officinalis* has also been recognized for its anti-malarial properties, showcasing its versatility in addressing a range of health concerns in traditional practices (22).

The historical use of the flowers and stems of *M. officinalis* L. in traditional medicine is extensive. These plant parts have been traditionally employed to address various health issues, including inflammation, throat infections, gastrointestinal disorders, spasms, anemia, and bacterial infections. The long-standing tradition of using *M. officinalis* highlights its perceived efficacy in addressing a diverse range of health conditions in traditional therapeutic practices (23). In addition to its traditional uses, both aqueous and ethanolic extracts of *M. officinalis* have demonstrated notable broad-spectrum anti-candidal activity. These extracts exhibited the capability to inhibit the growth of various *Candida* strains, showcasing their potential as antimicrobial agents against a range of fungal infections.

These results show that the "ethanol extract" of plant flowers had the highest content of TP, TF, coumarin, tannin, antioxidant, and antibacterial activity, whereas the water extract had the lowest content of these secondary metabolites and their biological effects, which is similar to another research (10). According to other studies, the scavenging of free radicals is very important in the treatment of many infectious and inflammatory diseases (24,25).

The chemical composition of *Melilotus* species has been thoroughly investigated, revealing that coumarin is frequently documented for its allelopathic activities (26). Indeed, coumarin, found in *Melilotus* species, exhibits diverse activities, encompassing antibacterial, nematocidal, insecticidal, and phytotoxic effects on other plants. Certain coumarins have demonstrated potent antibacterial effects against strains associated with animal pathogens (27).

It is fascinating to learn that the ethanol extract of *M. officinalis* L. is rich in catechin, a compound known for its phenolic acid and coumarin content. Catechin, being a natural phenol and antioxidant, contributes to the extract's potential health benefits. The presence of catechin in Melilotus species aligns with its known anti-inflammatory effects, as demonstrated in rats with colitis. This finding highlights the diverse array of bioactive compounds present in plant extracts and their potential therapeutic applications (28). The analysis of the results indicates that the ethanol extract of *M. officinalis* L. exhibited superior antibacterial and antioxidant activity compared to the aqueous extract. This observation suggests that phenolic compounds such as catechin, cinnamic acid, tannins, and coumarins, which are found in abundance in *Melilotus* species extracts, may play a pivotal role in conferring antimicrobial and antioxidant properties to these extracts. The presence and concentration of these specific phenolic compounds likely contribute to the observed variations in bioactivity between different solvent extracts, emphasizing the significance of extraction methods in harnessing the desired therapeutic potential of plant species (29,30).

The ethanol extracts derived from Melissa officinalis demonstrated relatively modest antibacterial effects when tested against gram-negative bacteria (*S. dysenteriae, S. typhimurium, Pseudomonas aeruginosa*, and *Escherichia coli*). However, they exhibited more pronounced activity against gram-positive bacteria (*S. aureus, S. epidermidis, B. cereus,* and *E. faecalis*). Notably, the ethanol extract from plant flowers showed the highest efficacy against gram-positive bacteria, with a MIC ranging from 24.5 to 78.1 µg/mL and an inhibition zone spanning 19.1 to 12.1 mm. This suggests a selective antibacterial impact, particularly potent against gram-positive bacterial strains.

Melilotus has a long history of use in traditional medicine as a sedative, anti-inflammatory, and anti-infection agent. However, there are only a handful of experimental reports in the literature on its antioxidant, antibacterial, and anti-inflammatory effects (31,32). As a herbal tea, it is used to treat a variety of conditions, including the common cold, respiratory and gastrointestinal catarrh, varicose veins and hemorrhoids, skin injuries, abscesses, swelling, and diabetic foot ulcers (3,32-34).

Our results demonstrated that the ethanol extract of plan flowers had the highest content of phenol, flavonoid, coumarin, and tannin content and, so it had the highest content of antioxidant and antibacterial activity. The findings of this study provide scientific validation for the traditional use of Melissa officinalis in northern Iran. The presence of secondary metabolites, including phenolic compounds such as flavonoids and phenolic acids, as well as terpenoids, underscores the plant's richness in natural antioxidants. These compounds play crucial roles in various biological processes, contributing to the plant's potential therapeutic benefits and supporting its traditional applications (35,36). Among the extracts we tested, the methanol extract of *M. officinalis* was the most studied and had the highest concentrations of flavonoids (57 ± 5.4 mgRU/g) and phenols (289.5±5 mgGAE/g) (8). In many phytochemical surveys, *Melilotus* species were characterized by high variation in components such as polyphenols, flavonoids, tannin, and coumarin which were also important constituents (35) and often varied in different plant parts, extraction method, and environmental conditions. So, others have confirmed the prevalence of these compounds from *Melilotus* species in many different plant parts from the Mediterranean regions of Turkey, which have also been reported in similar studies (25). *M. officinalis* is used in many traditional medicines as an expectorant, stimulant, antispasmodic, anti-inflammatory, digestive, sedative, and antiseptic (35-38).

Several studies have shown that the terpenoids, flavonoids, and coumarins present in certain wild species of plants commonly used in Iranian traditional medicine have significant antibacterial, anti-inflammatory, carminative, antioxidant, and anti-dermatophyte properties. This is mainly attributed to the high concentration of flavonoids, polyphenols, tannins, and coumarin in plants belonging to the *Melilotus*, *Mentha*, and *Calamintha* genera (38,39). Furthermore, there is a strong correlation between the phytochemistry of different parts of the *M. officinalis* L. plant and its antibacterial and antioxidant activity (39). The ethnobotanical survey aligns with traditional uses of Melilotus species across many regions, where they have been employed to address respiratory diseases, gastrointestinal issues, and diverse nervous system disorders. Several species have undergone investigations for their antioxidant, anti-infection, anti-inflammatory, and neuroprotective effects, utilizing various in vitro and in vivo methods. These therapeutic activities are often attributed to the presence of polyphenols and terpenoids, including compounds like pulegone, menthol, pinocamphone, isomenthone, 1,8-cineole, and limonene (37).

Conclusions

The antibacterial properties of *M. officinalis* L., conducted on different plant parts and extracts, underscore the particular importance of the ethanol extract from the blooming aerial parts. This specific extract showcased elevated levels of TP, TF, coumarins, and tannins, consequently leading to heightened antibacterial and antioxidant activity, especially in the DPPH method. These results align with numerous other studies that have consistently affirmed the antioxidant and antibacterial effectiveness of *M. officinalis* and its metabolites. The emphasis on the blooming aerial parts suggests a targeted approach to harnessing the plant's bioactive potential. Given the escalating concerns regarding the side effects and toxicity associated with synthetic antioxidants, there has been a significant surge in interest in natural alternatives like phenolics, terpenoids, coumarin, tannins, and other natural antioxidants. This shift is driven by their ability to scavenge free radicals linked to various human diseases. The examination of *M. officinalis* flower extracts leads to the conclusion that the ethanol extract serves as a significant source of biologically active compounds with both antioxidant and antimicrobial properties. This substantiates the traditional medicinal use of *M. officinalis* flowers, emphasizing the need for further research into their therapeutic potential.

Authors' Contribution

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Conflict of Interests

None to declare.

Ethical Issues

The ethics committee of Islamic Azad University Damghan Branch approved the study (project code: 14230523961001).

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