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The effects of solvent type and extraction method on phenolics content and antibacterial and antioxidant properties of Pennyroyal (Mentha pulegium L.) extract

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ABSTRACT

Two of the major factors that should be considered in leaves extraction are the solvent type and extraction method. In the current study, the effects of two solvents (Ethanol and Methanol) and two extraction methods (soaking and soxhlet) on phenolics and flavonoid contents, antibacterial and antiradical properties of extracts were investigated in a factorial experiment based on completely randomized block design. The analysis variance of results showed that the extract had significant effects on phenolic compounds and flavonoids ($p \leq 0.05$). The highest content of phenolics and flovonoids observed in methanolic extract obtained by soxhlet method. In the same way, the effects of pennyroyal extract on free radical scavenging indicated that the highest antiradical properties of extracts recorded in methanolic extract by soxhlet method ($p \leq 0.05$). The MIC and MBC experiments on gram positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, indicated that all extracts showed antibacterial properties and the highest sensitivity observed in *Bacillus cereus*. Also, the means comparison of results showed that the highest antiradical and antibacterial properties were observed in methanolic extract obtained by soaking method. It can be concluded that methanol as a more polar solvent has better ability to extract phenolic compounds than ethanol. According to results, while the extraction by soxhlet apparatus showed higher efficiency than soaking, but the probable destruction of phenolics in soxhlet method caused lower antibacterial properties of obtained extracts in comparison with soaking method.

1. Introduction

Medicinal plants have been used for centuries as remedies for human diseases because they contain therapeutically valuable components (Nostro et al., 2000). Essential oils are used in traditional medicine for their antiseptic action, are constituted 1% of plant secondary metabolites and are mainly represented by terpenoids, phenypropanoids or benzenoids, fatty acid derivatives and amino-acid derivatives (Dudareva et al., 2006).

The genus *Mentha* includes 25-30 species that grow in the temperate regions of Eurasia, Australia and South Africa. The *Mentha* genus represents by about 6 species in the flora of Iran (Mozaffarian, 1996). Mentha is generally known under the name *''na'na''* and ''*pooneh''* in Iran

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and commonly used as herbal tea, flavoring agent, and medicinal plant (Nickavar et al., 2008). The mint species have a great medicinal and commercial importance. Indeed, vegetative parts of *Mentha* spp. are frequently used as additives in commercial spice mixtures for many foods to offer flavor. In addition, *Mentha* spp. has been used to providing medical care of nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints due to its anti-inflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, and anti-catarrhal properties (Hadjlaoui et al., 2009). *Mentha pulegium* L. is one of the *Mentha* species commonly known as pennyroyal. It is native species of Europe, North Africa and in Asia Minor and near East. In Iran pennyroyal grows naturally now in Golestan, Gilan and Baluchestan provinces (Zargari, 1997). The flowering aerial parts of *Mentha pulegium* L. has been traditionally used for treatment of cold, sinusitis, cholera, food poisoning, bronchitis and tuberculosis because of its antiseptic activity, and also used as anti-flatulent, carminative, expectorant, diuretic, antitussive, menstruate. Some pharmacological effect of *Mentha pulegium* L. essential oil such as abortifacient effect in rat, cytotoxic activity against different human cell lines and its antioxidant effect were confirmed. The ingredients of *Mentha pulegium* L. oil have been subjected to a number of studies which have shown a difference in its constituents depending on the region of cultivation and there have been some variations in the constituents from different countries.

Extraction is an important step involved in the discovery of bioactive components from plant materials. Currently, conventional extraction techniques such as soaking and Soxhlet extraction have been used along with several novel extraction techniques, such as ultrasonic extraction, accelerated solvent extraction and microwave-assisted extraction. Several studies have been published regarding the variation in the biological activities of extracts obtained from different extraction techniques, emphasizing the importance of selecting the suitable extraction method (Hayouni et al., 2007). The main objective of this study was to investigate the effects of two extraction methods on antioxidant and antibacterial properties of pennyroyal extract using ethanol and methanol as solvents in order to select more suitable solvent and extraction method to accede noted purpose.

2. Materials and Methods

2.1. Plant material

The leaves of *Mentha pulegium* at full flowering phase have been collected during September 2013 in the region of Papkiadeh around Langroud city from Mazandarn province of Iran (latitude: 37° 13' 59" longitude: 50° 11' 33"). The collected leaves were then dried in the open air. The leaves were then isolated from the other specimen and conserved for extraction.

2.2. Essential oil extraction

The essential oils were extracted by hydro-distillation using an apparatus of Clevenger type. The extraction took 3 hours for mixing 100g of plants in 1000 ml of distilled water. The obtained oil was collected and stored in screw capped glass vials at 4°C until analysis.

2.3. Gas chromatography-mass spectrometry (GC-MS) analysis

The plant oils were injected into the Gas chromatography-mass spectrometry (GC-MS) system to determine the type of their constituents. An Agilent model 6890 GC interfaced to a 5973 mass selective detector was used for mass spectral identification of the components of the oils. HP-5MS capillary columns (30 m × 0.25 mm × 0.25 µm film thickness) were used for GC. GC-MS was performed using the following conditions: carrier gas, He; flow rate, 0.8 $ml.min^{-1}$; injection volume, 1.0 µl; injection temperature, 300°C; oven temperature programmed from 50°C (5 min hold) to 240°C at $3°C.min^{-1}$ (3 min hold) and from 240°C to 300°C at $15°C.min^{-1}$(5 min hold). In mass spectrometry electron-impact ionization was performed at electron energy of 70 eV. Components of the oils were identified from the GC retention time and MS fragmentation patterns by comparison with those of the National Institute of Standards and Technology (NIST) database library and with those of authentic compounds.

2.4. Soxhlet extraction

Twenty grams of dried leaves were filled in two sets of Soxhlet apparatus containing 500ml methanol or ethanol as extraction solvents. The percolation was carried out for 6h and then the extracts were discharged and filtered. Afterward the solvents were individually discarded at 40°C under reduced pressure in rotary evaporator. Once concentrated to a small volume, each extract was allowed to dry completely in laboratory condition (25°C) overnight.

2.5. Soaking extraction

Twenty grams of dried leaves were soaked in 500 ml of methanol or ethanol as extraction solvents for 24h in covered beachers, the obtained extracts were filtered through Whatman num.1 filter paper. Afterward the solvents were individually discarded at 40°C under reduced pressure in rotary evaporator. Once concentrated to a small volume, each extract was allowed to dry completely in laboratory condition (25°C) overnight.

2.6. Total phenol content

Total content of the phenolic compounds in the methanolic and ethanolic extracts were determined by the modified Folin-Ciocalteau reagent according to Mandana et al., (2012), with slight modifications. One ml of extracts samples (5 mg of dried extract in acetone: water (60:40 v/v) was mixed with 0.2 ml Folin-Ciocalteau reagent (1:5 diluted with water) and after 15 min, 1 ml of 2% Na_2CO_3 was added to the mixture. As control, reagent without adding extract was used. After incubation of the samples at room temperature in a dark place for 30 min, their absorbances were measured at 765 nm. The calculation of phenolics content was done on the basis of the gallic acid standard curve which construct by using the same procedure and concentrations of 0, 50, 100, 150, 250 and 500mg.ml^{-1}. The results were expressed as gallic acid equivalents (mg GAE/g extract sample).

2.7. *Total flavonoid content*

Total flavonoid content determined using Bahorun et al., (1996) method. Briefly, the crude extract was diluted and 1.5 ml of 2% (w/v) Aluminium chloride ($AlCl_3$) was added to 1.5 ml of diluted crude extract or quercetin (positive control) and then mixed thoroughly. The mixture was allowed to stand for 15 min. Absorbance of the mixture was determined at 430 nm versus the prepared blank. Total flavonoid content was expressed as mg quercetin equivalent per gram extract (mg QE/g extract sample).

2.8. DPPH radical scavenging assays

All extract solutions prepared by dissolving 50mg of dried extract in 100 ml methanol. The obtained solution diluted to preparing 100, 150, 200, 250 mg.L^{-1} concentrations. DPPH radical scavenging activity of extracts was determined according to the method described by Brand-Williams et al., (1995). The inhibition percentage of the samples was calculated using the following formula: inhibition percentage of samples = $[(A_0\text{-}A_1/A_0) \times 100]$ where A_0 is the absorbance of the control and A_1 is the absorbance of samples.

2.9. Tests for antibacterial activity

To investigate the antibacterial activities of extracts, two gram positive bacteria including *Staphylococcus aureus* and *Bacillus cereus* and two gram negative bacteria including *Pseudomonas aeruginosa* and *Escherichia coli* were used. The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 cfu.ml^{-1}. The extracts to be investigated were dissolved in 10% dimethyl sulfoxide/Muller Hinton Broth to achieve the wanted concentrations (0.24–200 µg.ml^{-1}). The microplates were incubated for 24 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation 50 µl of the suspensions from the wells which did not show any growth after incubation during MIC assays to 150 µl of fresh broth further incubation for 48 h. The lowest concentration with no visible growth was defined as the MBC. Gentamicin was used as a positive control (Mativandlela et al., 2006; Kuete et al., 2008).

2.10. Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± SE. Analysis of variances was performed with two way ANOVA method. The significant differences between mean values were determined by Duncan's multiple range test to discriminate significance (defined as $p \leq 0.05$). The statistical analysis was carried out using statistical analysis system software (version 9.2, SAS institute); graphs drew by Excel 2007.

3. Results and Discussion

3.1. The essential oil composition

In the essential oil which obtained of *Mentha pulegium* leaves at full flowering stage, thirteen different compounds were identified, which made up 97.97% of the total essential oil (Table1).

The higher percentages are as follows: 2-hydroxy-2-methyl butric acid (80.71%), Benzenthiol (4.3%), 2-Chloro1-(2-hydroxy-4-methoxy phenyl)-ethanon (2.94%), Menthone (2.02%), Pulegone (1.8%), n-hexadecanoic acid (1.44%) and O-ethyl dipropyl phosphorodithioic acid (1.38%) Fig.1. According to previous studies the highest yield for essential oil extraction observed in full flowering phase (Nouri and Esmaeilian, 2012; Hmiri et al., 2011; Toncer and Kizil, 2005). The highest yield of essential oils in this stage may be due to their ecological role in attracting pollinators and in being as antifungal defense mechanism (Verma et al., 2010).

The chemical compositions analysis of essential oils by GC/MC revealed that unlike to many studies from Iran and other countries such as Sardashti and Adhami (2013) and Agnihotri et al., (2005) which reported that Menthone and Pulegone made the main constituents of essential oils of pennyroyal leaves, we observed that the named compounds were less than 2.5% of essential oils components. It has been reported that the native *M. pulegium* in Iran contains less than 0.1% pulegone (Motamedi et al., 2009). The possible reason can be due to this fact the growth region of the plants have direct effect on their composition and active constituents. The differences in essential oil composition and yield of *Mentha* species arose of variation in temperature, relative humidity, duration of sunshine, air movement and rainfall of regions that have been collected (Abdullah-Ijaz, 2009).

Table 1. Chemical composition of essential oil of M. pulegium L. (97.97%)

Compound	RT	Percentage	Compound	RT	Percentage
Silanediol	4.45	0.72%	2,4-bis(1,1-dimethyl ethyl phenol)	27.79	0.35%
Ethyl acetat	5.16	0.68%	Diphenyl disulfide	34.25	0.45%
2-hydroxy-2-methyl butric acid(alpha-hydroxy-isovaleric acid)	5.76	80.71%	Hexadecanoic methyl ester	35.26	0.55%
Benzenthiol	14.36	4.3%	n-hexadecanoic acid	35.81	1.44%
2-Chloro1-(2-hydroxy-4-methoxy phenyl) ethanon	19.23	2.94%	3-(4-methoxy-2-propenoic acid)	39.97	0.63%
Menthone	19.7	2.02%	O-ethyl dipropyl phosphorodithioic acid	45.29	1.38%
Pulegone	21.89	1.8%			

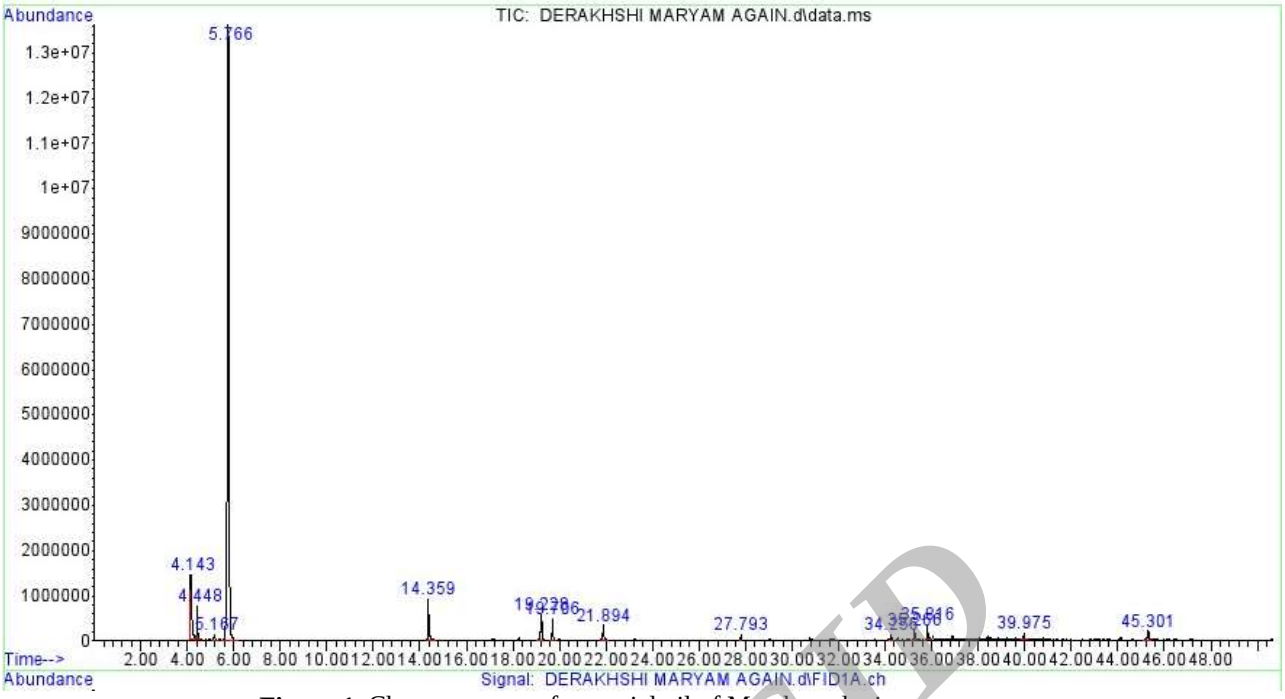

Figure 1. Chromatogram of essential oil of Mentha pulegium

3.2. The effect of extraction method on phenolics

The extraction method and solvent type effects on phenolics and flavonoids contents of pennyroyal extracts are shown in Fig 2 and Fig 3 respectively. Methanol has a polarity index of 5.1. Mostly methanol is used for extraction various polar compounds. Moreover methanol among all the alcohols has low boiling point of just 65 degree Celsius. So extraction and concentration of bioactive compounds is easy by using different extraction methods and rotary respectively. Methanol has been found to be more efficient in extraction of lower molecular weight phenolic compounds. This may be due to the fact that phenolics are often extracted in higher amounts in more polar solvents (Sultana et al., 2007; Guyot et al., 2001). According to results, the methanolic extract obtained by soaking method contained higher phenolics and flavonoids contents in comparison with others ($p \leq 0.05$).

The lower phenolics contents in extracts obtained by soxhelet method may be due to thermal decomposition of the target compounds which usually occurs at the boiling point of the solvent for a long time (Luque de Castro and Garcia-Ayuso, 1998). Our data are in accordance with those reported previously which has shown that methanol had better recoveries and is specifically effective in extracting polyphenols (Abaza et al., 2011). Also, Liu et al., (2007) found that phenolic and flavonoid contents of an endophytic Xylaria sp. were higher in methanol extracts than other extracts.

3.3. Free radicals scavenging activity

The free- radicals scavenging activity of *Mentha pulegium* extracts using the DPPH method is presented in Fig 4. Similar to phenolics and flavonoids contents of extracts, the highest antiradical properties of extracts recorded in methanolic extract obtained by soaking method. Antioxidant activities of extracts from aromatic plants are mainly attributed to the active compounds present in their extract. The phenolic compounds found naturally in Mentha were suggested to be the major contributors to the antioxidant activities of the plant extract. It has been reported that thermal processing conditions might result in the loss of natural antioxidants because heat may accelerate their oxidation and other degenerative reactions (Sultana et al., 2009). Thus, heating temperature may lead to lowered antioxidant activities of soxhelet obtained extracts. Similarly, Annegowda et al., (2011) reported

that soaking obtained extract showed higher antioxidant activity than soxhelet obtained extract.

3.4. Antibacterial activity

The antibacterial activities of pennyroyal extracts against four bacteria strains was determined by MIC and MBC method and shown in Table 2. As noted in previous section, the methanolic extract of pennyroyal which obtained with soaking method contained higher polyphenol and flavonoids than others. The antimicrobial power of plant phenolics is well known (Pereira et al., 2006). In this study, the main compounds of essential oil of pennyroyal was 2-hydroxy-2-methyl butyric acid (alpha-hydroxy-isovaleric acid), Benzenthiol, 2-Chloro1-(2-hydroxy-4-methoxy phenyl) ethanon, Menthone, Pulegone, n-hexadecanoic acid and O-ethyl dipropyl phosphorodithioic acid respectively (Table 1). It has been found that alpha-hydroxy acids such as alpha-hydroxy-isovaleric acid (2-hydroxy-2-methyl butyric acid) compounds are inhibitory to a large spectrum of micro-organisms (Hietala et al., 1979). Antibacterial activity of extracts can also be attributed to Menthone (Elansary and Ashmawy, 2013) and Pulegone (Sarrazin et al., 2015). In other studies, antibacterial properties of ocimene, α-pinene, 1,8 cineol have been previously reported (Glamoclija et al., 2001; Griffin et al., 2000; Pierangeli et al., 2009). According to results gram negative bacteria were less susceptible than gram positive ones. The outer membrane of gram-negative bacteria contains hydrophilic lipopolysaccharides (LPS), which create a barrier toward macromolecules and hydrophobic compounds, providing gram-negative bacteria with higher tolerance toward hydrophobic antimicrobial compounds like phenolic compounds. Our results are in good agreement with Motamedi et al., (2014) and Mahboubi and Haghi (2008) which reported that the main components of *M. pulegium* extracts and essential oil exhibited remarkable antibacterial activity against Gram positive bacteria than Gram negative ones.

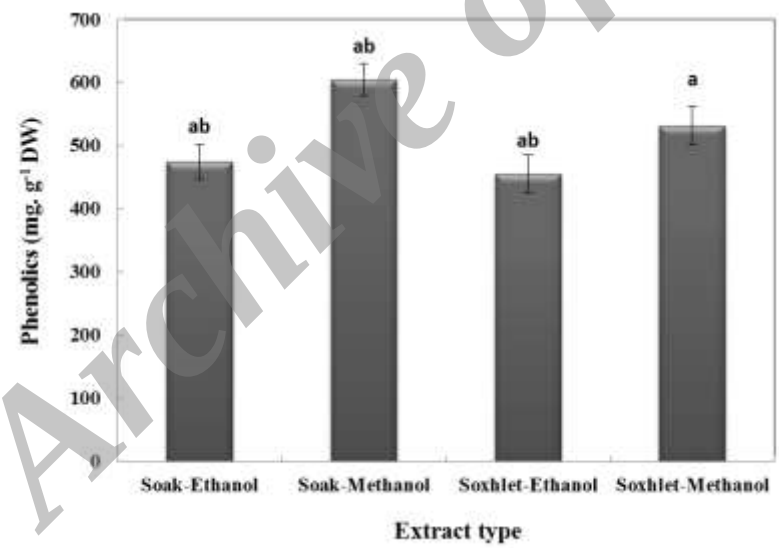

Figure 2. The effects of solvent type and extraction method on phenolic compounds content of pennyroyal extract. Values are expressed as means of three independent experiments ± standard error (SE). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p \leq 0.05$.

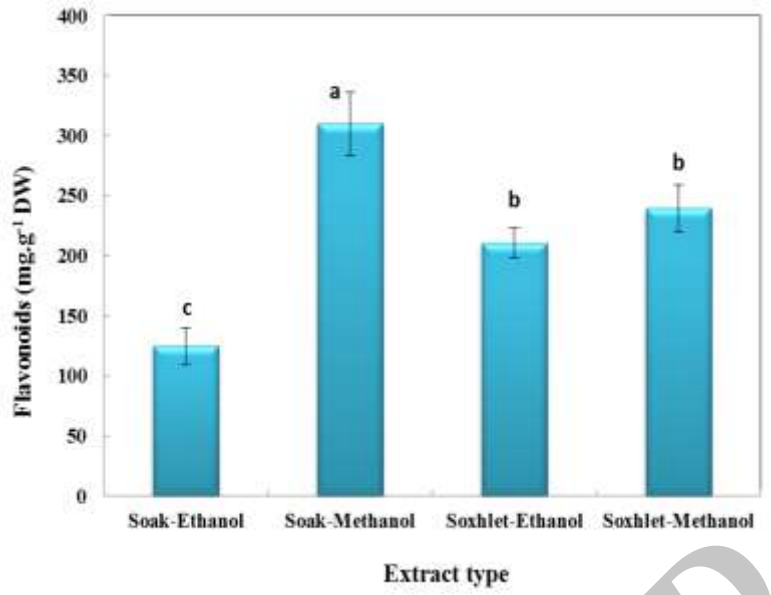

Figure 3. The effects of solvent type and extraction method on flavonoids content of pennyroyal extract. Values are expressed as means of three independent experiments ± standard error (SE). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p \leq 0.05$.

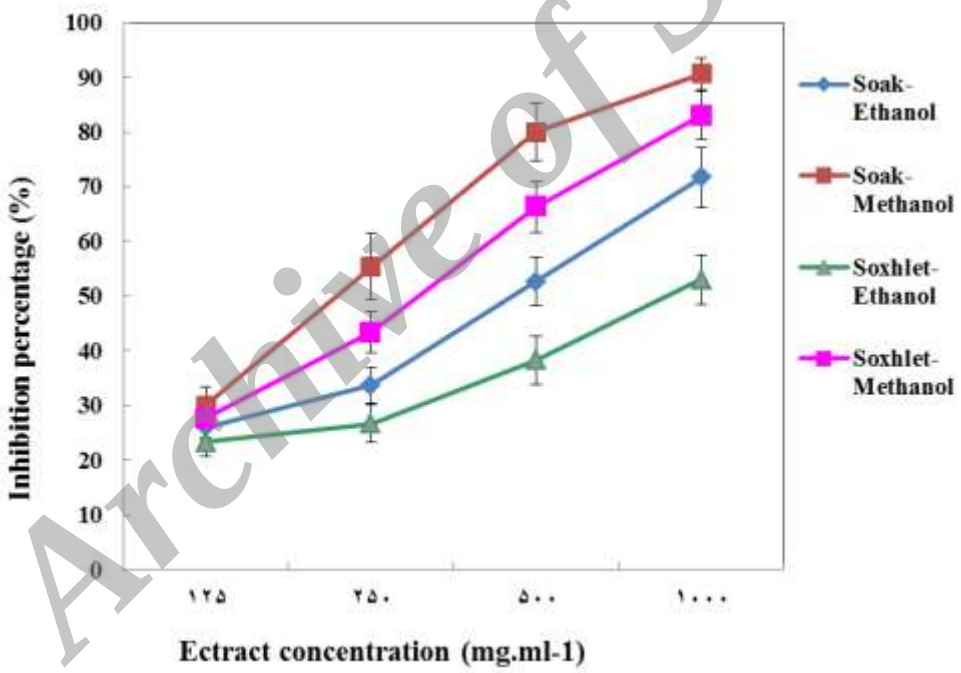

Figure 4. The effects of solvent type and extraction method on antibacterial properties of pennyroyal extract. Values are expressed as means of three independent experiments ± standard error (SE). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p \leq 0.05$.

Table 2. Antibacterial activity of different extracts (MIC and MBC – $\mu g.ml^{-1}$), microdilution method

Extraction method	Ethanol-Soxhlet		Ethanol-Soaking		Methanol-Soxhlet		Methanol-Soaking		Gentamicine	
Bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	62.5	125	62.5	125	62.5	125	15.62	31.25	3.906	7.81
Bacillus cereus	3.906	7.81	3.906	7.81	7.81	15.62	3.906	3.906	3.906	7.81
Escherichia coli	15.62	31.25	7.81	15.62	31.25	62.5	7.81	15.62	3.906	7.81
Pseudomonas aeruginosa	31.25	62.5	7.81	15.62	15.62	31.25	15.62	31.25	1.953	3.906

Conclusion

As conclusion remarks it can be concluded that more polar solvent (methanol) and soaking method solved phenolic compounds more efficiently than ethanol and soxhelet method. Because of higher phenolics in pennyroyal methanolic extract obtained with soaking method, this extract showed better antibacterial activity and antioxidant properties than other extracts.

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