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# Chemical content and antimicrobial properties of three different extracts of *Mentha pulegium* leaves from Mugla Region, Turkey

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**Abstract**

The extract of ethanol, methanol and DMSO of pennyroyal leaves from Mugla Region (Turkey) were tested for antimicrobial activity against eleven bacterial and one yeast strain by disc diffusion method. Among the extracts assayed, the methanol extract of pennyroyal leaves exhibited signifiant antimicrobial activity against *Salmonella typhimurium* and *Staphylococcus aureus* with 20 mm diameter inhibition zone. The DMSO extract of pennyroyal leaves displayed significant activity against *S.aureus* (19 mm) and *Bacillus subtilis* (21mm) showing inhibition zone of 19 mm and 21 mm diameter, while the ethanolic extract showed significant antimicrobial activity against *S.aureus* (17mm) and *Klebsiella pneumoniae* (20mm) inhibition zones. On comparint the MIC value of ethanol, methanol and DMSO leaf extract, methanolic extract of pennyroyal presented best activity (MIC 8 mg $ml^{-1}$) against *S.typhimurium* CCM 583 and *S.aureus* ATCC 6538/P. Analyses of GC/MS determined eleven compounds viz., neophytadiene (69.95%), Pulejon 7.85%, Pinane 4.81%, Bicyclo (3.1.1 Heptane 2.6.6.6 trimethyl) 4.68%. In conclusion, methanolic extracts of *M.pulegium* showed antimicrobial activity because of high neophytadiene content.

**Key words**

Antimicrobial activity, Inhibition zone, *Mentha pulegium*

## Introduction

Disease causing bacterias' have always been considered as a major cause of morbidity and mortality in humans. Occurrence of resistant microorganisms have paved a way for the infections that are only treated by a limited number of antimicrobial agents. The emergence of resistant Gram negative bacteria presents a major chalenge for the antimicrobial theraphy of infectious diseases and increases the incidence of mortality and morbidity. Bacterial resistance to antimicrobial agents is a medical problem with public health and socio-economic implications (Sharma *et al*., 2005). In search of new antibiotics, several studies have investigated the antimicrobial activity of different plant species in different geographical regions. (Abdul Sattar *et al.,* 2012 ). With increase in microbial resistance and limited shelf life of antibiotics, new sources especially plant sources are currently being investigated. Thousands of phytochemicals, especially, thymol, carvacrol, α-terpineol, terpinen-4-ol, eugenol, (±)-linalool, (–)-thujone, δ-3-carene, *cis*-hex-3-an-1-ol, geranyl acetate, (*cis*+□*trans*) citral, nerol, geraniol, menthone, β-pinene, *R*(+)-limonene, α-pinene, α-terpinene, borneol, (+)-sabinene, γ-terpinene, citronellal ~ terpinolene, 1,8-cineole, bornyl acetate with antimicrobial activity have already been identified but they should be subjected to animal and human studies to study their to toxicity and their effectiveness in organisms systems (Dorman and Deans, 2000). Several phytochemicals like carbohydrate, saponins, tannins, steroids, flavonoids and phlobatannin have already been tested in humans (Prohp an Onoagbe, 2012 ).

*Mentha pulegium* commonly known as pennyroyal belongs to family Lamiaceae native to Europe, North

America and the Middle East. The flowering aerial parts of *M. pulegium* have been used traditionally for treatment of cold, sinusitis, chlorera, food posioning, bronchitis and tuberculosis(Mahboubi and Haghi., 2008). The antifungal and antibacterial activities exhibited by *M. pulegium* essential oils have been demostrated previously by researchers (Sivropoulou *et al*., 1995; Bouchra *et al*., 2003; Marzouk *et al*., 2008; Boukhebti *et al*., 2011; Ait-Ouazzou *et al*., 2012; Teixeria *et al*., 2012; Ghazghazi *et al*., 2013) on these microorganisms (*E. coli* ATCC 8739, *S. typhimurium* NCTC 6017, *S. aureus* ATCC29213, *P. aeruginosa* ATCC 27853, *Aeromonas hydrophila*, *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC1247, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Staphylococcus epidermidis, Streptococcus pyogenes, Klebsiella pneumoniae, Salmonella typhi, Micrococcus luteus, Enterococcus faecalis* ATCC29212, *Micrococcus luteus* NCIMB 8166, *Citrobacter freundii*, *Enterobacter cloaceae*, *Salmonella enteritidis*, *Serratia marcescens, Shigella flexneri, Vibrio cholerae, Enterococcus faecium*.

In light of the above, the present study was carried out to investigate the antimicrobial activities of three different solvent extracts of *M. pulegium leaves* against bacterial and fungal strain.

## Materials and Methods

**Samples and storage :** Samples of *M. pulegium* was obtained from the market stall in Mugla, Turkey in 2013. Taxonomic identification of plant material was confirmed from the Department of Biology, Ege University, Turkey. The leaves were cut into small pieces and stored at 4°C for further analysis.

**Solvent extraction of *Mentha pulegium* leaves :** Solvent extraction of *Mentha pulegium* leaves was carried out using the standard methods developed by Yasmin *et al.* (2009) and Hussain *et al.* (2011). A 25 g of each dried powdered plant material was soaked separately in 250 ml DMSO (10%), ethanol and methanol. Extraction was carried out by maceration for 7 days in each solvent at room temperature (25±2°C). The solvents extracted material was filtered in separate flaks and then dried in a vacuum rotary evaporator, weighed and stored at 4°C until further analysis. Dried DMSO, ethanol and methanol extracts were then dissolved in their respective solvents in a proportion of 100 mg $ml^{-1}$.

**Test microorganisms :** The bacterial and fungal strains used in the study were as follows: Gram-negative bacteria; *Escherichia coli* ATCC 35218; *Pseudomonas aeruginosa* ATCC 27853; *Salmonella typhimurium* CCM 583; *Aeromonas hydrophila* ATCC 19570; *Klebsiella pneumoniae* CCM 2318. Gram-positive bacteria, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Staphylococcus aureus* ATCC 6538/P, *Streptococcus faecalis* ATCC 8043 and fungal strain *Candida albicans* ATCC 10239.

For preparation of stock cultures, each bacterial strain was inoculated in Nutrient Agar (Merck) and incubated for 24 hrs at 37°C, while *C. albicans* was inoculated in Potato-Dextrose Agar (Merck) and incubated for 24 hrs at 25°C, respectively.

**Determination of antimicrobial activity with disc diffusion method :** Paper disc diffusion method was used to determine the antimicrobial activity, following the method of National Committee for Clinical Laboratories Standards (2006). Sterile paper discs (6 mm; Oxoid) were loaded with 50 µl of different amount (0.25, 0.5 and 1 mg) of the extracts dissolved in 10 % DMSO (Lab-Scan) and were left to dry for 12 hrs at 37 °C in a sterile room. Microbial suspensions were diluted to match the 0.5 MacFarland standard scale (approximately $1.5 \times 10^8$ CFU $ml^{-1}$) and they were further diluted to obtain a final inoculum. After Mueller-Hinton agar (Merck) was poured into Petri dishes to give a solid plate and inoculated with 100 µl of suspension containing $1 \times 10^8$ CFU $ml^{-1}$ of microorganisms, the discs treated with extracts were applied to petri dishes. Tobramycin (10 µg $disc^{-1}$) (Oxoid), ampicillin (10 µg $disc^{-1}$) (Oxoid) and nystatin (30 µg $disc^{-1}$) (Oxoid) were used as positive control and paper discs treated with ethyl acetate, methanol and DMSO were used as negative control. The plates were then incubated at 35 °C for 24 hrs in an incubator. Diameter of inhibition zone around each disc were measured and recorded at the end of incubation time.

**Assay of susceptibility of broth microdilution :** MICs were determined by agar dilution method following the protocol of Clinical and Laboratory Standards Institute Standards (2006). The MICs of erithromycin (Oxoid) and chloramfenicol (Oxoid) were also determined. A final inoculum of $1 \times 10^4$ CFU $ml^{-1}$ was spotted onto agar plates. The plates were then incubated at 35°C for 24 hrs in an incubator. MIC was defined as the lowest concentration of extracts at which no visible growth was observed. The minimum concentration of extracts that inhibited 90% of the isolates tested was defined as $MIC_{90}$.

**GC/MS analysis** : The steam-distilled components were analysed by GC/MS. A HP 6890 gas chromatograph equipped with a HP-PTV and a 0.32mX0.60m HP-Innowax capillary column (0.5µm coating) was employed for GC analysis. GC/MS analysis was performed on a HP-5973 mass selective detector coupled with a 6890 gas chromatograph, equipped with a HP 6890 gas chromatograph, equipped with HP-1capillary column. The column temperature was

programmed from an initial temperature of 60 °C to a final temperature of 250 °C at 15 °C $min^{-1}$. Helium was used as a carrier gas (14.1ml $min^{-1}$). Identification of individual components was performed by comparing with mass spectra given in literature and by comparing their retention time (Rt) relative to $C_8$-$C_{32}$ *n*-alkanes mixture (Adams, 1995). A computerized search was carried out by Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples.

**Stastical analyses :** All the experiments were performed at least three times (n = 3). An average of three replicates and standard deviation from mean value were determined. Statistical analysis with *p* value was determined for the percentage calculation of inhibition zone diameters. Data were analyzed using the *t*-test seeking at least 95 % confidence.

## Results and Discussion

Among the extracts assayed, methanol extract of pennyroyal leaves exhibited significant antimicrobial activity against *S.typhimurium* and *S. aureus* with 20 mm diameter of inhibition zone (Table 1). A 10% DMSO extract of pennyroyal leaves displayed significant activity against *S. aureus* and *B. subtilis* with 19 mm and 21 mm diameter of inhibition zone, whereas ethanolic extract showed significant activity against *S. aureus* and *K. pneumoniae* with 17 mm and 20 mm diameter of inhibition zone, respectively. In Table 1, inhibition zones obtained from disc diffusion method were given as averages of three replicates. Standard deviations from mean values were calculated to range from 0.1 to 1.1 (These results were not given).

On comparing the antimicrobial activity of *M. pulegium* extracts with tobramycin and ampicillin, it was found that all the extracts showed higher antimicrobial activity than tobramycin and ampicillin, however, methanol, ethanol and DMSO extracts of pennyroyal leaves showed smaller than nystatin.

Mahboubi and Haghi (2008) reported that the volatile oil of Iranian *M. pulegium* showed potential antimicrobial activity especially against Gram positive bacteria *Listeria monocytogenes* with inhibition zone ranging between 8-21 mm, whereas least susceptible bacteria was *E. coli and S. Typhimurium.*

Hajlaoui *et al*. (2009) and Gulluce *et al*. (2007). reported that the methanol extract of aerial parts of *M. longifolia* and *M. pulegium* plants showed no antimicrobial activities However, in the present study essential oil of *M. pulegium* leaves produced strong antibacterial and antifungal activities due to the presenceof high content of menthone (41%), pulegone (31%) and isomethone (15%). These substances have earlier been reported as antibacterial and antifungal agents (Mimica-Dukic *et al*. 2003; Sahin *et al*. 2003; Gulluce *et al*.2007; Hajlaoui *et al*., 2008, 2009,2010).

Hajlaoui *et al*. (2009) evaluated the antimicrobial activity of *M. pulegium* essential oil from Mehdia. They found that this oil had great antimicrobial potential against all the ten bacterial species tested. The diameter of inhibition zone for bacterial strains, which were sensitive to *M. pulegium* oil ranged between 8–21 mm. Marzouk *et al*. (2008) demonstrated that *M. pulegium* oil was active against all the tested strains. The diameter of inhibition zone for most sensitive bacterial strains ranged between 10–20 mm. In addition, *L. monocytogenes* was the most sensitive strain. Similarly, Ait-Ouazzou *et al*. (2012) indicated that *L. monocytogenes*, *S. aureus* and *E. coli* were the most sensitive strains tested against essential oil of *M. pulegium* with

**Table 1 :** Antimicrobial activity of *Mentha pulegium* extracts against test microorganisms by disc diffusion method

| Microorganism | Methanol extract (mm)* | DMSO extract (mm)* | Ethanol extract (mm)* | Control (mm)* | | |
|---|---|---|---|---|---|---|
| | | | | Tobramycin (10 µg $disc^{-1}$) | Ampicillin (10 µg $disc^{-1}$) | Nystatin (30 µg $disc^{-1}$) |
| *S. faecalis* ATCC 8043 | 15±0.30 | 13±0.07 | 12±0.10 | 9±0.02 | 12±0.22 | NT |
| *S. typhimurium* CCM 583 | 20±0.15 | 14±0.15 | 16±0.14 | 10±0.02 | 12±0.18 | NT |
| *E. coli* ATCC 35218 | 13±0.09 | 13±0.20 | 11±0.08 | 11±0.15 | 9±0.06 | NT |
| *P. aeroginosa* ATCC 27853 | 16±0.11 | 16±0.13 | 11±0.30 | 10±0.06 | 10±0.09 | NT |
| *A. hydrophila* ATCC 19570 | 13±0.02 | 16±0.09 | 13±0.21 | 15±0.15 | 10±0.20 | NT |
| *S. epidermidis* ATCC 12228 | 16±0.02 | 8±0.10 | 15±0.15 | 16±0.20 | 12±0.15 | NT |
| *S.aureus* ATCC 6538/P | 20±0.10 | 19±0.22 | 17±0.17 | 10±0.11 | 12±0.32 | NT |
| *K. pneumoniae* CCM 2318 | 18±0.21 | 13±0.09 | 20±0.10 | 9±0.09 | 14±0.12 | NT |
| *B. cereus* CCM 99 | 13±0.25 | 11±0.11 | 10±0.09 | 11±0.08 | 10±0.8 | NT |
| *B.subtilis* ATCC 6633 | 16±0.14 | 21±0.10 | 12±0.04 | 11±0.05 | 9±0.12 | NT |
| *C. albicans* ATCC 10239 | 14±0.08 | 14±0.08 | 15+0.02 | 13+0.17 | 15+0.05 | 16+0.07 |

*Each inhibition zone value is an average of three replicates. ($p < 0.05$ for antimicrobial activity)

**Table 2 :** Minimum inhibitory concentration (mg ml$^{-1}$) of methanolic extract of *M. pulegium* and antibiotics against test microorganisms

| Microorganisms | Minimum inhibitory concentration | | | |
|---|---|---|---|---|
| | | Antibiotics | | |
| | Methanol | Eritromycin | Chloramfenicol | Nystatin |
| *S. faecalis* ATCC 8043 | 32 | 4 | 2 | - |
| *S. typhimurium* CCM 583 | 8 | 0,016 | 4 | - |
| *E. coli* ATCC 35218 | 64 | 2 | 0.008 | - |
| *P. aeruginosa* ATCC 27853 | 32 | 4 | 0.016 | - |
| *A. hydrophila* ATCC 19570 | 64 | 2 | 0.16 | - |
| *S. epidermidis* ATCC 12228 | 32 | 4 | 0.,16 | - |
| *S.aureus* ATCC 6538/P | 8 | 2 | 8 | - |
| *K. pneumoniae* CCM 2318 | 16 | 2 | 2 | - |
| *B. cereus* CCM 99 | 64 | 2 | 4 | - |
| *B.subtilis* ATCC 6633 | 32 | 32 | 0,008 | - |
| *C. albicans* ATCC 10239 | 64 | - | - | 16 |

**Table 3 :** Volatile components of methanolic extract of *M. pulegium*

| Component | Percent value | Rt[b] |
|---|---|---|
| n-Decane | 3.58 | 5.13 |
| N-Undecane | 0.95 | 6.48 |
| Benzene-1 ethyl- 3 methyl | 1.07 | 9.14 |
| Benzene 1 ethyl 2 methyl (CAS) | 0.89 | 10.06 |
| Benzen 1,2,4 Trimethyll ( CAS) | 1.61 | 10.60 |
| Isomenthone | 1.26 | 16.90 |
| Pinane | 4.81 | 20.34 |
| Pulegon | 7.85 | 21.60 |
| Bicyclo (3.1.1 Heptane, 2.6.6.6 trimethyl) | 4.68 | 21.82 |
| Neophytadiene | 69.95 | 23.06 |
| Piperitenone | 2.19 | 29.53 |
| Undefined | 1.13 | |

[a]Components listed in order of elution from a HP-1 capillary column; [b]Retention time (as min)

diameter of inhibition zone ranging from 12.6 mm (*E. coli*) to 35 mm (*L. monocytogenes*), however this essential oil did not show antibacterial activity against *P. aeruginosa*.

On comparing the results of MIC, it was found that the MIC value of eritromycin and chloramfenicol was more effective. Similarly, the MIC value of nystatin was less than extracts of methanol (Table 2).

Motamedi *et al.* (2014) reported that both ethanolic and methanolic extracts of *M. pulegium* showed significant antibacterial activity against Gram positive, *Bacillus cereus* and *Staphylococcus aureus,* and also Gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis*. Maximum effect was observed at all the concentrations of both ethanolic and methanolic extracts on *P. mirabilis* (25 mm), while lowest effect was found on *P. aeruginosa*. Minimum inhibitory concentration and maximum bactericidal concentration for both extracts against *S. aureus* was equal (MIC=MBC=8 mg ml$^{-1}$) and *P. mirabilis* were MIC=4 mg ml$^{-1}$ and MBC=8 mg ml$^{-1}$, respectively. Scanning electron microscopy showed deformation and cell wall disruption in affected bacteria.

GC-MS analysis revealed that the main constituents of different leaf extracts of *M. pulegium* were neophytadiene (69.95%), pulegon (7.85%), pinane (4.81%) and bicyclo 3.1.1 Heptane 2.6.6.6 trimethyl (4.68%) (Table 3).

Mahboubi and Haghi (2008) reported that piperitone (38.0%), piperitenone (33.0%), terpineol (4.7%) and pulegone (2.3%) are one of the main components of essential oil derived from plants *M. pulegium* of South Iran showed antimicrobial activity against *Staphylococcus aureus*. Cárdenas-Ortega *et al*. (2005) reported that piperitone completely inhibited *Aspergillus flavus* at low concentrations. Maggiore *et al*. (2012) demonstrated that the essential oils of *Mentha* spp., especially *M. pulegium* could

be a promising source of potential protoscolicidal agents. Lorenzo *et al*. (2002) reported that pulegeno, isomenthone and menthone are the major components in theoils of *M. pulegium*. Variation in the chemical composition of essential oil of mentha species from different geographical regions have been reported by several authors. These variations in chemical composition might be due to varied climatical, seasonal and geographical conditions of the regions, isolation regimes and metabolism of plants. Sokovic and Griensven (2006) reported carvone, menthone , limonene and 1,8-cineole as major constituents in the essential oil of M. *spicata* from Montenegro. Singh *et al*. (2005) from India reported high menthol content in essential oil of *M. arvensis*. Boukhebti *et al.* (2011) identified pulegone major chemical constituent and menthone, piperitenone, piperitone and isomenthone, limonene, octaan-3-ol in appreciable amount in essential oil of *M. pulegium.*

Similarly, several researches have reported neophytadien, pulegon, pinane, Bicylo (3.1.1 Heptane 2.6.6.6 trimethyl (4.68%) from essential oil of *Calamintha nepeta*, *Micromeria cilicica*, *Cyclotrichium niveum, Campomanesia adamantium* and *Artemisiats chernieviana* as well known antimicrobial compounds isolated from different plant species, (Flamini *et al*., 1999; Duru *et al*., 2004; Alim *et al*., 2009; Countinho *et al*., 2009; Kazemi *et al*., 2009).

In conclusion, the antibacterial and antifungal activities of *M. pulegium* leaves extracts obtained from plants growing in Mugla Region (Turkey) were more effective than traditional antibiotics to combat the microorganisms studied. The chemical composition of *M. pulegium* leaves the contained four compounds major phytochemicals, neophytadien, pulegon, pinane, Bicylo (3.1.1 Heptane 2.6.6.6 trimethyl). It is known that this four compunds have antimicrobial activity.

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