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Melissa officinalis: Antibacterial and Antioxidant Potential, Phenolic Profile and Enzyme Activities

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ABSTRACT
Popularly referred to as lemon balm, *Melissa officinalis* L., has been used as a cure for gastrointestinal disorders, respiratory and cardiovascular diseases, mental and central nervous system problems, various cancers, headache, nervousness, and rheumatism. In this study, the phenolic profile, antioxidant potential, antibacterial activity, and enzyme activity of lemon balm grown in nature in Bolu, Turkey were determined. Furthermore, comparisons were made with plants grown *in vitro*. Individual phenolic analysis with HPLC-DAD showed that the most prevalent phenol was rosmarinic acid in both extracts and naturally-grown plants had higher amount than *in vitro*-grown ones. Similarly, naturally-grown plants had considerably greater levels of total phenol-flavonoid, scavenging activity for free radicals (DPPH), and phenol synthesis related enzyme (PAL). As for the enzymatic antioxidant activity (SOD and CAT), naturally-grown plants were found to have higher CAT activity and lower SOD activity. As a remarkable result, although plants grown *in vitro* showed moderate antibacterial activity, no effect was observed in naturally-grown plants. In general, these results showed that the *M. officinalis* grown in nature is exposed to more biotic and abiotic stress and increases their phenolic content remarkably and consequently antioxidant capacity.

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ÖZET
Halk arasında oğul otu olarak bilinen *Melissa officinalis* L., mide bağırsak rahatsızlıkları, solunum ve kalp damar hastalıkları, zihinsel ve merkezi sinir sistemi problemleri, çeşitli kanserler, baş ağrısı, sinirlilik ve romatizma için şifa olarak kullanılmaktadır. Bu çalışmada, Bolu, Türkiye'de doğada yetişen oğul otu bitkisinin fenolik profili, antioksidan potansiyeli, antibakteriyel etkisi ve enzim aktivitesi belirlenmiştir. Ayrıca *in vitro* yetiştirilen bitkilerle de karşılaştırılma yapılmıştır. HPLC-DAD ile yapılan bireysel fenolik analiz her iki özütde en yaygın fenolün rosmarinik asit olduğunu ve doğal olarak yetişen bitkilerde *in vitro* yetiştirilenlerden daha yüksek miktarda rosmarinik asit bulunduğunu göstermiştir. Benzer şekilde, doğal olarak yetişen bitkilerin önemli ölçüde daha yüksek toplam fenol-flavonoid seviyelerine, serbest radikalleri temizleme aktivitesine (DPPH) ve fenol sentezi ile ilgili enzime (PAL) sahip olduğu görülmüştür. Enzimatik antioksidan aktivitesi olarak (SOD ve CAT), doğal olarak yetişen bitkilerin daha yüksek CAT aktivitesine ve daha düşük SOD aktivitesine sahip olduğu bulunmuştur. Dikkat çekici bir sonuç olarak *in vitro* yetiştirilen bitkiler orta derecede antibakteriyel aktivite göstermesine rağmen doğal olarak yetişen bitkilerde etki görülmemiştir. Genel olarak bu sonuçlar, doğada yetişen *M. officinalis' in* daha fazla biyotik ve abiyotik strese maruz kaldığını ve fenolik içeriğini ve dolayısıyla antioksidan kapasitesini

Bitki Fizyolojisi

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INTRODUCTION

The Lamiaceae plant *Melissa officinalis* L., commonly known as bee balm or lemon balm, is indigenous to the Mediterranean basin and southern Europe (Davis, 1978). It has been used as a medicinal plant in the remedy of headache, mental and central nervous system problems, respiratory and cardiovascular diseases, various cancers, gastrointestinal disorders, nervousness, and rheumatism (Adinee et al., 2008; Shakeri et al., 2016; Petrisor et al., 2022). It has a worldwide medical reputation, especially as an antioxidant, anxiolytic, antidepressant, antipyretic, carminative, anti-inflammatory, spasmolytic expectorant, antimicrobial, sedative, digestive, antitumor, hypoglycemic, antinociceptive, hypolipidemic, antihypertensive, hepatoprotective, and memory enhancer (Gbolade & Lockwood, 1989; Barros et al., 2013; Shakeri et al., 2016; Ulgen et al., 2020; Petrisor et al., 2022). According to phytochemical studies, the principal active components of *M. officinalis* include volatile substances, triterpenes, phenolic acids, and flavonoids (apigenin and luteolin derivatives) (Barros et al., 2013). According to WHO monographs on several medicinal plants, hydroxycinnamic acids, often known as rosmarinic acid, are among the recognized phytochemicals and are indicators of quality control (Shakeri et al., 2016). Essential oil, flavonoids, and phenolic acids, including rosmarinic and caffeic acids, have all been linked to these biological effects (Mencherini et al., 2007). Recently, the aqueous extracts and decoctions of lemon balm's aerial portions have attracted significant interest as potential food ingredients due to their antimicrobial and antioxidant properties. They give extra health advantages as well as good protection in a number of dietary compositions (Carocho et al., 2016; Caleja et al., 2018). It is also widely used for seasoning and flavoring (Gbolade & Lockwood, 1989; Carocho et al., 2015).

Phenolic compounds are the most significant secondary metabolites that accumulate spontaneously in plants and play a crucial role in the ability of plants to withstand stressful situations as non-enzymatic antioxidants (Selmar & Kleinwächter, 2013). Additionally, plants elevate antioxidant enzyme expression like SOD (superoxide dismutase), CAT (catalase) and ascorbate peroxidase to combat oxidative stress brought on by biotic and abitoc stresses, which generates reactive oxygen species (ROS) like hydrogen peroxide, hydroxyl radicals, singlet oxygen and superoxide (Sharma et al., 2012; Ighodaro et al., 2018). The primary enzyme in the manufacture of phenol is PAL (phenylalanine ammonia lyase), which serves in higher plants as a secondary metabolic route. The defensive mechanism PAL, which is responsible for the formation of phenolic constituents in plants, is the principle stimulating reaction in plants to numerous biotic and abiotic factors (MacDonald & D'Cunha, 2007).

M. officinalis attract particular attention due to their medicinal and health benefit properties. They have been used in food, nutraceutical, pharmaceutical, and cosmetic industries (Carović-Stanko et al., 2016). The purpose of this study is to indicate the phenolic profiles, antibacterial potential and antioxidant capacities (enzymatic and non-enzymatic) of *M. officinalis* making a comparison between naturally-grown and *in vitro*-grown shoots.

MATERIAL and METHOD

Plant Materials and Extraction

M. officinalis L. subsp. *officinalis* L. was collected from Bolu, Turkey and identified utilizing "Flora of Turkey and the East Aegean Islands" (Davis, 1978) keeping voucher specimens-AUT-2008 in Department of Biology (BAIBU). Field-grown shoots were obtained from the plant. *In vitro*-grown shoots were regenerated according to our prior research (Ulgen et al., 2020; Ulgen et al., 2021). Field-grown and *in vitro*-grown lemon balm shoots were collected, and the shoots were then lyophilized and crushed into a powder. Methanolic extract was obtained at 40 °C for 18 h and then vacuum-vaporized at 40 °C. The residual part was then lyophilized after being dissolved in distilled water.

Phenolic Constituents

Individual Phenolic Compounds

Two methanolic extracts obtained from naturally-grown and *in vitro*-grown *M. officinalis* were quantitatively evaluated for rosmarinic acid, quercetin, rutin hydrate, gallic acid monohydrate, myricetin and caffeic acid (Sigma®) using high performance liquid chromatography (HPLC) combined with a diode array detector (DAD) (VWR-Hitachi LaChrom Elite®). Operational parameters were

configured as previously reported in our study (Turker et al., 2021).

Total Phenol and Flavonoid Content

Colorimetric methods (Folin-Ciocalteu and aluminum chloride) were used respectively for the determination of phenolic and flavonoid content in methanol extracts according to our previous report (Turker et al., 2021). For total phenol content, they were given as mg g^{-1} gallic acid equivalent (GAE) g^{-1} dry extract, and for total flavonoid content, as mg g^{-1} catechol equivalent (CE) g^{-1} dry extract.

Antioxidant Activity

Scavenging activity of free radicals in *M. officinalis* methanol extracts was appraised using the DPPH (1,1-diphenyl-2-picrylhydrazyl) test according to our previous report (Turker et al., 2021). The method relies on the elimination of DPPH by substances at 517 nm using a UV-vis Spectrophotometer (Hitachi U-1900®). Ascorbic acid was utilized as an antioxidant reference.

Antibacterial Activity

Using 3 Gram-positive bacteria [*Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615)] as well as 7 Gram-negative bacteria [*Serratia marcescens* (ATCC 8100), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 13315), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883)], the disc diffusion assay was utilized to determine two distinct methanolic extracts of *M. officinalis* according to our previous report (Turker et al., 2021). Concentration of each bacterial culture was adjusted to the 0.5 McFarland with saline (0.9% NaCl) using densitometer McFarland Densitometer (Biosan®) and then cotton swabs were employed to inoculate Mueller Hinton agar plates. Filter paper discs containing 13 µl of the extracts (sterilized with 0.22 µm filter) were placed into inoculated petri plates. Positive controls include erythtomycin, tetracycline, and ampicillin (Bioanalyse®). Water and methanol were used as negative controls. Inhibitory zones after 16–18 hours of incubation at 37°C in petri dishes were determined.

Enzymatic Antioxidant Activity

Extraction of Enzymes and Protein Designation

To assess SOD and CAT enzyme activity, *M. officinalis* shoots obtained from 2 distinct sources were first processed for the extraction of enzymes and protein measurement. Lowry technique (Lowry et al., 1951) was used to calculate the protein content of the shoots employing bovine serum albumin as a reference protein. It was performed in regard to our preceding report (Ulgen et al., 2021).

Superoxide Dismutase (SOD) Enzyme Activity

The method mentioned in Ulgen et al. (2021) was used to monitor SOD activity. Absorbance was measured at 560 nm. One SOD unit was defined as the quantity of protein required to reduce nitroblue tetrazolium (NBT) by 50% in the reaction, and enzyme activity was reported as Unit mg^{-1} protein.

Catalase (CAT) Enzyme Activity

The method mentioned in Ulgen et al. (2021) was used to monitor CAT activity. CAT activity was evaluated by measuring the reduction in absorbance at 240 nm induced by catalase enzyme breakdown of H_2O_2. The H_2O_2 extinction coefficient of 0.0392 mM cm^{-1}, which is utilized to indicate activity, is given as mmol H_2O_2 mg^{-1} protein.

Phenylalanine Ammonium Lyase (PAL) Activity

The method mentioned in Ulgen et al. (2021) was used to monitor PAL activity. PAL activity was measured at 270 nm.

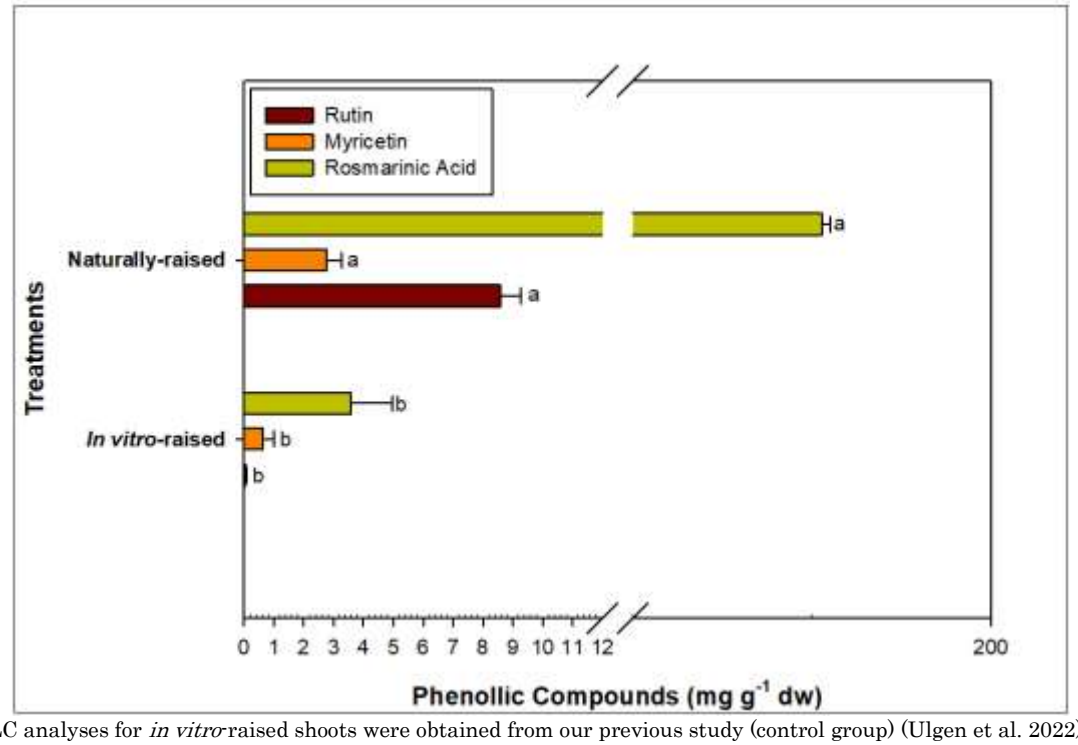
Data Analysis

All experiments had a totally randomized design. Data were analyzed using ANOVA as well as Duncan's multiple range tests in SPSS version 26 (SPSS Inc, Chicago, IL, USA). All of the data in the tables was provided as a mean value with a SE (standard error).

RESULTS and DISCUSSION

Six individual phenolic constituents included in methanol extracts of naturally-grown shoot materials were analyzed with HPLC-DAD system (Figure 1). HPLC analysis results for *in vitro*-raised shoots were collected from our previous work (control group) (Ulgen et al., 2021) to compare with naturally-raised ones. Rosmarinic acid was found to be the most prevalent phenol in both extracts and naturally-grown *M. officinalis* shoots contained higher amount of phenols than *in vitro*-grown shoots (Figure 1). In parallel to our results, the most prevalent phenolic in this species, according to several earlier studies was rosmarinic acid (Caniova & Brandsteterova, 2001; Fecka & Turek, 2007; Lee, 2010; Ziaková & Brandsteterová, 2003; Draginic et al., 2022; Petrisor et al., 2022; Abdellatif et al., 2023; Silva et. al., 2023). Naturally-grown and *in vitro*-grown plants involved rosmarinic acid (190.56±0.5 mg g^{-1} and 3.58±1.4 mg g^{-1}), rutin (8.58±0.7 mg g^{-1} and 0.07±0.01 mg g^{-1}) and myricetin (2.76±0.5 mg g^{-1} and 0.64±0.36 mg g^{-1}), respectively. Caffeic acid, quercetin and gallic acid were not detected in both extracts. Although the second most abundant phenol in natural plants was rutin, it was myricetin in *in*

vitro-grown plants. On the other hand, in another study, HPLC-DAD evaluation of an *M. officinalis* extract revealed the presence of rosmarinic acid (principal), caffeic acid, and m-coumaric acid (the least prevalent) (Dastmalchi et al., 2008). Pereira et al. (2014) listed caffeic acid, gallic acid, chlorogenic acid and ellagic acid in *M. officinalis* ethanol extract. Moreno et al. (2006) also detected rosmarinic acid, carnosic acid and carnosol in the leaves and flowers of *M. officinalis*, and they reported 5.5 g rosmarinic acid 100 g extract^{-1} in flowering plants. Barros et al. (2013) evaluated the phenolic constituents of garden grown and *in vitro*-cultured *M. officinalis* infusions. In parallel to our study, they found rosmarinic acid as the abundant phenolic being the lowest in *in vitro*-grown materials (15.46 mg g^{-1}) that is higher than our result (3.58 mg g^{-1}). On the contrary, naturally-grown sample in our study contained higher rosmarinic acid (190.56 mg g^{-1}) than garden-grown materials (20.96 mg g^{-1}) in the study of Barros et al. (2013). Spiridon et al. (2011) revealed the presence of caffeic acid (5.78%), p-coumaric acid (6.78%), rosmarinic acid (11.9%), quercetin glucoside (16.46%) and kaempherol diglucoside (59.09%) with HPLC-MS technique.



*Results of HPLC analyses for *in vitro*-raised shoots were obtained from our previous study (control group) (Ulgen et al. 2022) to make a comparison with naturally-raised shoots.

Figure 1. HPLC-DAD analysis of phenolic constituents in naturally-raised and *in vitro*-raised *M. officinalis*.

Şekil 1. Doğal olarak yetişen ve in vitro olarak yetiştirilen M. officinalis' in fenolik bileşenlerin HPLC-DAD analizi.

Total phenolic and flavonoid contents (Figure 3) and antioxidant capacity (Figure 4) of naturally-grown extracts were determined. Furthermore, to compare naturally-raised shoots to *in vitro*-raised shoots, we took the control group's total phenol-flavonoid and antioxidant results from our earlier work (Ulgen et al., 2021). It was observed that naturally-raised sample comprised higher total phenol (374.35±1.3 mg GAE g^{-1} dry extract) and flavonoid (327.61±1.6 mg CE g^{-1} dry extract) content than *in vitro*-raised extract (31.66±0.7 mg GAE g^{-1} and 32.50±0.6 mg CE g^{-1} dry extract, respectively). Significantly, the naturally-grown shoots had 12 and 6 times higher total phenol and flavonoid content, respectively, compared to *in vitro*-grown ones (Figure 3). The result obtained from natural habitat plant in our study was better than the findings of other previous studies. For example, Moreno et al. (2006) reported total phenolics of water, methanol and acetone extracts of *M. officinalis* as 3 g, 12 g and 19 g of GAE 100 g of extract^{-1}, respectively.

Atanassova et al. (2011) exhibited total phenol and flavonoid content in 80% aqueous methanol extract of *M. officinalis* as 48.86 mg GAE 100 g DW^{-1} and 45.06 mg CE 100 g DW^{-1}, respectively. Mabrouki et al. (2018) demonstrated total phenol and flavonoid content in ethanol extract of *M. officinalis* as 63.00 mg GAE g^{-1} and 3.61 mg QE g^{-1} dry extract. Draginic et al. (2022) found out total phenol and flavonoid level in ethanolic macerate as 73.39 mg GAE g^{-1} and 6.23 mg QE g^{-1} dry extract, respectively. Furthermore, Spiridon et al. (2011) indicated *M. officinalis* total phenol and flavonoid content as 54.9 mg GA g^{-1} and 25.8 mg R g^{-1}, respectively.

DPPH free radical scavenging activity of both extracts showed that naturally-grown plants were more

effective (Figure 4) and strong antioxidant capacity was observed with naturally-raised plants (IC_{50}=34.86 µg mL^{-1}). *In vitro* propagated plants exhibited very low antioxidant capacity (IC_{50}>200 µg mL^{-1}) comparing to naturally growing plants. The presence of phenolic acids, notably hydroxycinnamic acid derivatives like rosmarinic acid, is thought to be the cause of the antioxidant action of *M. officinalis* extracts (Caniova & Brandsteterova, 2001). Our result obtained from naturally-grown plants was in good agreement with some previous studies. For example, free radical scavenging activities (IC_{50}) of ethanolic macerate of *M. officinalis* was reported as 9.95 µg mL^{-1} by Draginic et al. (2022). Moreno et al. (2006) exhibited IC_{50} of methanol and acetone extracts of *M. officinalis* as 18 and 25.6 µg mL^{-1}, respectively. IC_{50} value of *M. officinalis* ethanol extract was determined as 18.16 µg mL^{-1} by Mabrouki et al. (2018). Atanassova et al. (2011) recorded IC_{50} value of 80 % aqueous methanol as 10.87 µg mL^{-1}. Moreover, Mencherini et al. (2007) reported considerable free radical scavenging action in a concentration-dependent manner for aqueous ethanol extract and rosmarinic acid showing IC_{50} values as 18.5 and 3.1 µg mL^{-1}, respectively. On the other hand, some studies reported the antioxidant capacity that is lower than our findings. Dastmalchi et al. (2008) reported IC_{50} value of naturally-grown *M. officinalis* leaves as 134.16 µg mL^{-1} and Spiridon et al. (2011) showed the effectiveness of *M. officinalis* for antioxidants capacity as IC_{50} = 87.28 µg mL^{-1}. *M. officinalis*'s therapeutic effects in the avoidance and treatment of oxidative stress-related illnesses, such as cardiovascular and neurological disorders, can be ascribed to its antioxidant power.

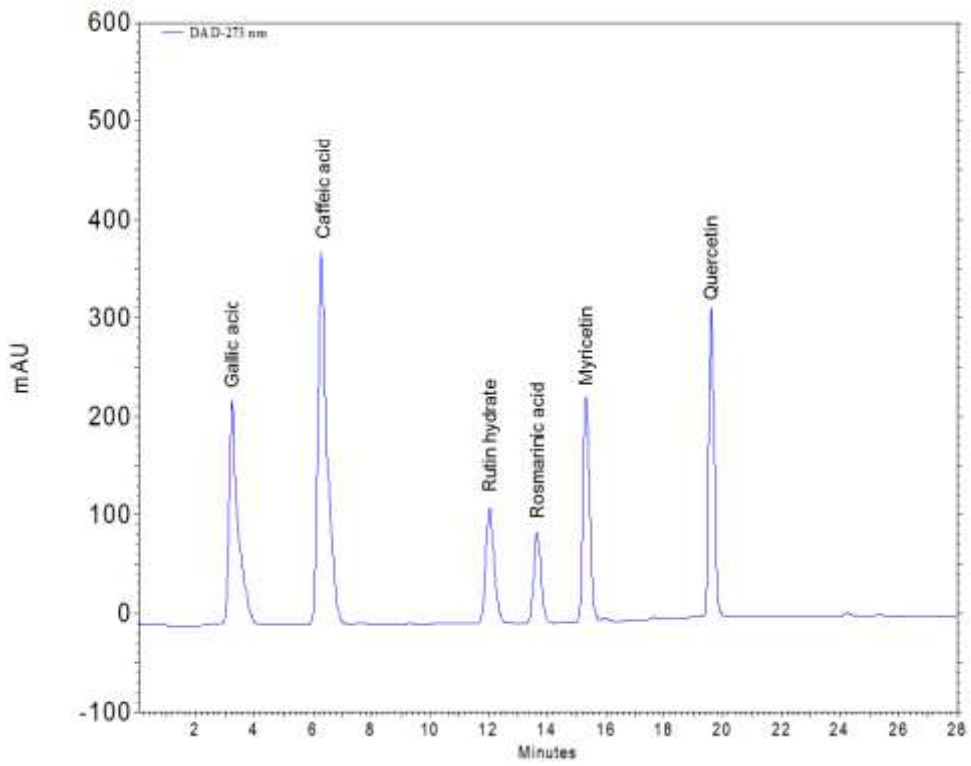
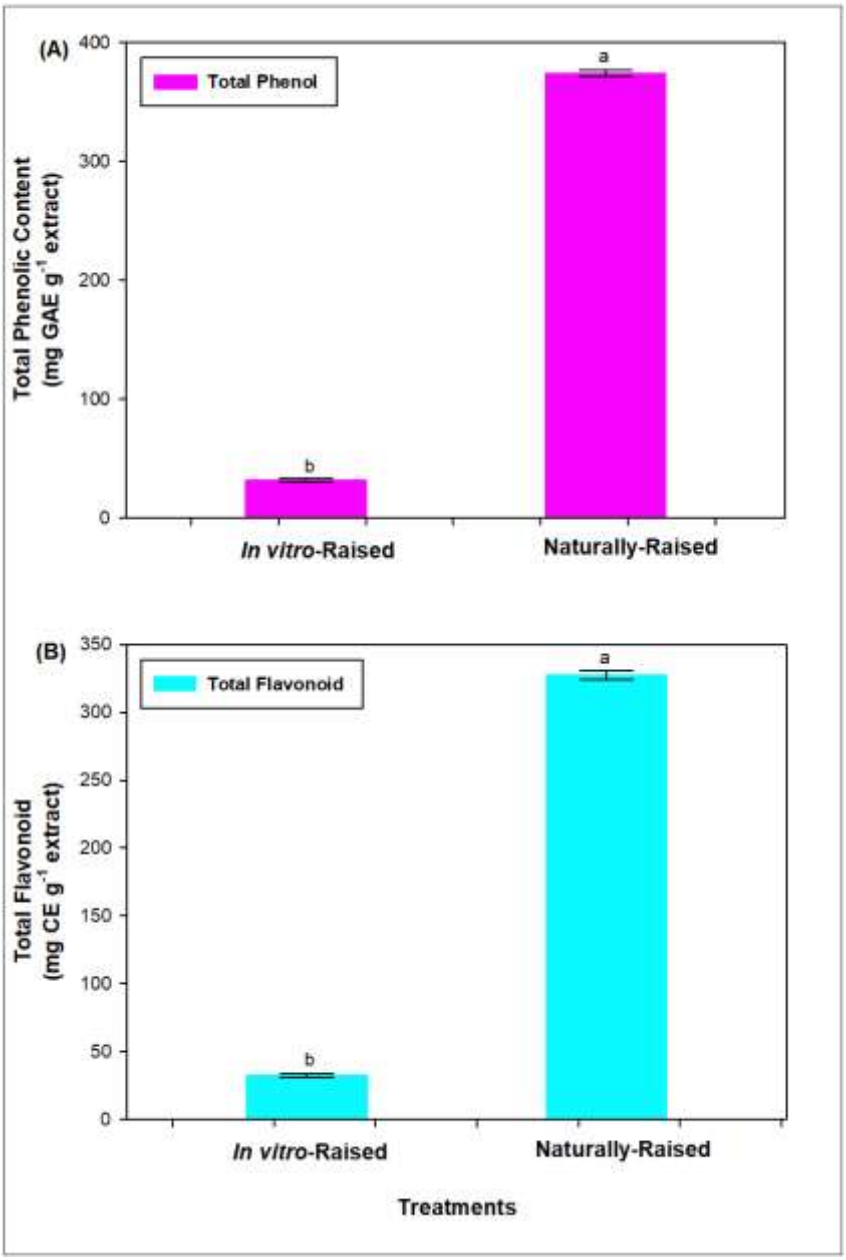


Figure 2. HPLC chromatogram of the phenolic standards. Retention times: 1. Gallic acid-3.2 min, 2. Caffeic acid-6.2 min, 3. Rutin hydrate-12.0 min, 4. Rosmarinic acid-13.6 min, 5. Myricetin-15.3 mi n, 6. Quercetin-19.6 min

Şekil 2. Fenolik standartların HPLC kromatogramı. Retensiyon zamanları: 1. Gallik asit-3.2 dk, 2. Kafeik asit-6.2 dk, 3. Rutin hidrat-12.0 dk, 4. Rosmarinik asit-13.6 dk, 5. Myricetin-15.3 dk, 6. Quercetin-19.6 dk.

Antibacterial activity of shoots obtained from nature and *in vitro*-propagation was presented in Table 1. Antibacterial assay showed the opposite result from antioxidant activity. It is quite intriguing because only the *in vitro*-grown *M. officinalis* showed antibacterial action against 8 pathogens and naturally-grown plants were not effective against tested bacteria (Table 1). Antibacterial activity of *in vitro*-grown *M. officinalis* may be related to the essential oil components involved in the plant. Because, it was observed that *in vitro* propagated plants were more aromatic than the shoots that were collected from nature. Rosmarinic acid is not thought to have a determinant effect on antibacterial activity of *M. officinalis* in our study because this phenolic acid is much less in *in vitro*-grown plants than in wild-collected ones. Similarly, Moreno et al. (2006) reported no antibacterial activity of rosmarinic acid at low concentrations (5–250 µg mL^{-1}). Mencherini et al.

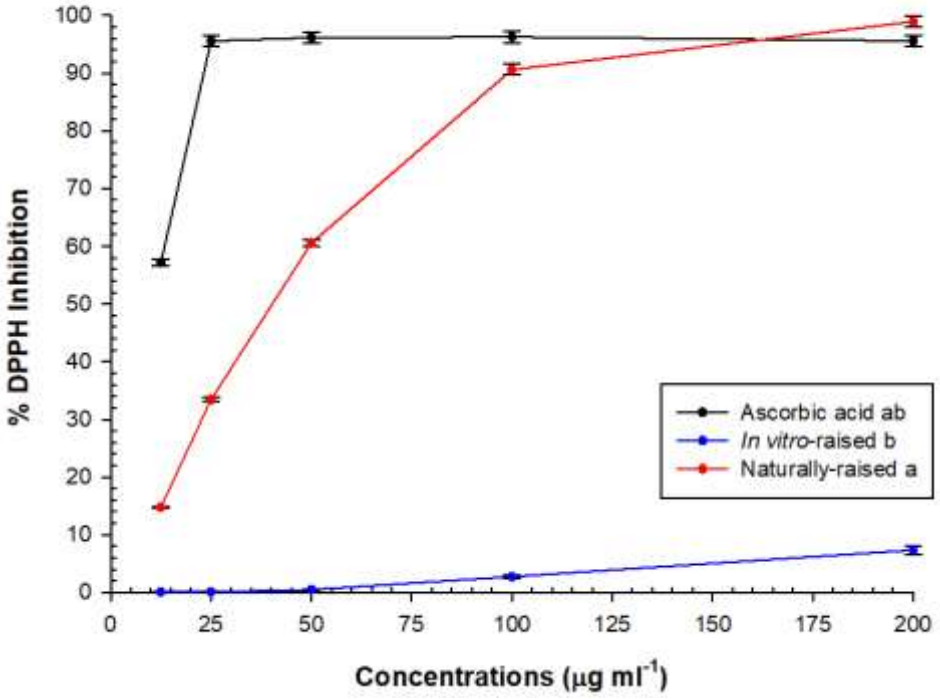
(2007) indicated the bacteriostatic effects of rosmarinic acid at higher concentrations like 0.12 mg mL µg mL^{-1} against Gram positive bacteria (*S. epidermidis* and *S. aureus*) and 2.0 mg mL µg mL^{-1} against Gram negative bacteria (*E. coli* and *P. aeruginosa*). According to prior studies, the antibacterial effect of rosmarinic acid was observed at very high concentrations. In our study, since the rosmarinic acid concentration in the crude extract was lower than its pure form, its antibacterial activity was probably not sufficient. Ivanov et al. (2022) reported that rosmarinic acid exhibited weak potency against *S. aureus* and moderate activity against *S. pyogenes* and *E. cloacae*. Mabrouki et al. (2018) indicated antibacterial activity of *M. officinalis* ethanol extract against *S. aureus* (25 mm inhibition zone), *P. aeruginosa* (17.5 mm) and *E. coli* (18.1 mm). Generally main components of *M. officinalis* essential oil were ß-caryophyllene, citral, geranial, neral, germacrene D, thymol and C-citronellal and they demonstrated antibacterial efficacy against the studied microorganisms (Ehsani et al. 2017; Çelebi et al., 2023; Petrisor et al., 2022).



*Results of total phenol and flavonoid content for *in vitro*-raised shoots were obtained from our previous study (control group) (Ulgen et al. 2022) to make a comparison with naturally-raised shoots.

Figure 3. Total phenol (A) and flavonoid (B) content of naturally-raised and *in vitro*-raised *M. officinalis*. GAE: Gallic acid equivalent, CE: Catechol equivalent.

Şekil 3. Doğal olarak yetişen ve in vitro olarak yetiştirilen M. officinalis' in toplam fenol (A) ve flavonoit (B) içeriği. GAE: Gallik asit eşdeğeri, CE: Katekol eşdeğeri.



*Results of % DPPH inhibition for *in vitro*-raised shoots were obtained from our previous study (control group) (Ulgen et al. 2022) to make a comparison with naturally-raised shoots.

Figure 4. DPPH free radical scavenging activity of naturally-raised and *in vitro*-raised *M. officinalis*. IC_{50} values: Ascorbic acid: <200 µg/ml, *In vitro*-raised: >200 µg/ml and Naturally-raised: 34.86 µg/ml.

Şekil 4. Doğal olarak yetişen ve in vitro olarak yetiştirilen M. officinalis' in DPPH serbest radikal süpürücü aktivitesi. IC_{50} değerleri: Askorbik asit: <200 µg/ml, in vitro yetiştirilen: >200 µg/ml and doğal olarak yetişen: 34.86 µg/ml.

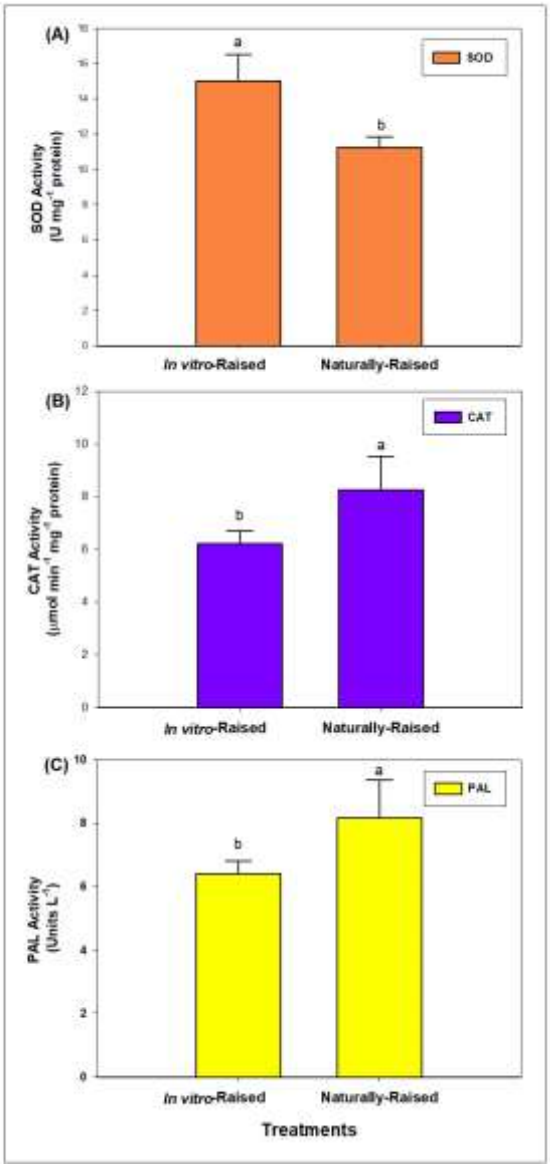
Table1. Antibacterial properties of naturally-raised and *in vitro*-raised *M. officinalis*. Different letters in each column indicate a significant difference ($P<0.05$).

Çizelge 1. Doğal olarak yetişen ve in vitro olarak yetiştirilen M. officinalis' in antibakteriyel özellikleri. Her sütundaki farklı harfler önemli bir fark gösterir (P <0.05).

Tested Bacteria	*In vitro*-raised	Naturally-raised	Ampicillin	Tetracycline	Erythtomycin
S.aureus	11±0.5^{b}	-	29±0.3^{b}	27±0.3^{b}	25±0.3^{c}
S.epidermidis	10±0.3^{c}	-	-	-	30±0.6^{b}
S.pyogenes	15±0.6^{a}	-	54±0.3^{a}	42±0.3^{a}	50±0.3^{a}
S.marcences	-	-	-	20±0.3^{e}	-
S.typhimurium	8±0.3^{d}	-	24±0.3^{c}	22±0.6^{d}	-
P.aeruginosa	8±0.3^{d}	-	-	11±0.3^{f}	-
P.vulgaris	10±0.1^{bc}	-	13±0.3^{f}	23±0.3^{d}	10±0.3^{d}
K.pneumonia	-	-	-	20±0.1^{e}	8±0.3^{e}
E.cloacae	9±0.3^{c}	-	21±0.3^{d}	25±0.1^{c}	-
E.coli	9±0.3^{c}	-	16±0.3^{e}	25±0.3^{c}	-

S. marcences and *K. pneumonia* were not susceptible to tested extracts. The best antibacterial activity was observed against *S. pyogenes* with 15 mm inhibiton zone. Generally, natural lemon balm inhibited Gram positive bacteria (*S. pyogenes*, *S. aureus* and *S. epidermidis*) more than Gram negative in our study. It might be due to the fact that gram-positive bacteria have single-layered cell walls, which differ from those of other bacteria. Gram-negative bacteria, on the other hand, have multi-layered cell walls, which may provide them resistance to plant extracts (Turker et al., 2021). Reviewed literature has demonstrated that *M. officinalis* extracts have greater antibacterial property against Gram-positive bacteria (Mencherini et al., 2007; Canadanović-Brunet et al., 2008; Stefanović and Comic, 2012; Abdellatif et al., 2023; Silva et. al., 2023). Some inhibitory potentials of lemon balm were also recorded against certain Gram-negative strains

(Carocho et al., 2015; Caleja et al., 2018; Abdellatif et al., 2023; Silva et. al., 2023). The majority of the tested microorganisms were susceptible to the reference antibiotic discs (ampicillin, tetracycline and erythtomycin) (Table 1). Methanol was employed as a negative control to alter the final concentrations of all extracts. Negative controls (methanol or water) had no inhibitory effect. Abdel-Naime et al. (2019) demonstrated antibacterial efficacy of *M. officinalis* crude extract and its derived fractions against a variety of human pathogenic microorganisms, which is consistent with the findings of our investigation. Their samples displayed varying degrees of inhibitory effectiveness against *P. aeruginosa* and *S. aureus*, with MICs ranging from 1.65 to 191.40 g ml^{-1}, whereas no antibacterial effects were noticed against *K. pneumoniae* and *E. coli*.



*Results of SOD, CAT and PAL activities for *in vitro*-raised shoots were obtained from our previous study (control group) (Ulgen et al. 2022) to make a comparison with naturally-raised shoots.

Figure 5. SOD, CAT and PAL activities of naturally-raised and *in vitro*-raised *M. officinalis*.

Şekil 5. Doğal olarak yetişen ve in vitro olarak yetiştirilen M. officinalis' in SOD, CAT ve PAL activiteleri.

SOD and CAT are two antioxidant enzymes that are not only essential but also crucial to the antioxidant defense of biological systems against free radical damage. SOD and CAT activities provide a first-line antioxidant defense system that plays a critical and basic role in overall defense mechanisms and tactics in biological systems (Ighodaro et al., 2018). Superoxide dismutase (SOD) is the cell's initial detoxifying enzyme and the most effective antioxidant. It catalyzes the dismutation of two molecules of superoxide anion (O_2) to hydrogen peroxide (H_2O_2) and molecular oxygen

(O_2), making the potentially hazardous superoxide anion less dangerous. CAT enzyme catalyzes the breakdown or reduction from hydrogen peroxide (H_2O_2) to water and molecular oxygen, providing the detoxification process beginning by SOD (Sharma et al., 2012). SOD and CAT activity of naturally-grown plants were detected and compared with *in vitro*-grown plants (Ulgen et al., 2021). We used enzyme activity values of SOD, CAT and PAL for *in vitro*-grown shoots from our previous study (Ulgen et al., 2021) to make a comparison between naturally-raised and *in vitro*-raised shoots. Although naturally-grown plants had higher CAT activity, *in vitro*-propagated plants exhibited higher SOD activity (Figure 5). Wild-grown plants are exposed to more biotic and abiotic stress so increased CAT activity was observed comparing to *in vitro*-propagated ones. SOD levels decrease with aging, although free radical production rises in biological systems (Ighodaro et al. 2018). The reason of lower SOD activity of the plant collected from nature may be related to the age of the wild plant that is relatively older than *in vitro*-grown ones. Many studies demonstrated increased SOD and CAT activity in *M. officinalis* that is exposed to different stress conditions like heat (Pistelli et al., 2019), salt (Ghasemian et al., 2021) and magnetic field (Ulgen et al., 2021).

An essential biosynthetic enzyme called phenylalanine ammonium lyase (PAL) catalyzes the initial step in the synthesis of phenolics, which are comprised in the body's defense system against a variety of biotic and abiotic stressors (Ghanati et al., 2007). Wild-raised shoots had higher PAL activity as expected comparing to *in vitro*-grown ones (Figure 5). It has been proven that the high phenol content of plants collected from nature is due to high PAL activity. Elevated PAL activity was exhibited with some stress factors like salt (Safari et al., 2020) and magnetic field (Ulgen et al., 2021).

It is obvious that different results have been obtained in different studies conducted in different parts of the world in terms of phenolic content and antioxidant activity of *M. officinalis*. The reason of this is the production and concentration of accumulated phenolics that are affected by a variety of internal and external variables, including plant physiology, harvesting time, geographical variation, age, developmental stage, soil composition, climate, elevation and pathogen attack type (Mabrouki et al., 2018; Pratyusha, 2022). Plants respond to environmental stress by increasing phenolic components (Turker & Yildirim, 2018; Turker et al., 2018; Thakur et al., 2019). Since plants grown *in vitro* are in a more controlled environment, they are relatively less exposed to stress than those collected from nature so *in vitro*-grown plants may not require a lot of phenol synthesis to survive.

CONCLUSION

Although many studies have been conducted on the antioxidant potency and phenolic constituents of *M. officinalis*, this study firstly ascertained the phenolic profile and antioxidant capacity of *M. officinalis* grown in Bolu, Turkey and antibacterial activity of *in vitro*-grown material was demonstrated for the first time. It has been determined that naturally-grown *M. officinalis* has strong antioxidant potency due to its high phenolic content, especially rosmarinic acid. High phenolic content of wild growing plants that are exposed to more stressful environmental conditions was verified with high enzyme activities of PAL and CAT. Essential oil of *in vitro*-grown materials that was more aromatic than plants collected from nature should be investigated in the next studies. Furthermore, subsequent studies should perform on enhancing the quantity of phenolics like rosmarinic acid in *in vitro*-grown plants by exerting various stress types.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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