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Promising Aromatic and Therapeutic Plants from Tunisia: Phytochemical Analysis, Antioxidant, and Antibacterial Properties

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Abstract

Melissa officinalis and *Stachys ocymastrum* are aromatic and therapeutic plants from the *Lamiaceae* family. This study aimed to analyze the phytochemical content and antioxidant and antibacterial properties of acetone and methanol extracts of *M. officinalis* and *S. ocymastrum*. The quantification included polyphenols, flavonoids, condensed tannins, flavonols, and proanthocyanidins. Antioxidant activities were evaluated through DPPH radical scavenging, ferric-reducing power, and ferrous chelation tests. Antibacterial effects were assessed using disk diffusion and microdilution methods. The findings revealed significant levels of polyphenols (ranging between 94.05 and 15.54 mg EGA/g DW), flavonoids, tannins, flavonols, and proanthocyanidins in both *M. officinalis* and *S. ocymastrum* extracts. Therefore, the two plants exhibited a notable ability to combat free radicals, reduce, and chelate iron, displaying their potent antioxidant properties. However, *S. ocymastrum* has significant DPPH˙ free radical scavenging activity (IC_{50} values: 0.06 mg/mL, 0.08 mg/mL, and 0.09 mg/mL, respectively). Moreover, both plants displayed substantial activity against Gram-positive and Gram-negative bacterial strains, underscoring *M. officinalis* and *S. ocymastrum*'s potential as effective antibacterial agents. This plant holds promise for future advancements in medicine and nutrition, reinforcing its traditional use as a health-promoting herbal infusion.

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Introduction

The therapeutic properties of medicinal plants have been exploited for thousands of years, and their phytochemical potential has been the subject of extensive research in recent years (Petrovska, 2012). Medicinal plants have been recognized as a promising source of new medicines and natural health products in the fields of natural medicine, pharmacology, and nutrition. Their effectiveness and their varied use are based on an exceptional variety of secondary metabolites that possess valuable biological properties (Abdallah *et al*., 2023). Alkaloids, terpenes, phenolic compounds, and many others are molecules with a variety of antimicrobial, anti-inflammatory, antioxidant, anticancer, and other activities. The phytochemical potential of medicinal plants is immense (Awuchi, 2019). Various phytochemical compounds have been found, each with its characteristics and mechanisms of action. Scientific research is constantly finding new elements and explaining their impact on people's health. This phytochemical diversity represents a promising opportunity to find new natural therapeutic agents to treat a variety of diseases (Dzobo, 2022). There remains much to be done in this field to develop and exclude new phytochemicals and their therapeutic applications. This will encourage more effective use of medicinal plants in the medical field, with fewer side effects and greater potential (Afzal *et al*., 2023). These are extensively used throughout the world, indicating a growing trend in the application of herbs in modern, cutting-edge treatments. The World Health Organisation (WHO) has recorded 21,000 different plants as being used medicinally in different countries (Kumar *et al*., 2021). Tunisia possesses a veritable botanical treasure trove of plant species with recognized therapeutic properties. Whether in the arid, semi-arid south or the mountainous, forested north, the local flora is rich in medicinal plants with properties so highly valued in traditional medicine. These ancestral practices are passed down from generation to generation within local communities. Around 2,163 plant species have been identified in Tunisia, of which 149 are thought to have therapeutic qualities (Wannes & Marzouk, 2016). One of the most varied and common plant families is the *Lamiaceae*. However, the potential therapeutic properties of the *Lamiaceae* family make them one of the most common groups in ethnomedicine (Moumni *et al*., 2020). *Melissa officinalis* and *Stachys ocymastrum* are aromatic and therapeutic plants from the *Lamiaceae* family (Tundis *et al*., 2014; Miraj *et al*., 2017).

As the demand for natural antioxidants to maintain health and protect against premature aging continues to grow, studying the phytochemical potential of medicinal plants is a major challenge. This study aims to reveal two real plant treasures that are still too little known: *Stachys ocymastrum* and *Melissa officinalis*. These two *Lamiaceae* come from Tunisia's rich biodiversity and contain a treasure trove of natural antioxidant molecules with a host of benefits yet to be discovered.

Materials and Methods

Sample Preparation and Extraction

Stachys ocymastrum was collected from its natural habitat in the "Jbal Fijel" area in Bazina-Joumine, Bizerte governorate, northeast Tunisia, in April 2017. *Melissa officinalis* was harvested from an experimental plot in the Tbaynia-Ain Drahem area, Jendouba governorate, northwest Tunisia, in March 2017.

After drying in the dark, the fine plant powder is ultrasonically extracted for one hour in acetone or methanol. The filtrates are evaporated to dryness, and then the extract powders are re-extracted in the extraction solvent to obtain the final extracts.

Preliminary Screening Tests to Characterize Active Compounds

The aerial parts of *Stachys ocymastrum* and *M. officinalis* are dried in the open air and shade. They are then ground and subjected to tests to characterize their active ingredients, using appropriate reagents.

Total Phenolic Content

Polyphenols are determined using the Folin-Ciocalteu method, which involves the oxidation of phenols, leading to the reduction of a mixture of acids to form a blue complex. The intensity of the resulting blue color, which is proportional to the polyphenol content, is measured spectrophotometrically at 760 nm after reacting with the Folin-Ciocalteu reagent. A standard range is established using gallic acid at concentrations of 50, 100, 200, 300, 400, and 500 µg/mL. The phenolic compound content is expressed in mg gallic acid equivalents per gram of dry matter (mg GA Eq. g^{-1} DM) (Dewanto *et al*., 2002).

Total Flavonoid Content

The flavonoid assay is based on the formation of a colored complex between flavonoids and aluminum chloride. The absorbance of this orange complex is measured at 510 nm, and the flavonoid content is expressed in catechin equivalents (mg C Eq.g^{-1} DM) (Dewanto *et al*., 2002).

Flavanol Content

Flavanols are measured by the formation of a colored complex with aluminum chloride, whose absorbance at 440 nm is used to determine their concentration in quercetin equivalents (mg Q Eq.g^{-1} DM) (Hechaichi *et al*., 2023).

Total Tannin Content

This determination is based on the binding of vanillin to condensed tannins to form a red chromophore complex whose absorbance at 500 nm is proportional to the tannin content expressed in catechin equivalents (mg C Eq. g^{-1} DM) (Sun *et al*., 1998).

Proanthocyanidins Content

Proanthocyanidins are assayed using the butanol-HCl method, in which they react with iron to give a red complex whose absorbance at 530 nm indicates their concentration in catechin equivalents (mg C Eq. g^{-1} DM) (Maksimović *et al*., 2005).

Antioxidant Capacity Assays

DPPH Radical Scavenging Assay

DPPH inhibition is a rapid colorimetric method for assessing the anti-free radical activity of an extract by measuring its ability to reduce the stable, violet-colored DPPH free radical to a colorless form. The percentage of DPPH inhibition is calculated for different extract concentrations to determine the IC_{50}, the concentration that inhibits 50% of the radical (Hatano *et al*., 1988).

Iron Chelating Power

Using spectrophotometry at 562 nm, ferrozine forms an intense violet complex with free ferrous ions, enabling the capacity of an extract to chelate iron to be quantified. The percentage of chelation is calculated for different concentrations to determine the EC_{50}, the concentration causing 50% chelation (Oyaizu, 1986).

Iron Reducing Power Assay

The antioxidants in an extract are determined by their ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which form a greenish-blue complex with the ferricyanide, whose absorbance at 700 nm is used to calculate the EC_{50}, the extract concentration with a reducing activity of 0.5 (Decker & Welch, 1990).

Antimicrobial Assays

Microorganisms

Acetone and methanol extracts of *S. ocymastrum and M. officinalis* were tested for their antibacterial activity against three pathogenic bacteria: *Escherichia coli, Staphylococcus aureus,* and *Klebsiella pneumoniae*. The bacterial strains were grown on Tryptone Soya Agar (TSA) and incubated at 37°C for 24 hours.

Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activity was assessed using the disk diffusion method. 108 CFU/ml suspension of each bacterial strain was spread at 100 µl on Petri dishes containing TSA medium. Paper discs (6 mm in diameter) were impregnated with 15 µl of the extracts to be tested and then placed on the previously inoculated agar. Blank discs were used as negative controls. After 1 hour at 4°C, the plates were incubated for 24 hours at 37 °C. Antimicrobial activity was assessed by measuring the diameter of the growth inhibition zones (in mm, including the 6 mm of the disc) around the discs compared with the controls. Three measurements were carried out, and the values correspond to the mean (Aouadhi *et al*., 2022).

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined by the liquid microdilution method (Aouadhi *et al*., 2022).

Statistical Analysis

Statistical tests such as the Person & Fischer test were applied to all the parameters studied, using SPSS 20 software to verify statistically the significance of the variability of the results obtained. The safety intervals for the means were calculated at the 5% threshold. Pearson's correlation coefficients were investigated using IBM SPSS 20, and interesting correlations ($P<0.01$) were obtained.

Results and Discussion

Preliminary Screening Tests to Characterize Active Compounds

Preliminary characterization tests were carried out to qualitatively determine the main active substances in *Stachys ocymastrum* and *Melissa officinalis*. The active ingredients tested were alkaloids, flavonoids, tannins, phenolic derivatives, saponosides, and essential oils. The results show that the two plants are rich in alkaloids, essential oils, and phenolic compounds (flavonoids, tannins, and phenolic derivatives), with low levels of saponosides **(Table 1)**.

Table 1. Phytochemical screening of two Lamiaceae (*Melissa officinalis* and *Stachys ocymastrum*).

Secondary metabolites		**RESULTS**	
		S. ocymastrum	***M. officinalis***
Alcaloïds	DR	+	+
	BR	+	+
	MR	-	-
Flavonoïds		+	+
Tanins		+	+
Phenolic derivatives		+	+
Saponosids		+	+
Essential oils		+	+

DR: Dragendorff reagent. BR: Bouchardat reagent. MR: Mayer reagent.
(-): absence; (+): presence.

Total Phenolic, Flavonoid, Flavanol, Proanthocyanidin, and Tannin Contents of M. Officinalis and S. Ocymastrum

The total phenolic, flavonoid, flavanol, proanthocyanidin, and tannin contents of *M. officinalis* and *S. ocymastrum* are summarized in **Table 2**. Quantification of the phytochemical compounds present in the acetone and methanol extracts of *S. ocymastrum* and *M. officinalis* revealed significant levels of these compounds. The phenolic compound content varied significantly depending on the species and the extraction solvent. However, for *S. ocymastrum*, its acetone extract was particularly concentrated, with 94.049 mg EAG/g of total polyphenols, 11.058 mg EQ/g of flavonoids, 10.904 mg EQ/g of flavanols, and 8.501 mg EC/g of proanthocyanidins. Its methanolic extract also contained high levels of polyphenols (85.265 mg EAG/g), especially proanthocyanidins (26.948 mg EC/g). Although *M. officinalis* was less rich, its extracts contained significant quantities of these beneficial compounds, notably 32.878 mg EAG/g of polyphenols and 7.587 mg EQ/g of condensed tannins for the acetone extract. The flavanol content reached 3.248 mg EQ/g in the methanolic extract **(Table 2)**.

Table 2. Total phenolic, flavonoid, flavanols, proanthocyanidin, and tannin contents of the acetonic and methanolic extracts of *Melissa officinalis* and *Stachys ocymastrum*.

Plants	**Extracts**	**Total phenolic content (mg GAE/g DW)**	**Total Flavonoid content (mg CE/gDW)**	**Flavanol content (mg CE/gDW)**	**Proanthocyanidin content (mg CE/g DW)**	**Total tannins content (mg TAE/g DW)**
Stachys ocymastrum	Acetone	94.05 ±0.81	11.06 ±0.02	10.90 ±0.1	8.50±0.01	5.79 ±0.01
Melissa officinalis		32.87 ±0.25	5.14 ±0.05	1.06 ±0.001	3.32 ±0.025	7.58 ±0.07
Stachys ocymastrum	Methanol	85.26 ±0.5	16.21 ±0.2	8.01 ±0.12	26.94 ±0.1	1.55 ±0.05
Melissa officinalis		15.53 ±0.014	4.523 ±0.05	3.24 ±0.01	1.12 ±0.005	1.92 ±0.01

Antioxidant Proprieties

The results of antioxidant tests including DPPH radical scavenging capacity, iron reducing power, and iron chelating power, expressed in terms of IC_{50}, of extracts and powders of *M. officinalis* and *S. ocymastrum* are presented in **Table 3**.

Table 3. Antioxidant activities of the acetonic and methanolic extracts of *Melissa officinalis* and *Stachys ocymastrum*. Values given are means (error bars represent standard deviations) of three independent experiments.

		S. ocymastrum		***M. officinalis***	
		Acetonic extract	Methanolic extract	Acetonic extract	Methanolic extract
Antioxidant activity	DPPH test IC_{50} (mg/mL)	0.06±0.00	0.14±0.02	0.48±0.01	0.74±0.03
	Iron chelating IC_{50} (mg/ml)	0.03±0.00	0.01±0.00	0.34±0.06	1.06±0.1
	Reducing power IC_{50} (mg/mL)	0.89±0.06	0.96±0.08	0.56±0.03	1.54±0.80

S. ocymastrum stood out for its remarkable ability to trap DPPH free radicals, particularly with its extremely potent acetone extract ($IC_{50} = 0.06$ mg/mL), as well as its excellent ferrous iron chelating capacity for both types of extract with $IC_{50} = 0.03$ and $IC_{50} = 0.01$ mg/mL, respectively. On the other hand, *M. officinalis* showed outstanding ferric iron-reducing power, with its methanolic extract achieving the highest absorbance value ($IC_{50} = 1.54$ mg/mL).

Although modest, its radical scavenging and chelation activities were not insignificant as well.

Antimicrobial Activity

The inhibition zone diameter (mm) and average inhibitory and bactericidal concentrations against the three bacterial strains (*Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus*) and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are shown in **Tables 4 and 5**.

Table 4. Diameters of the inhibition zones of acetone and methanol extracts of *Melissa officinalis* and *Stachys ocymastrum* relative to the strains tested using the disc method.

Bacteria	***S. ocymastrum***		***M. officinalis***		**Antibiotic reference (Gentamicin)**
	Acetonic extract	**Methanolic extract**	**Acetonic extract**	**Methanolic extract**	
S. aureus	13	14	12	14	20
E. coli	17	15	15	13	24
K. pneumoniae	14	18	14	14	25

Table 5. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for acetone and methanol extracts of *Melissa officinalis* and *Stachys ocymastrum*

Bacteria	***S. ocymastrum***				***M. officinalis***			
	Acetonic extract		**Methanolic extract**		**Acetonic extract**		**Methanolic extract**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	0.31	0.62	0.31	0.62	0.625	1.25	0.31	0.62
E. coli	0.15	0.31	0.31	0.62	0.31	0.62	0.31	0.625
K. pneumoniae	0.31	0.62	0.078	0.15	0.31	0.62	0.62	1.25

The inhibiting power of acetone and methanol extracts of the leaves of *S. ocymastrum* and *M. officinalis* was tested against the three strains: two Gram-negative and one Gram-positive bacteria. All the extracts showed high antibacterial activity against all the bacterial strains, with an MBC/MIC ratio of <4, which explains their bactericidal effect on the bacteria tested.

According to the table, we can deduce that the extracts of *Stachys ocymastrum* and *Melissa officinalis* have significant antibacterial activity against the three pathogenic strains, and this was reflected by the diameter of the zone of inhibition (varying between 12 mm and 18 mm), and the average inhibitory and bactericidal concentration. We found that the methanolic extract of *Stachys ocymastrum* had the greatest antibacterial activity against *Klebsiella pneumoniae*, with a diameter of 18 mm and a very low MIC (0.078 mg/ml). We also note that the acetone extract of *Stachys ocymastrum* induces a large inhibition diameter (17 mm) with a very low MIC (0.15 mg/ml) against *E. coli*. Similarly, the acetone extract of *Melissa officinalis* has significant antibacterial activity against *E. coli* (15 mm).

Aromatic and medicinal plants are a precious natural treasure trove of bioactive substances with health benefits. These plant resources have been used by civilizations for thousands of years for therapeutic, dietary, and cosmetic purposes. Today, this heritage is of increasing interest to scientists and industrialists, who are looking for safe and effective natural active ingredients (Chaachouay & Zidane, 2024). In addition, these plants are now of great interest to science because of their rich composition of secondary metabolites with remarkable biological properties (Afzal *et al*., 2023). Medicinal plants from the *Lamiaceae* family native to Tunisia, *Melissa officinalis* and *Stachys ocymastrum*, were found to have remarkable phytochemical potential. An in-depth study of their acetone and methanol extracts revealed high levels of various categories of phenolic compounds known for their active antioxidant properties. These findings agree with those obtained by Duda *et al*. (2015), who highlighted the richness of these plants in secondary metabolites. In addition, our results showed that the phenolic compound content varies according to the species and the extraction solvent. These results corroborate those obtained by Duda *et al*. (2015), who reported that phenolic compound contents depend on the species and extraction solvents. Thus, Bourgou *et al*. (2016) showed that the ultrasound method improves the extraction of phenolic compounds.

Stachys ocymastrum showed exceptional richness in total polyphenols, flavonoids, flavanols, and proanthocyanidins. The acetonic extract showed the highest concentrations of these compounds. The genus *Stachys* is known for its high content of polyphenolic secondary metabolites, as shown by several previous studies (Tomou *et al*., 2020; Benedec *et al*., 2023).

As well as these high concentrations of phenolic compounds, extracts of *S. ocymastrum* and *M. officinalis* are highly effective in various studies evaluating their antioxidant capacity. Several activities of the *Stachys* genus (*Lamiaceae*) have been demonstrated, including anti-inflammatory, antimicrobial, and antioxidant properties. Because the secondary metabolites (flavonoids, diterpenes, and phenylethanoid glycosides) of this type have different biological interests (Lakhal *et al*., 2011), their antioxidant properties could be explained by differences in phytochemical composition between the two *Lamiaceae* studied.

The antioxidant potential of both plants seems to be linked to the presence of secondary metabolites. Our results are compatible with research that mentions the high antioxidant potential of other species in the *Lamiaceae* family, often linked to their diversity of phenolic compounds (Jebali *et al*., 2022; Campinho *et al*., 2023; Moshari-Nasirkandi *et al*., 2023). For example, Jebali *et al*. (2022) indicate that the activity of *Mentha pulegium* (*Lamiaceae* family) is due to the presence of secondary metabolites. *Melissa officinalis* is a medicinal plant known for its antioxidant properties, although studies have shown that the antioxidant activity is due to phenolic compounds and flavonoids (Virchea *et al*., 2021).

S. ocymastrum is richer overall in polyphenols, flavonoids, and related compounds. These promising results reinforce the potential of these local aromatic plants as natural sources of antioxidants with numerous applications.

There was a highly and negatively significant correlation between antioxidant activity and the phenolic compounds. Moreover, a positive correlation between flavanol content and iron reduction power was observed **(Table 6)**.

Table 6. Correlation coefficients between the phenolic composition and antioxidant capacity of *Melissa officinalis* and *Stachys ocymastrum* extracts.

	DPPH	Iron chelating power	Iron reducing power
Polyphenol	-0.987*	-0.892	-0.360
Flavonoids	-0.839	-0.773	-0.182
Flavanols	-0.843	-0.631	0.010
Proanthocyanidins	-0.673	-0.667	-0.165
Tannins	-0.127	-0.291	-0.772

*. Correlation is significant at the 0.05 level (2-tailed).

Although known for their medicinal properties, the detailed phytochemical exploration of *Stachys ocymastrum* and *Melissa officinalis* carried out here has confirmed and clarified their very high antioxidant potential because of their richness in a variety of phenolic compounds.

In evaluating the antimicrobial activity of different extracts of *S. ocymastrum* and *M. officinalis*, the results were very promising. They confirm the already suspected antimicrobial potential of these two medicinal plants in the traditional pharmacopeia. This powerful antibacterial activity can be attributed to the presence of phenolic compounds such as flavonoids and proanthocyanidins, which are known for their antimicrobial properties. Numerous previous studies have highlighted the potential of polyphenols to inhibit bacteria (Bouarab-Chibane *et al*., 2019; Ivanov *et al*., 2022; Liu *et al*., 2023). However, these extremely promising results mean that further research can be carried out to isolate and identify the bioactive molecules responsible. These two medicinal plants also reinforce interest in using them as potential sources of safe and environmentally friendly natural antimicrobial agents in response to the current problems of antibiotic resistance. Their high potential can be exploited in a variety of sectors, including agri-food, cosmetics, and therapeutic and animal health solutions.

Conclusion

The phytochemical analysis revealed significant concentrations of phenolic compounds, such as total polyphenols, flavonoids, flavanols, proanthocyanidins, and condensed tannins, in extracts from both plants. However, variations were observed depending on the extraction solvent used. The acetone extract of *S. ocymastrum* was particularly rich in natural antioxidants, with a high DPPH radical scavenging capacity. This exceptional antioxidant activity may be linked to high levels of polyphenols. In addition, the extracts showed significant iron chelation and reduction capacities, highlighting their promising antioxidant potential through various mechanisms. Interesting applications of these two medicinal *Lamiaceae* as natural sources of antioxidants are suggested by these preliminary results.

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