

## CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF *MENTHA PIPERITA* VAR. *CITRATA* EXTRACTS OBTAINED BY DIFFERENT EXTRACTION SOLVENTS

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### ABSTRACT

The main aim of this study was to evaluate the phytochemical composition of extracts of *Mentha piperita* var. *citrata* obtained by using three different solvents as well as their antimicrobial activity against a panel of Gram positive and Gram negative bacteria. The antibacterial activities of the extracts (hexane, chloroform, and 70% aqueous methanol) were tested against a panel of bacteria using broth microdilution method. Rosmarinic acid (10.505 mg/g extract), cynaroside (8.545 mg/g extract) and cosmosiin (8.489 mg/g extract) appeared to be the major components of methanolic extract, whereas acetin was the most abundant (8.438 mg/g extract) component of chloroform extract. *M. citrata* extracts showed significant antimicrobial activity against Gram positive bacteria at different concentrations. Chloroform extract from *M. citrata* showed antimicrobial activity at concentration of 512-4096 µg/mL, whereas hexane and methanolic extracts had activity at 1024-4096 µg/mL and 2048-4096 µg/mL, respectively. This study shows the different chemical composition and antimicrobial activities of *M. citrata* extracts obtained by using different extraction solvents.

**Keywords:** *Mentha citrata*, antimicrobial activity, phytochemicals, methanol extract

### **MENTHA PIPERITA VAR. CITRATA'NIN FARKLI SOLVENTLER KULLANILARAK HAZIRLANAN EKSTRAKTLARININ KİMYASAL KOMPOZİSYONLARI VE ANTİMİKROBİYAL AKTİVİTELERİ**

### ÖZ

Bu çalışma farklı çözücüler kullanılarak *Mentha piperita* var. *citrata* bitkisinden elde edilen ekstraktların fitokimyasal kompozisyonunun belirlenmesi, Gram pozitif ve Gram negatif bakterilere karşı antimikrobiyal etkinliğinin belirlenmesini amaçlamaktadır. Hekzan, kloroform, ve %70'lik metanol ile hazırlanan ekstraktların antimikrobiyal aktiviteleri sıvı mikrodilüsyon metodu kullanılarak test edilmiştir. Rosmarinik asit (10.505 mg/g ekstrakt), sinarosid (8.545 mg/g ekstrakt) ve kosmosiin (8.489 mg/g ekstrakt) metanolik ekstraktta baskın olarak bulunan fitokimyasallar olurken, akasetin

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(8.438 mg/g ekstrakt) kloroform ekstrakta en fazla bulunan fitokimyasadır. *M. citrata* ekstraktları Gram pozitif bakterilere karşı farklı konsantrasyonlarda antimikrobiyal aktivite göstermişlerdir. *M. citrata* bitkisinden elde edilen kloroform ekstraktının antimikrobiyal aktivite aralığı 512-4096 µg/mL iken, hekzan ve metanolik ekstraktlarının aktivitelelerinin sırasıyla 1024-4096 µg/mL ve 2048-4096 µg/mL olduğu bulunmuştur. Bu çalışma farklı ekstraksiyon çözücüleri kullanıldığında *M. citrata* ekstraktlarının farklı kimyasal kompozisyona ve farklı antimikrobiyal aktiviteye sahip olduğunu göstermiştir.

**Anahtar kelimeler:** *Mentha citrata*, antimikrobiyal aktivite, fitokimyasallar, methanol ekstrakt

## INTRODUCTION

Antimicrobial resistance has been identified as one of the most serious public health threat worldwide (Tacconelli and Pezzani, 2019). Goff vd., (2017) recently estimated that 700.000 people die from infections globally each year and somberly warned that annual death toll will keep going over 10 million by 2050. Therefore, not surprisingly, it is argued that there is a great need to find effective alternatives to combat with this issue (Stanton, 2013). One of these alternatives is the use of the medicinal plants, which have been widely used and proven as promising alternatives as an ancient practice for thousands of years (Lillehoj vd., 2018; Sumner, 2000). Therefore, in recent years, extracts and essential oils originated from plant sources have gained more urgency for the search of the antimicrobial properties.

Among the potential medicinal plants of antimicrobial properties, the genus *Mentha* (Lamiaceae) is of particular interest due to its essential oil rich characteristics (İşcan vs., 2002). *Mentha piperita* var. *citrata* (Ehrh.) Briq. (*M. citrata*; Orange mint), known as Eau de Cologne mint or bergamot mint, has a citrus fruit scent. Unlike the commercial *Mentha* species such as peppermint, for which the main essential oil patterns are menthol and menthone (İşcan vd., 2002; Singh and Pandey 2018), the predominant essential oil patterns of *M. citrata* consist of mainly linalool and linalyl acetate (Hendawy vd., 2015; Murray and Lincoln 1970). Not only can *Mentha citrata* be used commercially for food, cosmetic and fragrance industries, it can also be used as tea for the treatment of fevers, headaches, digestive disorders and various minor ailments (Hendawy vd., 2015).

Although previous studies have described the phytochemical properties of other *Mentha piperita*

extracts and their antimicrobial and cytotoxic activities (Elansary vd., 2020; Pramila vd., 2012), no detailed investigation on the antimicrobial activity of *M. citrata* extracts has been presented so far. In view of all these, we aimed to establish the concentrations of phytochemicals and antimicrobial activity of *M. citrata* extracts obtained by different extraction solvents against Gram positive and Gram negative bacteria.

## MATERIAL AND METHODS

### Plant material and extract preparations

*M. citrata* leaves used in this study were harvested from the wild and species identification was carried out by Assoc. Prof. Dr. Yelda Güzel from Faculty of Arts and Sciences at Hatay Mustafa Kemal University. Before use, fresh leaf material was shade dried and sieved (using a sieve mill) to a particle size 3-5 mm.

Ground leaf material (2.5 g) was extracted with 50 mL of methanol (70% prepared with deionized water), hexane (100%) and chloroform (100%) in a glass beaker as described previously (Kemp and McSweeney, 2010). These mixtures were mixed at room temperature for 3 hours on a shaker machine. Extracts were filtered through paper filter (Wattman No: 2) and the procedure was repeated two times. All collected supernatants were combined and organic phases from the leaf extract was dried with a rotary evaporator under reduced pressure (Heidolph, Germany). Extracts were then freeze-dried (Telstar Lyoquest -85, Austria) and stored in amber glass bottles at 4°C in the dark until microbiological studies.

### Qualitative Analysis of Phytochemicals

The qualitative and quantitative analysis of phytochemicals in methanol and chloroform extracts was determined by ultrahigh performance liquid chromatography (UHPLC) according to

the method of as previously described by Yilmaz (2020). The method used herein was previously validated by Yilmaz (2020). The chromatographic system (Shimadzu-Nexera model UHPLC coupled with a tandem mass spectrometer, Japan) was composed of an auto-sampler (SIL-30AC model), a column oven (CTO-10ASvp model), binary pumps (LC-30AD model) and a degasser (DGU- 20A3R model). Briefly, chromatographic separation was conducted on a reversed phase Agilent Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) analytical column. The following MS operating conditions were used: drying gas (N<sub>2</sub>) flow, 15 L/min; nebulizing gas (N<sub>2</sub>) flow, 3 L/min; DL temperature, 250°C; heat block temperature, 400°C, and interface temperature, 350°C. The temperature of the column was maintained at 40°C. Eluent A (water+5 mM ammonium formate+0.1% formic acid) and eluent B (methanol+5 mM ammonium formate+0.1% formic acid) were used to separate the phytochemicals. The step gradient patterns were 20-100% B for 0-25 min, 100% B for 25-35 min and 20% B for 35-45 min. The solvent flow rate was set at 0.5 mL/min and injection volume was 5 µL. LabSolutions software (Shimadzu,

Japan) was used for LC-ESI-MS/MS data acquisition and processing.

#### Antimicrobial activity

The antimicrobial activity of extracts against a panel of organisms given in Table 1 was tested by broth microdilution assay as described by Kemp and McSweeney, (2010). The extracts were dissolved in dimethyl sulfoxide (DMSO; a stock concentration of 41 mg/mL). Strains were cultured from the glycerol stocks in blood agar plates overnight growth at 37 °C. Bacterial suspensions (10 µL; 1x10<sup>6</sup> cfu/mL) were subsequently transferred to U bottom 96 well plates with increasing concentrations of extract (from 128 to 4096 µg/mL). Two controls, one with no bacteria (sterility control) and the other without addition of extract (bacterial growth control), were also included in each assay. DMSO concentration used in the test was 2.5% and this concentration did not show any antimicrobial activity for test organisms. After 24 h incubation, 30 µL of resazurin (2.2 mg/mL) was added to each well. The 96-well plate was incubated at 37°C for 30 min, then the color change was observed by visually (Sarker et al. 2007). The experiments were repeated three times.

Table 1. Test microorganisms

Microorganisms	Antimicrobial resistance profile
<i>Escherichia coli</i> ATCC 25922	None
<i>Escherichia coli</i> NCTC 13476	Carbapenemase producer (IMP type)
<i>Escherichia coli</i> NCTC 14476	Plasmid-mediated AmpC beta-lactamase and ESBL producer, also resistant to quinolones, trimethoprim
Gram negative <i>Escherichia coli</i> NCTC 14477	ESBL producer
<i>Klebsiella pneumoniae</i> NCTC 13443	Carbapenemase producer (NDM-1 type)
<i>Klebsiella pneumoniae</i> NCTC 13438	Carbapenemase producer (KPC-3 type)
<i>Klebsiella pneumoniae</i> NCTC 13440	Carbapenemase producer (VIM type)
<i>Salmonella</i> Typhimurium ATCC 14028	None
<i>Staphylococcus aureus</i> ATCC 25923	None
<i>Staphylococcus aureus</i> NCTC 13552	Methicillin resistant (MRSA)
<i>Staphylococcus aureus</i> ATCC 29213	None
Gram positive <i>Enterococcus casseliflavus</i> ATCC 700327	None
<i>Enterococcus faecium</i> NCTC 10202	None
<i>Enterococcus faecium</i> (RSKK 623)	None
<i>Enterococcus faecalis</i> ATCC 51299	Vancomycin resistant
<i>Bacillus cereus</i> ATCC 13061	None

**RESULTS AND DISCUSSION**

All the phytochemical components of the extracts of *M. citrata* differed significantly between the two organic solvents, methanol and chloroform, as presented in Table 2 and Figure 1. 26 and 17 different phytochemicals (out of 56 tested) were determined in *M. citrata* methanolic and chloroform leaf extracts, respectively (Table 2). Among the different phytochemicals in methanol extracts, the highest concentration was found to be rosmarinic acid (10.505 mg/g extract), that was followed by cynaroside (8.545 mg/g extract) and

cosmosiin (8.489 mg/g extract). Similar observations were reported for other *Mentha* species such as *M. piperita* and *M. longifolia* by Elansary vd., (2020), who reported that the main phytochemical found was rosmarinic acid. The other phytochemicals were also found in high concentrations, hesperidin (2.878 mg/g extract), quinic acid (2.243 mg/g extract), acacetin (1.571 mg/g extract), tannic acid (0.877 mg/g extract), caffeic acid (0.625 mg/g extract) and luteolin (0.555 mg/g extract).

Table 2. Phytochemical composition (mg analyte/g extract) of extracts of *M. citrata* leaf

Analyte	Methanol	Chloroform
Vanillin	0.203	0.259
Coumarin	0.004	0.026
Hesperidin	2.878	0.320
Quinic acid	2.243	N.D.
Fumaric acid	0.204	0.122
Gallic acid	0.051	N.D.
Protocatechuic acid	0.363	0.102
Gentisic acid	0.042	N.D.
Protocatechuic aldehyde	0.048	0.285
Chlorogenic acid	0.125	N.D.
Tannic acid	0.877	N.D.
Vanilic acid	N.D.	0.256
Caffeic acid	0.625	0.079
Syringic aldehyde	N.D.	0.018
p-Coumaric acid	0.194	0.317
Salicylic acid	0.099	0.102
Cynaroside	8.545	N.D.
Miquelianin	0.101	N.D.
isoquercitrin	0.134	N.D.
Rosmarinic acid	10.505	N.D.
Cosmosiin	8.489	0.634
Astragalin	0.151	N.D.
Luteolin	0.555	0.3
Hesperetin	0.136	0.673
Naringenin	0.161	1.013
Kaempferol	0.009	N.D.
Apigenin	0.387	0.628
Acacetin	1.571	8.438

N.D.: Not detected

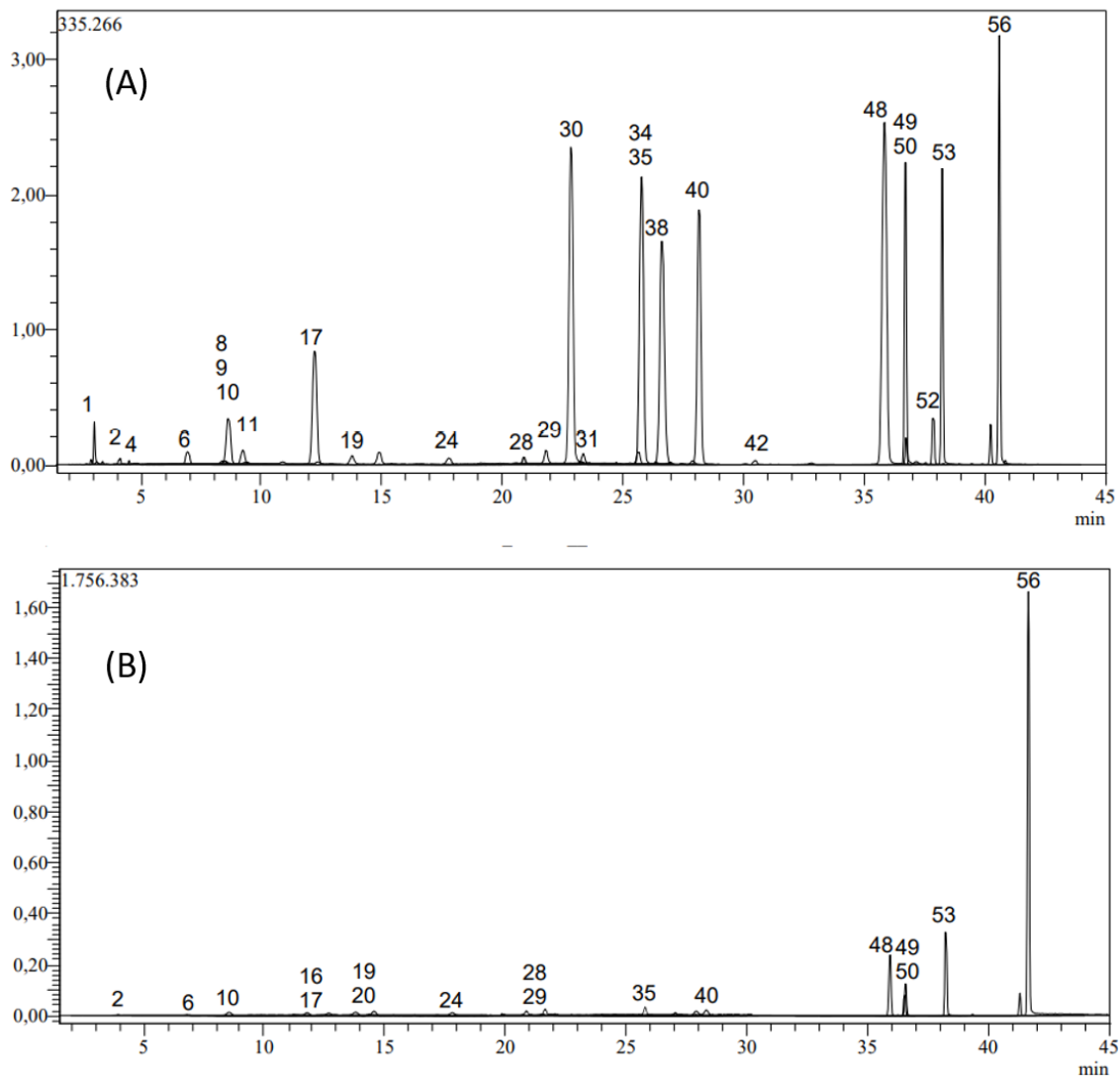


Figure 1. Chromatograms of methanol (A) and chloroform (B) extracts. Peaks: (1: Quinic acid, 2: Fumaric acid, 4: Gallic acid, 6: Protocatechuic acid, 8: Gentisic acid, 9: Chlorogenic acid, 10: Protocatechuic aldehyde, 11: Tannic acid, 16: Vanilic acid, 17: Caffeic acid, 19: Vanillin, 20: Syringic aldehyde, 24: *p*-Coumaric acid, 28: Coumarin, 29: Salicylic acid, 30: Cynaroside, 31: Miquelianin, 34: isoquercitrin, 35: Hesperidin, 38: Rosmarinic acid, 40: Cosmosiin, 42: Astragalin, 48: Naringenin, 49: Hesperetin, 50: Luteolin, 52: Kaempferol, 53: Apigenin, 56: Acacetin)

On the other hand, acacetin was the most abundant (8.438 mg/ g extract) phytochemical in chloroform extract of this plant. The following phytochemicals were also identified; naringenin (1.013 mg/ g extract), hesperetin (0.673 mg/ g extract), cosmosiin (0.634 mg/ g extract), apigenin (0.628 mg/ g extract), hesperidin (0.320 mg/ g extract), *p*-Coumaric acid (0.317 mg/ g extract) and vanillin (0.259 mg/ g extract) in

chloroform extract. It is well known fact that the use of different organic solvents (polar and nonpolar) affected the chemical composition in extracts (Ghosh vd., 2012; Khanam vd., 2015). Thus, the different recoveries of phytochemicals observed in the present study can be explained by the polar characteristics of the solvents and also suggests the importance of using different types of extraction solvent.

As seen from analysis of the MIC results, the growth of Gram-positive bacteria was influenced by all type of extracts (Table 3). Regardless of the species, all *Enterococcus* spp., including vancomycin resistant, exhibited high susceptibility to chloroform extract of *M. citrata*, with a MIC of 512-1024 µg/mL. In contrast, the MICs of methanol (2048->4096 µg/mL) and hexane (1024-4096 µg/mL) extracts for the strains of *Enterococcus* were higher. The MIC of chloroform extract for *B. cereus* was found to be 1024 µg/mL, whereas it was determined to be 2048 µg/mL for methanol and hexane extracts. The respective MICs for the *S. aureus* strains ranged from 1024 to 4096 µg/mL for all extracts. Several studies have confirmed that antimicrobial activity has influenced by the organic solvent used for extraction and these studies shown that the antimicrobial activity of chloroform extracts was superior to aqueous, ethanol and hexane extracts of different plants (Hassan vd., 2011; Yilmaz vd.,

2004), even though there are conflicting data revealing the different antimicrobial activity of polar and non-polar solvent extracts (Gul and Bakht, 2015; Khanam vd., 2015). On the other hand, Gram negative bacteria tested in the current study showed resistant to all extract types at the highest concentration level of 4096 µg/mL. The results of the current study agree with Kurekci vd., (2012) who also reported that Gram negative bacteria such as *E. coli* and *S. Typhimurium* are less susceptible to antimicrobial activity of ethanolic extracts of Australian medicinal plants when compared to Gram positive bacteria like *B. cereus* and *E. faecalis*. In addition, İşcan vd., (2002) reported that the essential oil of *M. piperita* exhibited weaker activity against Gram negative bacteria than Gram positive ones. These differences in sensitivity to the extract was previously attributed to the structural and compositional differences in membranes between the two groups (Friedman vd., 2002).

Table 3. The minimum inhibitory concentration (µg/mL) of extracts of *M. citrata* against microorganism

Microorganisms	MIC (µg/mL)		
	Hexane	Methanol	Cloroform
<i>S. aureus</i> ATCC 25923	2048	4096	4096
<i>S. aureus</i> NCTC 13552	4096	4096	4096
<i>S. aureus</i> ATCC 29213	4096	4096	4096
<i>E. casseliflavus</i> ATCC 700327	4096	4096	1024
<i>E. faecium</i> NCTC 10202	4096	NA	512
<i>E. faecium</i> (RSKK)	2048	2048	1024
<i>E. faecalis</i> ATCC 51299	1024	2048	512
<i>B. cereus</i> ATCC 13061	2048	2048	1024

NA: No activity at the highest concentration (4096 µg/mL), The results for Gram negative organisms are not included in the table as there has been no activity obtained at the highest concentration at all.

Extracts and essential oils of *Mentha* species including *M. piperita* and *M. longifolia* have been known to exert antimicrobial and antioxidant activity against a variety of organisms (Elansary vd., 2020; Gholamipourfard vd., 2021; İşcan vd., 2002). The results of the current study are higher than extracts of *M. piperita* and *M. longifolia* (Elansary vd., 2020). It is well-known fact that biological activities of plants are greatly influenced

by external factors such as extraction types and products including whole plant, extracts, essential oils. Additionally, chemical composition alone has a strong impact on the biological activities of plant extracts. For example, rosmarinic acid, acacetin and cosmosiin, highly detected compounds in the current study, have shown to had antioxidant, anticancer and anti-inflammatory properties (Al-Dhabi vd., 2014; Patel, 2021; Singh vd., 2020). A

recent study conducted by Elansary vd., (2020) reported the antiproliferative and antibacterial activities of rosmarinic acid by using *in vitro* studies. Since previous studies demonstrated the effectiveness of *Mentha* leaves to enhance the shelf life of food products due to the reduced microbial growth (Gholamipourfard vd., 2021), *M. citrata* extracts might be beneficial as food preservation agent due to their antimicrobial activity towards Gram positive organisms, though antioxidant activity was not investigated.

## CONCLUSION

Our findings suggest that the composition and phytochemical profile of *M. citrata* are somewhat influenced by the polarity of extraction solvent. In addition, the different solvent extracts of *M. citrata* have antibacterial properties against Gram positive bacteria including strains with antibiotic resistance, but also serve polyphenolic chemicals such as rosmarinic acid and hesperidin highlighting the potential of this plant to be used for food preservation.

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## CONFLICTING INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Cemil Kürekci designed research, performed antimicrobial activity and wrote the paper; Neslihan Beyazid extracted plant, analyzed HPLC data.

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