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Phytochemical composition and antioxidant activities of *Mentha spicata* L. and *Melissa officinalis* L. from the Settat region of Morocco: A comparative study of extraction methods

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ABSTRACT

The growing interest in medicinal plants as natural antioxidant sources has intensified due to their potential applications in pharmaceuticals and nutraceuticals. This study uses various extraction methods to examine the phytochemical composition and antioxidant properties of *Mentha spicata* and *Melissa officinalis* from Morocco's Settat region. The research revealed these plants contain rich bioactive compounds, including flavonoids, tannins, polyphenols, glycosides, and alkaloids. *M. spicata* demonstrated impressive total phenolic content through maceration (398.76 ± 25.45 µg GAE/mg), while its flavonoid content reached 3437.79 ± 485.32 µg QE/mg through reflux heating. *M. officinalis* showed significant tannin levels (234.71 ± 24.33 µg ATE/mg) using infusion. Antioxidant activities were evaluated using DPPH, ABTS, and FRAP assays. The reflux heating extract showed remarkable free radical scavenging capacity, with a DPPH IC_{50} value of 13.51 ± 1.11 µg/mL. Combining both plant extracts produced synergistic effects, with the maceration mixture achieving the highest FRAP value (95.19 ± 6.59 µg/mL). These findings suggest that *M. spicata* and *M. officinalis*, especially in combination, offer promising potential as natural antioxidant sources for food preservation and health applications.

Keywords: *Mentha spicata*, *Melissa officinalis*, phytochemical composition, antioxidants activities.

INTRODUCTION

Researchers are increasingly focused on identifying natural antioxidants that are safe for human health, aiming to discover new, effective alternatives that can be used in food and pharmaceutical products instead of synthetic options.

Medicinal plants are a key source of bioactive compounds with antioxidant properties. Studies on Moroccan medicinal plants have highlighted their rich content of health-promoting compounds such as phenolic acids, flavonoids, and tannins, which are recognized for their antioxidant benefits (El Jemli et al., 2017; Frei et al., 2003). Morocco's flora is diverse, with over 4.200 vascular plant species, yet only a few have been extensively studied for their chemical and pharmacological properties. Among these, the Lamiaceae family is notably widespread, particularly in the Mediterranean region, which is a global biodiversity hotspot.In Moroccan traditional medicine, Lamiaceae plants hold significant importance due to their aromatic properties and therapeutic uses. (Bouyahya et al., 2020; Goudjil et al., 2020)

Mentha spicata and *Melissa officinalis* are perennial herbaceous plants of the *Lamiaceae family*, originating from the Mediterranean, East Asia, and Southeast Siberia, but have adapted worldwide (Bouhadi et al., 2021; Silva et al., 2023). These plants have been used for centuries in traditional medicine and culinary applications, with recent scientific studies increasingly validating their therapeutic potential (Koliopoulos et al., 2010).

Mentha spicata, known as spearmint, is a hardy perennial herb known for its distinctive aroma and flavor. In Moroccan traditional medicine, this plant has been extensively used, particularly as an infusion with tea, to address a variety of ailments (El Hassani, 2020). Its applications range from managing digestive disorders and respiratory issues to alleviating sore throats and treating skin conditions (Menyiy et al., 2021). This plant is rich in essential oils, primarily composed of carvone, which contributes to its characteristic scent and potential medicinal properties. Recent studies have begun to explore the antioxidant, antimicrobial, and anti-inflammatory properties of *M. spicata*, suggesting a scientific basis for its traditional uses (Boukhebti et al., 2011).

Melissa officinalis often referred to as lemon balm, is another important medicinal plant with a long history of use (Shakeri et al., 2016). This perennial herb is noted for its mild lemon scent and calming properties. This plant has been the subject of numerous studies investigating its potential benefits in treating gastrointestinal disturbances, reducing anxiety, and improving sleep quality (Soltanpour et al., 2019). Its antioxidant properties are particularly interesting, which have been linked to high levels of polyphenolic compounds such as rosmarinic acid, quercetin, and caffeic acid. These compounds have shown promise in addressing oxidative stress-related conditions, including certain neurological diseases (Miraj et al., 2017).

In recent years, there has been growing interest in natural antioxidants for food preservation and health applications. This trend has led to increased research into various herb species, including *M. spicata* and *M. officinalis*, focusing on their chemical composition, antioxidant activity, and potential antimicrobial properties (Silva et al., 2023; Gómez-Bellot et al., 2022; Koksal et al., 2011). The shift towards natural alternatives is driven by consumer demand for safer, more sustainable options in food and medicine (Shaikh et al., 2014).

Although several studies have evaluated the Phytochemical and antioxidant effects of *M. spicata* and *M. officinalis* individually, none have investigated the Phytochemical and antioxidant activity of their combination. This gap in the literature underscores the significance of the present study, which explore in addition the potential synergistic effects of these two plants when used together, as they are commonly combined in Moroccan traditional medicine. Therefore, the present study aims to determine and compare the phytochemical and antioxidant potential of *M. spicata* and *M. officinalis* extracts alone or in a mixture, from the Settat region of Morocco.

MATERIAL AND METHODS

Plant material

The plants used in this study, *M. spicata* and *M. officinalis* were harvested in the province of Settat (33°06’35.6”N 7°43’01.5” W), Morocco, in 2022. *Mentha spicata* was collected in February, while *M. officinalis* was gathered in January. The plant species were identified by the Department of Botany at the Scientific Institute of Rabat, Morocco, and voucher specimens were deposited at the herbarium of the same Institute. These specimens were assigned the following reference numbers: RAB 114746 for *M. spicata* and RAB 114745 for *M. officinalis.* After collection, the plant material was subjected to air-drying at room temperature, ranging between 20 and 24 °C. After drying, the leaves were carefully separated from the rest of the plant material. These isolated leaves were then crushed with a laboratory grinder and stored in tightly sealed, dark containers to protect them from light exposure. The storage period lasted for three weeks, ensuring the preservation of the plant material’s integrity before further processing and analysis.

This careful collection, identification, drying, and storage process ensures the authenticity and quality of the plant material used in the study, providing a solid foundation for the subsequent phytochemical and antioxidant analysis.

Extraction procedure

The extraction of bioactive compounds from *M. spicata*, *M. officinalis,* and their mixture (50% *M. spicata* + 50% *M. officinalis)* was carried out

using three different methods: Infusion, Maceration, and Reflux heating. These methods were chosen to compare their efficiency in extracting the plant's active components and to mimic various traditional and modern extraction techniques.

Infusion method

The infusion method was selected to simulate the traditional tea-making process. For each plant species and their mixture, 10 g of dried, powdered plant material was placed in a beaker. 100 mL of distilled water, heated to its boiling point (100 °C), was poured over the plant material. The mixture was then covered and allowed to steep at room temperature (20–24 °C) for 30 min. After steeping, the infusion was filtered through Whatman filter paper to remove plant debris (Ervina et al., 2016; Sussman et al., 1980).

Maceration method

The Maceration method, a cold extraction method, was performed to extract compounds that might be sensitive to heat. For this process, 10 g of dried, powdered plant material was placed in a glass container with 100 mL of distilled water at room temperature (20–24 °C). The mixture was left to macerate for 24 hours with occasional stirring to ensure thorough extraction (Abdelbaky, 2021). After the maceration period, the extract was filtered through the Whatman filter paper.

Reflux heating method

The Reflux heating method was employed as a more intensive extraction technique. A quantity of 10 g of dried, powdered plant material was placed in a round-bottom flask with 100 mL of distilled water. The flask was connected to a reflux condenser and heated to maintain a gentle boil for one hour. This method allows for continuous extraction at the solvent's boiling point while preventing loss of solvent through evaporation. After cooling, the extract was filtered through the Whatman filter paper (El Jemli et al., 2016; Shikob et al., 2022).

For all methods, the filtered extracts were concentrated under a vacuum using a rotary evaporator at 60 °C. The dry extract was collected in an opaque glass vial and then stored in the refrigerator at 4 °C until us. These diverse extraction methods allow for a comprehensive comparison of the efficiency of different techniques in extracting bioactive compounds from *M. spicata* and *M. officinalis*, providing valuable insights into the optimal method for obtaining these plant extracts. The extraction yield (%) was calculated according to the following formula (1):

$$Yield\ (\%) = \frac{M_{rot}}{M_p} \times 100 \quad (1)$$

where: M_{rot} is the mass of rotary evaporated extract and M_p is the mass of the plant material used.

Phytochemical screening

Phytochemical screening assays were conducted to identify various families of secondary metabolites present in the aerial portions of *M. spicata* and *M. officinalis* and their mixture. This qualitative analysis offers valuable insights into the chemical composition of these plants and their potential biological activities. These processes rely on precipitation or coloration reactions using reagents specific to each chemical family. Our study focused on the following compounds: flavonoids, tannins, polyphenols, alkaloids, saponins, reducing sugars, and terpenoids.

Flavonoids, tannins and polyphenols

Flavonoids and tannins are two important classes of plant secondary metabolites known for their diverse biological activities. Flavonoids are a large group of polyphenolic compounds characterized by a benzo-γ-pyrone structure. They are widely distributed in plants and are known for their antioxidant, anti-inflammatory, and antimicrobial properties (Nea et al., 2021). The cyanidin reaction was used to identify flavonoids. 10 mg of each extract was taken in 5 mL of hydrochloric alcohol diluted 2 times. The heat was released by adding 2 to 3 magnesium chips, followed by observation of a pink-orange or purplish coloration. The addition of 3 drops of isoamyl alcohol intensified this coloration, confirming the presence of flavonoids (Bouslamti et al., 2024).

Tannins are complex polyphenolic compounds that can precipitate proteins. They are found in many plant species and are known for their astringent properties and potential health benefits. Tannins were identified by adding $FeCl_3$ (Sheel et al., 2014). 5 mL of each aqueous extract was filtered. The filtrate was collected and saturated with sodium acetate. The addition of

3 drops of 2% $FeCl_3$ produced an intense blue-black color, indicating the presence of tannins (Benzidia et al., 2019).

Polyphenols are a large and diverse group of plant-based compounds characterized by the presence of multiple phenol structures. These compounds are widely distributed in plants and are known for their potent antioxidant properties. For the qualitative detection of polyphenols in the phytochemical screening, the ferric chloride test was employed. In this test, a few drops of 1% ferric chloride solution were added to 1 mL of the plant extract. The formation of a blue, blue-black, green, or purple coloration indicated the presence of polyphenols. This color change occurs due to the formation of iron (III)-phenol complexes (Nea et al., 2021).

Alkaloids and saponins

The Dragendorff reagent was used to detect alkaloids. 6 mL of each extract was evaporated to dryness. The residue was redissolved in 6 mL of 60 °C alcohol. The addition of two drops of the Dragendorff reagent to the alcohol solution caused either a precipitate to form or an orange coloration, indicating a positive reaction (Zhang et al., 2021). To detect saponins, 10 mL of each aqueous extract was placed in a test tube. The tube was shaken for 15 s and then left to stand for 15 min. A persistent foam height greater than 1 cm indicates the presence of saponins (Sama et al., 2022).

Reducing sugars and terpenoids

Fehling's test was used to detect the presence of reducing sugars. In a test tube, 1 mL of Fehling's A solution and 1 mL of Fehling's B solution were combined. This mixed solution was boiled for one minute. Then an equal volume (2 mL) of the test solution was added. The observation of a brick-red precipitate confirmed the presence of reducing sugars (Das et al., 2014).

The Salkowski test was used to detect the presence of terpenoids. 5 mL of the extract was mixed with 2 mL of chloroform, and 3 mL of concentrated sulfuric acid was carefully added to form a separate layer. The formation of a reddish-brown coloration at the interface between the layers indicated a positive result for the presence of terpenoids (Sheel et al., 2014).

These phytochemical screening methods provide a comprehensive qualitative analysis of the major secondary metabolites present in the plant extracts, forming a foundation for further quantitative analysis and exploration of their potential bioactivities.

Quantitative determination assays

Total phenolic content

The total phenolic contents (TPC) were determined spectrophotometrically using the Folin-Ciocalteu colorimetric method. To determine the TPC, a gallic acid standard curve (0–100 µg/mL) was prepared in distilled water. In a test tube, 20 µL of the extract solution was mixed with 1.16 mL of distilled water. 100 µL of Folin-Ciocalteu reagent was added to this mixture and mixed well. Immediately after, 300 µL of sodium carbonate (Na_2CO_3) solution (20%) was added. The solution was vortexed to ensure thorough mixing. The reaction mixture was incubated at 40 °C for 30 min. The absorbance of the solution was measured at 760 nm using a spectrophotometer (Hosu et al., 2014).

Total flavonoid content

To determine the total flavonoid content (TFC), a quercetin standard curve was prepared in an appropriate range. In a test tube, 1 mL of the extract solution or quercetin standard was mixed with 1 mL of 2% aluminum chloride ($AlCl_3$) solution. Ensure thorough mixing of the components. The reaction mixture was allowed to stand at room temperature for 1 hour. After the incubation period, the absorbance of the solution was measured at 420 nm using a spectrophotometer (Ordonez et al., 2006). The TFC was calculated using the quercetin standard curve prepared earlier. The results were expressed as mg Quercetin Equivalents (mg QE)/g of sample.

Total tannin content

To determine the total tannin content (TTC), a standard curve was prepared using an appropriate tannin standard such as tannic acid. In a test tube, the sample extract was combined with 0.5 mL of Folin-Ciocalteu reagent (0.2 mol/L) and 1 mL of sodium carbonate solution (0.7 mol/L). The components were Mixed thoroughly, then dilute the mixture to a predetermined volume with distilled water. The reaction mixture was allowed to stand at room temperature for 30 min for color development. After the incubation period, the absorbance was measured of each sample at 760 nm using a spectrophotometer. The TTC was calculated using

the previously prepared standard curve. Express the results as mg of tannin standard equivalents per gram of sample (mg TAE)/g (Sington, 1999).

Antioxidant activity

The antioxidant activity of *M. spicata* and *M. officinalis* extracts was evaluated using three methods: DPPH, ABTS, and FRAP assay.

Radical scavenging assay (DPPH)

The ability of the plant extracts to scavenge the DPPH radical was estimated using the method described by Şahin et al. (2004). A 60 µM DPPH solution was prepared fresh in methanol. Sample extracts were serially diluted to various concentrations. 0.1 mL of each diluted sample was mixed with 3.9 mL of DPPH solution in test tubes. The mixtures were vortexed and incubated in darkness at room temperature for 30 min. Absorbance was measured at 517 nm using an UV-Vis spectrophotometer. Butylated hydroxytoluene (BHT) was used as a positive control and prepared in the same manner as the samples. Radical scavenging activity DPPH was determined as the inhibition percentage and the following formula (2):

$$Inhibition\ (\%) = \frac{Abs_{DPPH} - Abs_{Sample}}{Abs_{DPPH}} \times 100 \quad (2)$$

Dose-response curves were constructed and IC_{50} values were determined. IC_{50} represents the sample concentration required to scavenge 50% of DPPH radicals. This assay quantified the free radical scavenging capacity of the samples relative to BHT, indicating their antioxidant potential.

ABTS radical cation decolorization assay

The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 70 mM potassium persulfate in water and allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. The $ABTS^{\bullet+}$ solution was then diluted with ethanol or phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 (± 0.02) at 734 nm. Sample extracts were diluted to various concentrations. 20 µL of each diluted sample was mixed with 2 mL of diluted $ABTS^{\bullet+}$ solution. The mixtures were allowed to react at room temperature for 6 min. Absorbance was measured at 734 nm using an UV-Vis spectrophotometer (Pukalskas et al., 2002). A control was prepared using the solvent instead of the sample. Trolox was used as a standard antioxidant for comparison. The percentage inhibition was calculated according to the following formula (3):

$$Inhibition\ (\%) = \frac{Abs_{ATBS} - Abs_{Sample}}{Abs_{ATBS}} \times 100 \quad (3)$$

Ferric reducing antioxidant power (FRAP) assay

The assay was conducted by mixing 0.2 mL of sample extracts or positive control at various concentrations with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide $K_3Fe\ (CN)_6$ (1%). The mixture was incubated at 50 °C for 20 min to reduce ferricyanide to ferrocyanide. Subsequently, 2.5 mL of trichloro acetic acid (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 10 min. Following centrifugation, 2.5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%). The absorbance of the resulting solution was measured at 700 nm using an UV-Vis spectrophotometer (Oyaizu et al., 1986).

Statistics

Measurements were carried out in triplicate and analyzed using one-way analysis of variance (ANOVA). ANOVA performed the statistical analysis at a 95% confidence level with IBM-SPSS version 25 to determine significant differences between treatments. Tukey's post-hoc test was then used to compare group means.

RESULTS AND DISCUSSION

In the context of the valorization of medicinal plants, this work aims to study the phytochemical composition and antioxidant activities of *M. spicata* and *M. officinalis* of Morocco. 3 methods of extraction were used: infusion, maceration, and reflux heating to extract the maximum of bioactive components. The results of this study are as follows:

Phytochemical screening

The phytochemical screening of *M. spicata*, *M. officinalis*, and their mixture is shown in Table 1. The cyaniding reaction test revealed a pink-orange coloration, confirming the presence of

Table 1. Results of phytochemical screening of *M. spicata*, *M. officinalis*, and their mixture

Parameters	*M. spicata*	*M. officinalis*	Mixture
Flavonoids	+	++	+++
Terpenoids	+	-	-
Polyphenols	+	++	++
Tannins	+++	+	+
Reducing sugars	+	-	-
Alkaloids	-	+	+
Saponins	-	-	-

Note: +++ strongly, ++ positive, + medium, – negative.

flavonoids in all samples, with the highest concentration observed in the plant mixture (+++), followed by *M. officinalis* (++), and *M. spicata* (+). The enhanced flavonoid content in the mixture suggests a possible synergistic effect when combining the two plants, potentially leading to increased antioxidant properties.

The Salkowski test for terpenoids resulted in a reddish-brown coloration at the interface between the layers, while Fehling's test for reducing sugars produced a brick-red precipitate. Interestingly, terpenoids and reducing compounds were only detected in *M. spicata* (+). This suggests that *M. spicata* may possess unique aromatic and reducing properties compared to *M. officinalis* and the mixture.

The ferric chloride test produced a blue-black coloration, indicating the presence of these compounds. Polyphenols were present in moderate amounts (++) in *M. officinalis* and the plant mixture, while *M. spicata* had a lower concentration (+). This suggests that all samples contain significant amounts of these important antioxidants, with *M. officinalis* and the mixture possibly offering greater antioxidant benefits.

Adding ferric chloride to the filtered extracts resulted in an intense blue-black color, confirming the presence of tannins. Tannins were most abundant in *M. spicata* (+++), with lower levels detected in *M. officinalis* and the mixture (+). This high tannin content in *M. spicata* could contribute to its astringent properties and potential antimicrobial activities.

The Dragendorff reagent produced an orange coloration or precipitate, indicating the presence of these nitrogen-containing compounds. Alkaloids were detected in *M. officinalis* and the plant mixture (+) but were absent in *M. spicata*. This suggests that *M. officinalis* contributes alkaloids to the mixture, which could be associated with various pharmacological activities. Notably, saponins were not detected in any of the samples, as evidenced by the absence of persistent foam formation in the froth test. This indicates that these plants may not be significant sources of saponins

Overall, the phytochemical screening revealed the presence of various classes of bioactive compounds in *M. spicata*, *M. officinalis*, and their mixture. Flavonoids, polyphenols, tannins, and reducing sugars were detected in all samples. The mixture exhibited the highest flavonoid content, suggesting a potential synergistic effect between the two plants. *Mentha spicata* showed a notable abundance of tannins and was the only sample containing terpenoids and reducing sugars, whereas *M. officinalis* contributed alkaloids that were absent in *M. spicata*. Notably, saponins were absent from all tested extracts.

These findings are consistent with previous studies that also reported the presence of flavonoids, polyphenols, and tannins in both *M. spicata* and *M. officinalis*, as well as a general absence or very low levels of alkaloids. For instance, Chatterjee et al. (2022) found that a methanolic leaf extract of *M. spicata* collected from Kyamgei Shantipur contained several phytochemical classes, including reducing sugars, phenolic compounds, flavonoids, glycosides, sterols, saponins, alkaloids, coumarins, tannins, carbohydrates, and proteins, while anthraquinones, lignins, and anthocyanins were absent. In contrast to our results, their study reported the presence of alkaloids and saponins in *M. spicata*, which were not detected in our samples. These discrepancies may be attributed to differences in extraction solvents, environmental growing conditions, or the sensitivity of phytochemical assays (Pouyanfar et al., 2018; Bowyer et al., 2015; Ghasemzadeh et al., 2018).

According to a review by El Menyiy et al. (2022), various phenolic compounds have been

identified in *M. spicata*, further supporting its richness in bioactive molecules. Consistent with these findings, *M. spicata* has been reported to be rich in flavonoids, glycosides, sugars, saponins, alkaloids, anthraquinones, vitamins, and minerals (Farahbakhsh et al., 2021; Zaidi and Dahiya, 2015).

Regarding *M. officinalis*, several studies have demonstrated the presence of various phytochemicals, including flavonoids, phenolic acids, triterpenoids, and volatile constituents (Shakeri et al., 2016). In another study, *M. officinalis* collected from Brazil was found to be rich in bioactive compounds with antioxidant properties (Pereira et al., 2014). The presence of key antioxidant compounds such as flavonoids, phenolic compounds, and tannins in M. spicata and M. officinalis has been confirmed by multiple studies, thus supporting their therapeutic potential (Pereira et al., 2014; Shakeri et al., 2016; El Menyiy et al., 2022; Chatterjee et al., 2022).

Quantitative determination assays

Table 2 presents a detailed quantitative analysis of total polyphenols, flavonoids, and tannins in the infusion extracts of *M. spicata, M. officinalis,* and their mixture. The results provide valuable insights into the phytochemical profiles of these plants and their potential antioxidant activities.

The infusion of the mixture exhibited the highest total polyphenol content at 119.32 µg GAE/mg, suggesting that combining the two species enhances the extraction of polyphenolic compounds. This enhancement may be due to synergistic interactions between their respective phytochemicals. Research indicates that such synergistic effects can arise from the complementary actions of different bioactive compounds found in various plants *M. officinalis* showed a total polyphenol content of 114.70 µg GAE/mg, indicating a strong presence of these compounds, which aligns with its known high levels of beneficial substances like rosmarinic acid (Hashim et al., 2024). *Mentha spicata* recorded the lowest concentration at 108.37 µg GAE/mg, highlighting the variability in phytochemical accumulation among different species.

Flavonoid content further emphasizes the benefits of combining these plants. The mixture reached 380.26 µg QE/mg, significantly exceeding the flavonoid levels in both individual plants. The synergistic effects can be attributed to several mechanisms, including enhanced solubility and stability of bioactive compounds when combined. Croft et al. (1998) suggest that interactions between flavonoids and phenolic acids can form more stable complexes, which may improve their biological activities.

When examining total tannins, *M. officinalis* exhibited the highest content at 234.71 µg ATE/mg, which may be linked to its astringent properties and potential antimicrobial activities. Interestingly, the tannin content in the mixture was lower at 108.94 µg ATE/mg, indicating that while combining these plants enhances polyphenol and flavonoid extraction, it may not significantly boost tannin levels. *M. spicata* recorded a tannins content of 169.02 µg ATE/mg, contributing to the overall tannins profile but still falling short of *M. officinalis*.

The analysis of total polyphenols, flavonoids, and tannins in the extracts with maceration from *M. spicata, M. officinalis*, and their mixture reveals significant differences in phytochemical content (Table 3). In terms of polyphenol content, *M. spicata* demonstrated remarkably high levels (398.76 µg GAE/mg), significantly exceeding both *M. officinalis* (128.66 µg GAE/mg) and the mixture (115.79 µg GAE/mg). This higher polyphenol content in *M. spicata* could be attributed to its unique cellular structure or the specific composition of its cell walls, which might be more susceptible to the maceration process (Aleixandre-Tudo et al., 2018). The lower polyphenol content in the mixture suggests that combining the plants might create interactions that potentially inhibit polyphenol extraction during maceration.

The flavonoid content analysis revealed a striking synergistic effect in the mixture, which

Table 2. Total polyphenols, flavonoids, and tannins contents plant extracts with infusion

Assays	Infusion		
	M. spicata	*M. officinalis*	Mixture
Polyphenols (GAE/mg)	108.37 ± 14.96a	114.70 ± 36.49a	119.32 ± 7.98a
Flavonoids (QE/mg)	196.63 ± 1.93b	202.75 ± 36.03b	380.26 ± 47.36d
Tannins (ATE/mg)	169.02 ± 48.79b	234.71 ± 24.33b	108.94 ± 12.14a

Note: different letters represent significant differences between treatment groups.

Table 3. Total polyphenols, flavonoids, and tannins contents plant extracts with maceration

Assays	Maceration		
	M. spicata	*M. officinalis*	Mixture
Polyphenols (GAE/mg)	398.76 ± 25.45c	128.66 ± 24.26b	115.79 ± 64.65ab
Flavonoids (QE/mg)	559.70 ± 56.98c	469.24 ± 141.15c	1879.12 ± 94.40d
Tannins (ATE/mg)	99.93 ± 14.73a	70.87 ± 11.64a	60.89 ± 56.00a

Note: different letters represent significant differences between treatment groups.

exhibited an exceptionally high concentration (1879.12 µg QE/mg) compared to the individual plants. This represents more than a threefold increase over *M. spicata* (559.70 µg QE/mg) and about four times the content found in *M. officinalis* (469.24 µg QE/mg). This enhancement in flavonoid extraction when the plants are combined suggests positive interactions between the plant matrices during the maceration. Such synergy could be explained by the complementary nature of the plants' chemical compositions, possibly creating more favorable conditions for flavonoid extraction when combined (Chaves et al., 2020).

Regarding tannins content, the results showed relatively modest levels across all samples, with no statistically significant differences between them. *M. spicata* contained the highest amount (99.93 µg ATE/mg), followed by *M. officinalis* (70.87 µg ATE/mg), and the mixture (60.89 µg ATE/mg). The lack of significant variation in tannin content suggests that the Maceration might not be optimal for tannin extraction, or that these plants naturally contain lower levels of tannins compared to other phytochemical compounds (polyphenols and flavonoids).

The Reflux Heating extraction method revealed interesting patterns in the phytochemical composition of *M. spicata, M. officinalis,* and their mixture (Table 4). For polyphenols, the mixture demonstrated the highest content of 208.40 µg GAE/mg, significantly higher than both individual plants. This suggests a synergistic effect when combining the two species, potentially due to the complementary nature of their phenolic compounds. *M. spicata* showed a moderate polyphenol content of 156.04 µg GAE/mg, while *M. officinalis* had the lowest at 79.10 µg GAE/mg.

Regarding flavonoid content, all samples showed remarkably high values under Reflux heating extraction. The mixture again exhibited the highest concentration at 3437.79 µg QE/mg, substantially exceeding the individual plants. *M. officinalis and M. spicata* also showed impressive flavonoid contents of 2414.76 µg QE/mg and 2297.12 µg QE/mg respectively. These elevated levels suggest that Reflux heating is particularly effective at extracting flavonoids, possibly due to the sustained high temperature enabling better solubility and extraction of these compounds (Biesaga, 2011).

The tannins content followed a different pattern, with the mixture showing the highest concentration at 121.62 µg ATE/mg. This was notably higher than both individual plants, where *M. officinalis* contained 54.89 µg ATE/mg and *M. spicata* showed the lowest content at 44.19 µg ATE/mg. The enhanced tannins content in the mixture might be attributed to the interaction between compounds from both plants during the heating process, potentially creating conditions more favorable for tannins extraction (De Hoyos-Martínez et al., 2019).

Several studies have reported the high polyphenol content of *M. spicata* and *M. officinalis* extracts, highlighting their potential as natural sources of antioxidants (Silva et al., 2923; García-Risco et al., 2017; Abootalebian et al., 2016). Literature data on the phenolic content of *Mentha* species are often heterogeneous and difficult

Table 4. Total polyphenols, flavonoids, and tannins contents of plant extract with reflux heating

Assays	Reflux heating		
	M. spicata	*M. officinalis*	Mixture
Polyphenols (GAE/mg)	156.04 ± 10.70e	79.10 ± 13.92c	208.40 ± 3.31f
Flavonoids (QE/mg)	2297.12 ± 468.54g	2414.76 ± 120.10g	3437.79 ± 485.32h
Tannins (ATE/mg)	44.19 ± 6.97a	54.89 ± 7.81b	121.62 ± 9.13d

Note: different letters represent significant differences between treatment groups.

to compare, due to methodological variations as well as factors such as geographical origin, harvest period, climatic conditions, and extraction protocols (Pouyanfar et al., 2018; Bowyer et al., 2015; Ghasemzadeh et al., 2018). These parameters may account for the discrepancies observed with our results.

Evaluation of antioxidant activity

The antioxidant activity evaluation using three different assays (DPPH, ABTS, and FRAP) revealed varying levels of effectiveness among *M. spicata, M. officinalis,* and their mixture when using the infusion method (Table 5). For the DPPH assay, which measures free radical scavenging ability, the mixture demonstrated the strongest antioxidant activity with an IC_{50} value of 179.19 µg/mL, significantly lower than both individual plants. *M. spicata* showed moderate activity with an IC_{50} of 754.57 µg/mL, while *M. officinalis* exhibited the weakest DPPH scavenging activity with an IC_{50} of 1513.38 µg/mL.

The ABTS radical scavenging assay revealed a similar pattern, with the mixture showing the most potent activity (IC_{50} = 44.43 µg/mL). *M. spicata* demonstrated moderate activity with an IC_{50} of 317.82 µg/mL, while *M. officinalis* showed the lowest activity with an IC_{50} of 817.01 µg/mL.In the FRAP assay, which measures reducing power, the mixture again showed the strongest activity with an IC_{50} of 30.77 µg/mL, followed closely by *M. spicata.* at 38.39 µg/mL. *M. officinalis* showed significantly lower reducing power with an IC_{50} of 559.07 µg/mL. A study by Wojdyło et al. (2007) noted that combining flavonoids and phenolic acids from different plant sources often results in enhanced antioxidant activity compared to individual components. Similarly, multiple compounds can lead to a more robust defense against oxidative stress, as they may work together to neutralize free radicals more effectively. Also, this finding underscores the potential for enhanced antioxidant properties through combination, as flavonoids are well-recognized for their health benefits, including reducing oxidative stress.

Table 6 presents a comparative analysis of the antioxidant activities of *M. spicata, M. officinalis.,* and their mixture using three different assays (DPPH, ABTS, and FRAP) with the Maceration method. The results were compared with standard antioxidants BHT and Trolox as positive controls. The DPPH assay results showed that *M. spicata* exhibited the strongest antioxidant activity among the individual plant extracts, with an IC_{50} value of 17.08 ± 0.70 µg/mL. This value was significantly lower than that of *M. officinalis* (55.02 ± 18.88 µg/mL), indicating better radical scavenging ability. Also, the IC_{50} of *M. spicata, M. officinalis,* and their mixture is higher than the positive control.

For the ABTS assay, *M. spicata* again demonstrated superior antioxidant activity with an IC_{50} value of 53.72 µg/mL, compared to *M. officinalis* (85.86 µg/mL). The mixture showed intermediate activity with an IC_{50} value of 70.64 µg/mL. The FRAP assay results revealed that *M. spicata* had the most potent ferric reducing power among the individual extracts, with an IC_{50} value of 11.34 µg/mL. This was followed by *M. officinalis*

Table 5. IC_{50} values of Plants with the infusion method

Assays		Infusion (µg/mL)		Positive control	
Parameter	*M. spicata*	*M. officinalis*	Mixture	BHT	Trolox
DPPH	754.57 ± 13.68g	1513.38 ± 380.59i	179.19 ±7.20d	8.98 ±0.01b	—
ABTS	317.82 ± 24.31e	817.01 ± 38.45h	44.43 ±13.66c	—	2.07 ± 0.03a
FRAP	38.39 ± 9.90c	559.07 ± 19.71f	30.77 ±19.80c	5.30 ±1.41b	—

Note: different letters represent significant differences between treatment groups.

Table 6. IC_{50} values of plants with the maceration method

Assays	Maceration (µg/mL)			Positive control	
	M. spicata	*M. officinalis*	Mixture	BHT	Trolox
DPPH	17.08 ± 0.70d	55.02±18.88f	179.19 ± 7.20h	8.98±0.01c	—
ABTS	53.72 ± 3.50f	85.86 ± 7.63g	70.64 ± 11.80g	—	2.07 ± 0.03a
FRAP	11.34 ± 2.75d	40.76 ± 2.28e	95.19 ± 6.59g	5.30±1.41b	—

Note: different letters represent significant differences between treatment groups.

(40.76 µg/mL), while the mixture showed the highest IC_{50} value (95.19 µg/mL). The weak antioxidant effect of *M. spicata* could be due to the high values of polyphenols flavonoids and tannins observed during extraction (Table 3).

Table 7 presents the antioxidant activities of *M. spicata, M. officinalis,* and their mixture using DPPH, ABTS, and FRAP assays under Reflux Heating conditions. The results were compared with standard antioxidants BHT and Trolox as reference compounds. The DPPH radical scavenging assay revealed that the mixture exhibited the strongest antioxidant activity with an IC_{50} value of 13.51 µg/mL, which was notably close to the synthetic antioxidant BHT (8.98 µg/mL). *M. spicata* showed moderate activity with an IC_{50} of 42.69 µg/mL, while *M. officinalis* demonstrated the lowest activity with an IC_{50} of 226.90 µg/mL. This suggests a significant synergistic effect when combining the two plants, as the mixture's antioxidant capacity was substantially higher than either individual plant.

In the ABTS assay, the mixture demonstrated slightly better activity with an IC_{50} value of 94.99 µg/mL, followed by *M. spicata* (101.96 µg/mL). *M. officinalis* showed the lowest activity with an IC_{50} of 172.58 µg/mL.

The FRAP assay results showed close reducing power among all samples, with IC_{50} values ranging from 48.50 µg/mL for the mixture to 51.21 µg/mL for *M. spicata* and 51.01 µg/mL for *M. officinalis.*

Numerous *in vitro* and *in vivo* studies have demonstrated the antioxidant properties of *Mentha spicata* and *Melissa officinalis* extracts, supporting their potential as natural sources of therapeutic agents (Bayat et al., 2012; Carocho et al., 2015; Canadanovic-Brunet et al., 2008; Lopez et al., 2009; Ferreira et al., 2006; Zeraatpishe et al., 2011; Luno et al., 2014; Mimica-Dukic et al., 2004).

A comprehensive study by Dastmalchi et al. (2008) assessed the antioxidant activity of an aqueous ethanol extract of *M. officinalis* using various assays, including ferric reducing power, ferrous ion chelation, DPPH and ABTS radical scavenging, superoxide and nitric oxide quenching, and inhibition of *β*-carotene-linoleic acid oxidation. The extract demonstrated significant antioxidant capacity (90.43 ± 1.55 µg/mL), surpassing that of gallic and caffeic acids and exhibiting comparable effectiveness to quercetin (98.46 ± 0.89%) and BHA (96.08 ± 1.58%). These findings suggest that the high phenolic content is largely responsible for the extract's strong antioxidant performance.

Further evidence from Pereira et al. (2014) revealed that different fractions of *M. officinalis* collected in Brazil displayed potent DPPH radical scavenging activity. Likewise, its essential oil exhibited notable antioxidant potential with an IC_{50} of 7.58 µg/mL, attributed to the presence of bioactive volatile compounds such as monoterpene aldehydes and ketones (e.g., citrals, citronellal, isomenthone, and menthone), as well as a mixture of mono- and sesquiterpene hydrocarbons (Mimica-Dukic et al., 2004).

In the case of *M. spicata*, several studies have reported significant antioxidant activities. For instance, Fatiha et al. (2015) demonstrated that leaves collected in Algeria exhibited strong radical scavenging capacities in both ABTS and DPPH assays, with IC_{50} values of 10.3 ± 0.9 and 16.2 ± 0.2 µg/mL, respectively.

Indeed, our results showed a positive correlation between antioxidant effect and phenolic compounds. It has been demonstrated that phenolic substances act as antioxidants by reacting with a variety of free radicals. The mechanisms of action of phenolic antioxidants involve either hydrogen atom transfer, single electron transfer, sequential proton loss electron transfer, or transition metal chelation (Zeb, Alam. 2020)

Similarly, Nickavar et al. (2008) studied *M. spicata* harvested in Tehran during its flowering stage and found a direct correlation between TPC and antioxidant activity, reinforcing the pivotal

Table 7. IC_{50} values of plants with the reflux heating method, extraction

Assays	Reflux heating (µg/mL)			Positive control	
	M. spicata	*M. officinalis*	Mixture	BHT	Trolox
DPPH	42.69 ± 2.36e	226.90 ± 20.51i	13.51 ± 1.11d	8.98 ± 0.01c	—
ABTS	101.96 ± 0.22a	172.58 ± 18.01h	94.99 ± 12.91g	—	2.07 ± 0.03a
FRAP	51.21 ± 1.70f	51.01 ± 5.90f	48.50 ± 5.20e	5.30 ± 1.41b	—

Note: different letters represent significant differences between treatment groups.

role of phenolic compounds in scavenging free radicals. These findings are consistent with earlier observations by Dorman et al. (2003), who reported similar associations in aqueous extracts.

Summarize, oxidation, or oxidative stress, is a critical event in all cells and can become a serious problem for living organisms. It is one of the main causes of the development and progression of several life-threatening diseases (Covarrubias et al., 2008; Kasote et al.,2015). Additionally, oxidative stress can also lead to food spoilage through lipid oxidation (Gray, 1978; Halliwell and Gutteridge, 1999). One alternative to counteract oxidative stress is the use of extracts from aromatic and medicinal plants. Given the close relationship between antioxidant activity and phenolic compounds content (El Jemli et al., 2016), a quantification of phenolic compounds was carried out, and the antioxidant activity of *M. spicata*, *M. officinalis,* and their mixture was subsequently evaluated. This study successfully demonstrated the phytochemical richness and significant antioxidant potential of *M. spicata* and *M. officinalis* and their mixture from the Settat region of Morocco. Phytochemical analyses revealed the presence of key bioactive compounds, particularly polyphenols, flavonoids, and tannins, in both plants. The three extraction methods employed infusion, maceration, and reflux heating showed varying efficiencies in extracting bioactive compounds. Reflux heating was generally the most effective method for obtaining extracts rich in phenolic compounds and exhibiting high antioxidant activity. A particularly noteworthy finding of this study was the synergistic effect observed when combining the two plants. In several cases, the mixture demonstrated higher contents of bioactive compounds and antioxidant activities compared to the individual plants, suggesting the promising potential of combining these plants for various applications. The antioxidant activities evaluated using DPPH, ABTS, and FRAP methods confirmed the high antioxidant potential of both plants, especially their mixture.

Collectively, these results underscore the capacity of *M. officinalis* and *M. spicata* to exert antioxidant effects through various mechanisms, including neutralization of free radicals, inhibition of lipid peroxidation, and enhancement of endogenous antioxidant defenses. Such properties may contribute to their therapeutic potential in mitigating oxidative stress-related

CONCLUSIONS

This study conclusively demonstrated the exceptional phytochemical richness and significant antioxidant potential of *M. spicata* and *M. officinalis* from Morocco's Settat region, revealing the presence of key bioactive compounds including polyphenols, flavonoids, and tannins in both plants. Among the three extraction methods employed (infusion, maceration, and reflux heating), reflux heating proved most effective for obtaining extracts rich in phenolic compounds and exhibiting high antioxidant activity, notably achieving the highest total flavonoid content (3437.79 µg QE/mg) in the mixture. A particularly significant finding was the synergistic effect observed when combining the two plants, with the mixture demonstrating superior bioactive compound content and antioxidant activities compared to individual plants, especially evident in the FRAP assay where the mixture's macerate extracts showed the highest value (95.19 µg/mL). The antioxidant activities, evaluated using DPPH, ABTS, and FRAP methods, confirmed the high antioxidant potential of both plants and their mixture, with the DPPH assay revealing an impressively low IC50 value of 13.51 µg/mL for the mixture's reflux heating extract, indicating potent free radical scavenging ability. These comprehensive results not only support the traditional use of these plants but also suggest their significant potential as natural antioxidant sources for various applications in the food and pharmaceutical industries, particularly when used in combination.

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