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RESEARCH PAPER

Phytochemical Screening and Antibacterial Activity of the Vacuum Liquid Chromatography Fractions of *Mentha pulegium* Growing in Iraq

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Abstract

Mentha pulegium, a species of Lamiaceae family, is flowering plant native to Europe, North Africa and the Middle East. Crushed *M. pulegium* leaves exhibit a very strong fragrance like spearmint. The objective of the study was to investigate the phytochemical constituents and antibacterial activity of *M. pulegium* stems and leaves vacuum liquid chromatography (VLC) fractions. The powdered leaves and stems were macerated with absolute methanol then filtered using Buchner system. The filtrate was evaporated, then submitted to VLC using different solvents, and their fractions were collected. Phytochemical screening tests were done to investigate the chemical composition of methanol extract. The antibacterial effect of both methanol crude and the five extracts was studied on gram positive (*S. aureus and S. haemolyticus*) and gram negative (*P. aeruginosa* and *K. pneumoniae*) bacteria using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results indicate the presence of flavonoids, saponins, tannins in plant aerial parts with the presence of alkaloids but in a lesser degree. The result of antibacterial assay showed that MICs of methanol fraction were approximately 900 µg/ml for gram positive bacteria (*S. aureus* and *S. haemolyticus*), 225 µg/mL for *P. aeruginosa* and 450 µg/mL for *K. pneumonia*. While the best MBCs of the five fractions along with MOHM extract gave, the bactericidal effect in concentrations 450 µg/mL for *P. aeruginosa***.** Our study is the first report on phytochemical screening and antibacterial activity of VLC fractions of *Mentha pulegium*.

Keywords: *Mentha pulegium*, phytochemical screening, vacuum liquid chromatography, antibacterial

INTRODUCTION

Mentha pulegium, a member of the Lamiaceae family, is a flowering, aromatic, herbaceous, perennial plant (Tutar et al., 2016). In Europe, it is commonly known as pennyroyal or pennyrile (Zekkri et al., 2013), also called mosquito plant, squaw mint, and pudding grass (Hadi et al., 2017). It grows in most of the world, including north and east Africa, America, western, southern, and central Europe, Asia, and Arab countries, including Egypt, Algeria, and Iraq (Teixeira et al., 2012). In humid, sunny areas of the plains, mountains and riverbanks, it grows wildly (Zekri et al., 2013). A wide range of medical disorders are traditionally treated by the aerial parts of this plant, such as flatulent dyspepsia and intestinal colic, due to its antispasmodic

and carminative properties (Brahmi et al., 2014). It has a wide range of biological activities, including antiseptic, antioxidant, anti-inflammatory, anti-bacterial, insecticidal, and fungicidal properties (Khaled-Khodja et al., 2014; Foganholi et al., 2015). The dry parts and essential oil of *M. pulegium* have been traditionally used in medicine to treat digestive, liver and gallbladder disorders, gout, colds, amenorrhea, increased micturition, skin diseases, and abortifacients, gastronomy (culinary herb), aromatherapy, and cosmetics (Aires et al., 2016). A wide diversity of secondary metabolites such as tannins, resins, flavonoids, pectin, bitter principles, and essential oils were found in the aerial parts of pennyroyal (Zekri et al., 2013). It is well documented that the environmental and growing conditions in different planting regions significantly influence the quantity and quality of this plant's active constituents (Brahmi et al., 2014). While major components of the extraction products and essential oils are similar, other active constituents may vary according to geographic regions, where the plant is harvested or due to seasonal climate conditions, soil composition, age, and the different vegetative phases of the plant (Teixeira et al., 2012; Foganholi et al., 2015).

In Iraq, *M. pulegium* grows as a wild plant, especially near the river. The aroma of this plant is thought to be due to interaction between different compounds (Daz-Maroto et al., 2007), such as citrus compounds (limonene, octanal, nonanal), minty compounds (pulegone, piperitol, isopulegone, 1,8-cineol), herbaceous (hexanal, isopulegol), fruity (damascenone, methyl-2-methylbutanoate, ethyl-3-methylbutanoate), and floral notes. High concentrations of pectolinarigenin, pedalitin, chrysoeriol and 5-hydroxy-3,4,6,7-tetramethoxy flavone are present in *M. pulegium* (Zaidi et al., 1998), which are excellent antispasmodics, antioxidants, anti-secretory, anti-allergic, anti-diarrheal, anti-inflammatory, blood pressure, antiulcer, and protect against cataract and cancer (Zekri et al., 2013). In this study, the extract of stems and leaves of *M. pulegium* was submitted to VLC and the resultant fractions were studied for their antibacterial effect.

MATERIALS AND METHODS

Plant Material

The aerial parts of *M. pulegium* (leaves and stems, 470 g) were collected from Karbala City, Iraq during the summer mornings of July 2019.

Extraction of Plant Materials

The leaves and stems of *M. pulegium* were ground using an electric grinder. The powdered materials were macerated in absolute MeOH for 5 days at room temperature. Afterwards, it was filtered under vacuum using the Buchner system. The filtrate was evaporated over a water bath to obtain a viscous extract, MOHM (33.9 g). VLC was carried out on Methanol extract over silica gel powder, using different polar solvents: petroleum ether, dichloromethane, EtOAc, acetone, and MeOH. The fractions were collected separately, evaporated, and weighed in each case. Thin layer chromatography analysis was performed for all five fractions and the original extract using pre-coated TLC sheets with $CHCl_3$ as the mobile phase. TLC spots were visualised using a UV_{254} lamp and vanillin reagent.

Phytochemical Analysis

The MeOH extract was submitted for phytochemical screening to investigate the presence of the following plant secondary metabolites: flavonoids, saponins, tannins, and alkaloids.

Flavonoid test: The dilute NaOH solution (1 mL) was added to 1.5 mL of the extracts. The mixture was inspected for the production of yellow color, which is a positive result (Hossain et al., 2013).

Saponin Test: 2 mL of the extract was dissolved in 10 mL of distilled water. The test tube was stopped and then shaken vigorously for 30 sec. It was then allowed to stand for 30 min. A honeycomb froth above the surface that stays after 30 min is taken as a positive result (Hossain et al., 2013).

***Tannin Test*:** 1 mL of the extract and a few drops of freshly prepared 1% lead acetate were dissolved together. The yellow precipitate shows a positive result (Mamta & Jyoti, 2012).

Test for Alkaloid (Dragendorff's): 1 mL of the extract was mixed in a test tube with 0.3 mL of dilute HCl and 1 mL of Dragendorff's reagent and left for a few minutes. A positive result is indicated by the presence of an orange-to-brown precipitate (Chetri & Khatri, 2017).

Test for Alkaloid (Mayer's): 0.5 mL of the extract was mixed with a few drops of ethanol, 2 drops of dilute HCl (10%) and 2 drops of Mayer's reagent. A positive result for the presence of alkaloids is a yellow precipitate (Chetri & Khatri, 2017).

Antibacterial Assay

The antibacterial activity of *M. pulegium* aerial parts was investigated by the determination of its MIC and MBC against gram-positive (*Staphylococcus aureus* and *Staphylococcus heamolyticus*) and gram-negative bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). All bacteria strains were isolated and diagnosed using VITEK 2 Compact in Al-Kafeel speciality hospital, lab unit at Karbala.

Preparation of Cultural Media

Nutrient broth medium was prepared according to the manufacturer by dissolving 13 g of media in 1 L of D.W. This medium was sterilised in an autoclave at 15 lbs of pressure and 121°C for 15 min, then kept in clean sterilised test tubes.

Preparation of Bacterial Inoculum and Stock Solution

Bacterial inocula were prepared from a single pure colony inoculated in nutrient broth medium and incubated at 37°C for 16-18 hrs. The concentration of bacterial inoculum was adjusted by comparison with 5% McFarland solution (9.95 mL of H_2SO_4 solution, 1% in broth and 0.05 mL of $BaCl_2$ solution 1% in broth) equivalent to 150106 colony-forming units (CFU) per mL. Crude extracts (1800 g/mL) were prepared by dissolving 1.8 mg in 1.0 mL of DMSO.

Determination of MIC and MBC

The MIC of each sample was achieved according to the reported procedure in the literature (Magina et al., 2009) with minor modifications. Each sample (1.8 mg) was dissolved in 1.0 mL of DMSO. The concentration of stock solution, 1800 g/mL, was gradually diluted twofold to get a concentration for each sample in the range of 1800 to 14.07 g/mL in the 96-well plate. Wells were impregnated with 100 L of bacteria and were covered for incubation overnight at 37°C. For MBC, 10 L of solution was pipetted into the surface of agar in a petri dish and gently spread with a glass rod; the dish was sealed and incubated overnight at 37°C.

RESULTS AND DISCUSSION

M. pulegium belongs to the Lamiaceae, or mint family, and there are few studies on the biological activities of this species. The original MOHM extract was fractionated by VLC with increasing solvent polarity as follows: petroleum ether, dichloromethane, ethyl acetate, acetone,

and finally with methanol. Fractions were evaporated to remove solvents, weighed and were recorded in Table 1.

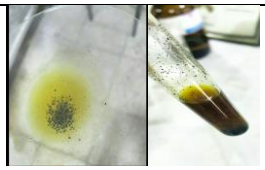
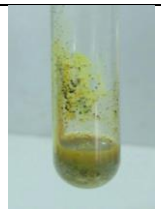
Table 1. Weight of Fractions

Name of Fractions	Weight of fractions (g)
Petroleum ether fraction	0.4
Dichloromethane fraction	3.04
Ethyl acetate fraction	2.94
Acetone fraction	3.62
Methanol fraction	18.05

The extraction process was done using absolute methanol as a solvent in the cold maceration process. As it is considered as a polar solvent, it dissolves polar and some semi-polar compounds, and thus most of the compounds that are present in this plant, such as flavonoids, triterpenes, alkaloids, saponins, and tannins, were extracted. These compounds have significant biological activity, such as antioxidant, antifungal, and antibacterial activities. Vacuum liquid chromatography was done in order to fractionate methanol extract into different fractions according to the polarity of the solvents; petroleum ether for nonpolar, dichloromethane and ethyl acetate for semi-polar, acetone and then methanol for more polar compounds.

Extraction of the plant with a methanol extractor gave 33.97 g of MOHM extract. To identify the presence of active constituents, phytochemical investigation was done using Mayer's reagent and Dragendorff's reagent for alkaloids, lead acetate test for tannins, froth test for saponins, and sodium hydroxide test for flavonoids. Results of chemical investigation showed that the aerial parts of the plant contain flavonoids, tannins, and saponins (the froth last half an hour) in high amounts, while alkaloids appear in low amounts, as indicated in Table 2. Thin layer chromatography was performed to confirm the presence of these active constituents in each extract.

Table 2. Phytochemical Screening of *M. pulegium*

Active constituents	Result	Active constituents	Result
Alkaloid	+ 	**Saponins**	+++ 
Flavonoid	+++ 	**Tannins**	+++ 

+ = presence of compounds

The crude extract MOHM and five fractions were submitted to investigate their antibacterial activity. The MIC and MBC results are reported in Table 3. Antibacterial activity was assessed using MIC and MBC as quantitative assays. MIC results show that the methanol fraction (F5) gave the highest antibacterial activity against four tested bacteria, with MIC concentrations of 900 g/mL for both *S. aureus* and *S. haemolyticus*, 225 g/mL for *P. aeruginosa,* and 450 g/mL for *K. pneumoniae.* This may be attributed to polar compounds such as flavonoids eluted by methanol and reported to have antibacterial effects (Cushnie & Lamb, 2005). Flavonoids inhibit or interfere with many essential bacterial processes, including nucleic acid

synthesis, cytoplasmic membrane function, energy metabolism, attachment and biofilm formation, and porin on the cell membrane. This led to alteration of the membrane permeability and attenuation of bacterial pathogenicity (Cushnie & Lamb, 2005). MBC results show that all five fractions along with MOHM extract exert an antibactericidal effect at 1800 g/mL for *S. aurous, S. haemolyticus, and K. pneumoniae* and at 450 g/mL for *P. aeruginosa.*

Table 3. Antibacterial activity results of MIC and MBC

Concentration	**MIC**						**MBC**					
	C	**F1**	**F2**	**F3**	**F4**	**F5**	**C**	**F1**	**F2**	**F3**	**F4**	**F5**
	Staphylococcus aureus						***Staphylococcus aureus***					
1800 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
900 µg/mL	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve
	Staphylococcus haemolyticus						***Staphylococcus haemolyticus***					
1800 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
900 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve
	Pseudomonas aeruginosa						***Pseudomonas aeruginosa***					
1800 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
900 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
450 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
225 µg/mL	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve
	Klebsiella pneumonia						***Klebsiella pneumonia***					
1800 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	-ve	- ve	-ve	-ve	- ve	- ve
900 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve
450 µg/mL	-ve	- ve	-ve	-ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve

+ ve = no inhibition of bacterial growth - ve = inhibition of bacterial growth; C: Original crude of MOHM, F1: Petroleum Ether Fraction, F2: Dichloromethane Fraction, F3: Ethyl Acetate Fraction, F4: Acetone Fraction, F5: Methanol Fraction.

Staphylococcus aureus is a virulent gram-positive bacteria that is currently the most common cause of infection in hospitalised patients. *S. aureus* infections can involve any organ system. It is a leading cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir et al., 2003). This bacteria is inhibited with MIC 1800 g/mL of crude extract and the five fractions but with MIC 900 g/mL of the F5 fraction, which indicates that the F5-containing compounds act as bacteriostatic even with concentrations lower than the other fractions. The MBC for this bacterium was 1800 g/mL of all fractions and crude extract, which may indicate the bactericidal activity through disruption of the cellular wall.

Staphylococcus haemolyticus is an opportunistic bacterial pathogen that colonises human skin. It is frequently cultured from hospitalised patients and is characterised by resistance to multiple antimicrobial agents. Infections most frequently occur after the implantation of medical devices and are attributed to their biofilm-forming potential (Archer et al., 1996). According to results shown in table 3, this bacteria is inhibited with MIC (1800 and 900) g/mL by crude extract and all five fractions with MBC at 1800 g/mL. This indicates that the extracts contain strong bacteriostatic agents that are able to penetrate the thick layer of peptidoglycan and inhibit the growth even with a low concentration, while a higher concentration is needed to disrupt the cell wall and cause bacterial lysis.

Pseudomonas aeruginosa is a motile, non-fermenting, gram-negative organism belonging to the family Pseudomonaceae. A rod-shaped and blue-green pigmented bacterium (Alhazmi, 2015). It has the ability to cause both severe acute and chronic infections among patients with burn wounds, cystic fibrosis, leukemia, and organ transplants (Lambert, 2002). It needed an MIC of 450 g/ml to be inhibited by crude extract and the fractions, except F5 fraction (methanol fraction), which inhibits bacteria at a MIC of 225 g/mL. This methanol fraction contains bacteriostatic agents capable of inhibiting the growth of this bacteria.

Klebsiella pneumoniae, a member of the family Enterobacteriaceae, is a rod-shaped, gram-negative, lactose-fermenting bacillus with a prominent capsule. It is an opportunistic

pathogen that is widely found in the mouth, skin, and intestines, as well as in hospital settings and medical devices, and mostly affects those with compromised immune systems or who are weakened by other infections (Li et al., 2014). This bacteria needed an MIC of 450 g/mL to be inhibited by all extracts, and this indicates the strong antibacterial activity of these extracts. While it needed a high MBC of 1800 g/mL to be penetrated and killed by all extracts, this may be attributed to its protective outer membrane that permeates only small hydrophilic molecules through porins.

Gram-positive bacteria were slightly more sensitive to the flavonoids than gram-negative bacteria. Active flavonoids against gram-negative bacteria showed a narrower range of lipophilicity compared to active flavonoids against gram-positive bacteria, which showed a wide range of lipophilicity and cell lysis. In addition, gram-positive bacteria possess a thick peptidoglycan layer, and in the case of gram-negative bacteria, in addition to the inner membrane and the peptidoglycan layer, an outer membrane represents an additional barrier for many small molecules. Such an asymmetric bilayer is a highly effective barrier for polar molecules. Permeation of small molecules through porins is considered the main pathway for the entry of polar compounds into gram-negative bacteria.

CONCLUSION

The phytochemical screening of *M. pulegium* aerial parts indicates the presence of flavonoids, saponins, and tannins in high amounts, while alkaloids appear in low amounts. The antibacterial assay on gram-positive and negative bacteria, indicated by MIC, shows that the methanol fraction is the most effective fraction, having MIC values lower than those of other fractions. However, the antibacterial assay by MBC was of the same values for each crude and fractions with a concentration of (1800 g/mL) for each of *S. aurous*, *S. haemolyticus*, and *K. pneumoniae*, and (450 g/mL) for *P. aeruginosa.*

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