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Original Research

ANTI-BIOFILM POTENTIAL OF MENTHOL PURIFIED FROM *MENTHA PIPERITA L.* (MINT)

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Abstract: *Menthol, a bioactive compound of Mentha piperita (mint) with antibacterial properties was purified by column chromatography to determine its anti-biofilm potential. After phytochemical analysis, TLC was carried out using n-hexane: ethyl acetate: methanol: water (2:2:2:1) as the solvent system for ethanolic extract of mint. TLC achieved the maximum separation of mint constituents with Rf value of 0.68. A purified menthol fraction was obtained after silica gel column chromatography using four different eluting solvents. The menthol obtained was then used to perform biofilm inhibition assay to establish its antibacterial potential. Percentage inhibition was highest for bacillus subtilis (79.4%), as opposed to Pseudomonas aeruginosa (33.6%) and the combination of both bacteria (20%). ELISA reader was used to measure absorbance at 450-620nm and 630 nm. Using 450-620nm filter the values for percentage inhibition lies between 48.6-95% for standard and crude menthol samples. Similarly, at 630nm the values of inhibition lie between 23.4-70.6%. This anti-biofilm property of menthol can be utilized in antibacterial drug formulations.*

Keywords: menthol, *Mentha piperita*, anti-bioflim, *Pseudomonas aeruginosa*, inhibition

Introduction

Herbal medicines are an effective remedy for many infectious diseases since ancient times. Even today, several drug constituents are obtained from plant sources. One such plant of medicinal importance is *Mentha piperita* which is commonly referred as peppermint or mint (Al-Bayati, 2009; Snoussi *et al*., 2015). *M. piperita* (peppermint) is a member of family *Lamiaceae* and is found growing in moist habitats. Mint not only serves as the flavoring agent for many food products and drinks, but it also imparts fragrance. It has been used to treat common colds, inflammation and gastrointestinal tract problems even by earlier civilizations (Bupesh *et al*., 2007). Menthol is a terpenoid which is the major bioactive agent of mint family. It is a waxy, crystalline, white color substance which is solid at room temperature and has a sweet, minty, refreshing odor (Mikaili *et al*., 2013). The extraction of menthol for pharmaceutical purposes is made possible using different techniques; like colorimetric methods, gas-liquid chromatography, column chromatography and High-performance liquid chromatography (HPLC). Its quantification using UV-spectrophotometer has not been reported (Parkin, 1984).

The researchers have investigated different methods by which terpenoids can be isolated. Several different solvents were used for extraction process like acetone, 80% methanol, ethanol, hexane, ethyl acetate and chloroform. However, gas liquid chromatography proved to be the most efficient method for purification of terpenoids (Liu *et al*., 2014). Menthol has antiseptic, antibacterial, antitumor and antiallergenic properties. Its antibacterial property has been extensively studied against a few pathogenic and non-pathogenic strains. Due to this property, it has been used for the treatment of sore throat, common cold, coughing and mouth, throat irritation (Chandki *et al*., 2011; Husain *et al*., 2015). A breakthrough in the antibacterial properties of menthol is its ability to disrupt or inhibit biofilms. This has been confirmed by the work of Husain and his colleagues who reported 64.8 % retardation of *A. hydrophila* biofilm at a concentration of about 800 µg/mL of menthol (Husain *et al*., 2015). Similarly, The researchers have assessed the formation of *S. aureus* biofilm through biofilm assay at different pH levels (Doughari, 2012; Zahra *et al*., 2011). Qualitative analysis showed that the percentage of biofilm formation was about 60 %. It also showed that the formation of biofilm was dependent on pH. Very acidic pH (Sun *et al*., 2013) and very basic pH (Skalicka-Woźniak and Walasek, 2014) showed lower growth of biofilms whereas as neutral pH showed moderate growth. The bacteria living in biofilms interact with each other via a mechanism known as Quorum sensing. Menthol extracted from peppermint inhibits quorum sensing mechanism in bacteria. Inhibition of quorum sensing can reduce pathogenicity and modify antibiotic resistance of bacteria. Owing to its antibiofilm potential menthol can be used to modify the antibiotics used to treat oral problems (Chusri *et al*., 2012; Saharkhiz *et al*., 2012; Wakimoto *et al*., 2004).

Materials and methods

Preparation of crude extract

Fresh mint was air dried for about two days and the leaves were finely grounded to powder. Ethanolic extract was prepared by dissolving 10g of the mint powder in 80ml of the solute. The extract was kept overnight before performing phytochemical analysis; which included the detection of alkaloids, terpenoids, phenols and flavonoids.

Thin layer chromatography

For TLC, mint extract was prepared by dissolving 50 g of mint powder in 500 ml of 70% ethanol. The extract was placed in a shaking incubator for 24 hours at 37^oC and 120 rpm. After shaking for 24 hours the solution was filtered with Whatman's filter paper. Further washing was done with 20 ml ethanol and 10 ml distilled water. The filtrate obtained was then concentrated at 72^0C in a rotary evaporator (Rao *et al*., 2007; Shaikh and Patil, 2010; Still *et al*., 1978). The 50-100 ml concentrated crude extract obtained had high concentration of menthol. This concentrated extract was subjected to preparative thin layer chromatography using n-Hexane: Ethyl acetate: Methanol: Water (2:2:2:1) as the solvent system. The retention factor (R_f value) was calculated:

$$R_f = \frac{\text{Distance travelled by the active fraction}}{\text{Distance travelled by the solvent}}$$

Purification of menthol by column chromatography

Purification of menthol from the crude sample was carried out using silica gel column chromatography. A 20-cm long plastic, reusable column with a filter paper disc at the bottom and a yellow plastic plug which fits in the nozzle stopping the flow of solvent was used. Slurry was prepared with a 70-230 mesh column grated silica gel. About 20 grams of silica was dissolved in n-hexane to make free-flowing slurry. 400µl of sample was loaded in the column. The adsorbed compound was eluted using four different solvents *i.e.* n-Hexane, Chloroform: Ethanol (10:1), Chloroform: Methanol (10:1) and Methanol under the flow rate of 2ml/min. Seventeen different The solvent system used for TLC consisted of n-Hexane: Ethyl acetate: Methanol: Water (2:2:2:1). The R_f value was calculated to be 0.68. This Rf value was similar to the Rf value reported for menthol (Lugemwa, 2012). It is also important to note that fractions (2ml volume each) were collected after eluting the column with four different solutes. First four fractions were obtained using n-hexane, fraction 5-9 using chloroform: ethanol (10:1), fraction 10 to 12 using chloroform: methanol (10:1) and fraction 13 to 17 using methanol. The collected fractions were pooled together and were subjected to TLC(n-Hexane: Ethyl acetate: Methanol: Water (2:2:2:1)) against commercially available standard menthol for further confirmation (Çitoğlu and Acıkara, 2012; Tang *et al*., 2011).

Determination of antibiofilm potential of menthol

The antibiofilm property of menthol was established using menthol fraction obtained after column chromatography, crude ethanolic extract of *Mentha piperita* and commercially purified menthol standard. Then the efficiency of their percentage inhibitions was compared. Biofilms were prepared using two bacterial strains *of P. aeruginosa* and *B. subtilis.* To perform the biofilm inhibition assay, the biofilms were grown on freshly prepared LB media in 300µl capacity microtiter plate. Single strain biofilms as well as biofilms formed by the combination of *P. aeruginosa* and *B. subtilis* were subjected to inhibition by standard menthol, crude sample of *Mentha piperita* and purified fraction from column chromatography. 5% crystal violet was used as method of staining. Two filters of ELISA reader i.e. 630nm and a bi-chromatic filter of 450-620nm was used to obtain absorbance. Percentage inhibition was calculated using:

$$\%\ \text{Inhibition} = \frac{\text{OD control} - \text{OD of Biofilm} \times 100}{\text{OD control}}$$

Results and Discussion

Phytochemical analysis

The phytochemical analysis for ethanolic extract showed positive results for flavonoids, phenols and terpenoids whereas the test was negative for alkaloids. Menthol is a monoterpene having one phenolic group. The presence of this phenolic group makes it slightly polar. Therefore, it is soluble is non-polar solvents like chloroform, n-hexane, toluene, ether, petroleum ether and polar solvents like glacial acetic acid, methanol and ethanol (both absolute and 70%). Its solubility in alcohols is due to the presence of phenolic group. It is insoluble in water because water is a highly polar solvent which forms hydrogen bonds. Menthol being weakly polar itself, does not dissolve in water (Lugemwa, 2012; Still *et al*., 1978).

Thin layer chromatography

different solvent systems give different Rf values. The difference in R_f value is because of different solubility of menthol in different solvent system. It also depends on the amount of the sample spotted, temperature and the thickness of the TLC plate or

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card (Kuehler and Lindsten, 1983). Menthol being very slightly polar moves rapidly up the TLC card. It is because it is more readily soluble in non-polar solvents therefore; it has a higher R_f value. The polar compounds in the extract bind to the adsorbent (silica), so they move slowly and have low R_f values (Kuehler and Lindsten, 1983; Lugemwa, 2012).

Table 1: The R_f value of standard and all the fractions pooled

FRACTION NUMBER	R_f Value
Standard	2.4/3.5 = 0.68
Combined Fractions	2.4/3.5 = 0.68

Purification of menthol by column chromatography

TLC was followed by column chromatography to isolate and purify menthol from the crude extract. Seventeen different fractions of about 2 ml each were collected in vials using four different dilution solvents. The fractions were labelled as M1- M17 and were subjected to TLC. The same solvent system was used i.e. n-hexane: ethyl acetate: methanol: water (2:2:2:1). The fractions having same Rf value were pooled (Table 1).The presence of menthol was then further made by a test. All those spots on the TLC card were sprayed with Folin-Ciocalteu reagent. Purple color appears due to oxidation reaction. Addition of folin reagent is a confirmatory test for the detection of phenolic compounds or group present (Figure 1).

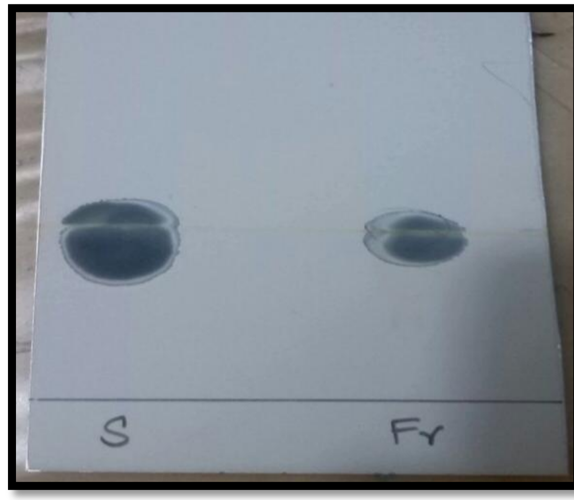

Figure 1: TLC card showing the result of standard and all the fractions combined. Purple spot appeared after spraying Folin-Ciocalteu.

Determination of antibiofilm potential of menthol:
Anti-biofilm potential of the fraction purified by column chromatography

The absorbance was taken at 630 nm. Acetic acid served as blank this was because acetic acid was present in each well as biofilm dissolving agent. 0.0294 was the reading obtained for blank. The results are depicted in table 2.

Table 2. Biofilm inhibition showed by purified menthol fraction and standard menthol

BIOFILM	ABSORBANCE 630nm Control	Menthol Fraction	Menthol Standard	PERCENTAGE INHIBITION % Menthol Fraction	Menthol Standard
P. aeruginosa	0.1625	0.1078	0.0622	33.6 %	62%
B. subtilis	0.9552	0.1966	0.1423	79.4%	85.1%
P. aeruginosa* + *B.subtilis	1.1241	0.9021	0.6720	20%	40.2%

Biofilm Inhibition assay

The biofilm ring was visible on the microtiter plate after drying. The absorbance for the inhibition was taken using two filters of the ELISA reader *i.e.*, one on a bichromatic filter and the other one using 630nm filter.

Using 630nm wavelength

The results obtained with 630nm filter (Table 3) showed that 100µl of each sample either crude, standard menthol, or purified menthol fraction showed maximum inhibition. Also, the biofilms formed by single strains are more easily inhibited than the biofilm formed by both *Pseudomonas* and *Bacillus.* Crude extract has better ability to inhibit biofilms because it has severalphytochemical constituents like phenols, terpenoids and flavonoids all of which exhibit antimicrobial property (Djordjevic *et al*., 2002; Sasidharan *et al*., 2011).

Table 3. Mean percentage inhibition values of Biofilm inhibition assay using 630nm Eliza filter

	***P.aeruginosa* + *B.subtilis* Biofilm Percentage Inhibition % with sample volume**			***P.aeruginosa* Biofilm Percentage Inhibition % with sample volume**			***B.subtilis* Biofilm Percentage Inhibition % with sample volume**		
	50µl	**75 µl**	**100 µl**	**50 µl**	**75 µl**	**100 µl**	**50 µl**	**75 µl**	**100 µl**
Crude Sample	29.7	34.8	52	66	70.3	70.6	20.1	28.5	33.2

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Menthol Standard	39.5	42	43	37	58.7	62	13.1	20.1	23.4

Using a bi-chromatic filter (450-620nm)

The second set of absorbance was taken with a bi-chromatic filter (450-620nm), showed almost similar results (Table 4). The working of a bichromatic lens is different than that of a single wavelength filter. The bichromatic lens takes the absorbance at two wavelengths which in this case is 450 and 620 nm. The ELISA reader disperses the light at both wavelengths then shows the result after subtraction. It is because of this reason, the values of absorbance obtained are much lower than those obtained using a single wavelength filter (Donlan, 2001; Elvers *et al*., 1998; Odeyemi and Oluwajoba, 2011). A higher percentage inhibition was therefore calculated using a bichromatic lens, but a similar trend of inhibition was observed.

Table 4. Mean percentage inhibition values of Biofilm inhibition assay using a bichromatic filter (450-620nm)

	***P.aeruginosa* + *B.subtilis* Biofilm Percentage Inhibition % with sample volume**			***P.aeruginosa* Biofilm Percentage Inhibition % with sample volume**			***B.subtilis* Biofilm Percentage Inhibition % with sample volume**		
	50µl	**75 µl**	**100 µl**	**50 µl**	**75 µl**	**100 µl**	**50 µl**	**75 µl**	**100 µl**
Crude Sample	5.5	45.8	48.6	42	68.4	80	80	93	93.5
Menthol Standard	54	94	94	71.5	75	95	16	89.7	94.4

The basic mechanism behind the inhibition of biofilms has been studied for many medicinal plant extracts and their components like alkaloids, flavonoids *etc*. Biofilms are inhibited because these antimicrobial compounds either inhibit the mechanism of Quorum sensing within the biofilm, prevent the formation of flagella, pili or fimbriae, or prevent the formation of nucleotides (Kelly *et al*., 1979; Sun *et al*., 2013). Antimicrobial agents also affect the membrane fluidity and permeability of bacterial cell wall resulting in the disruption of cells (Delwiche and Gaines, 2005). Menthol also works in similar fashion. It prevents the attachment of bacteria to the surface of the substratum by affecting the flagella of the bacterial strains of *Pseudomonas* and *Bacillus.* It also interferes with the extracellular matrix (consisting of DNA, proteins and polymers) necessary for biofilm formation and its attachment, thus preventing the formation of biofilm. It also interferes with the quorum sensing mechanism (Qiu *et al*., 2011; Uzair *et al*., 2008). Hence, it was established after extensive and repeated research that crude extract of mint as well as the purified form of menthol exhibit antibiofilm property. This property can be exploited to produce number of antimicrobial drugs as well as for the modification of antibiotics. Using compounds derived from medicinally important plants can redefine and reshape the formation of drugs in future.

Conclusion

The qualitative analysis proved ethanol to be the best solvent for the detection of phytochemicals. The purified menthol fraction obtained after column chromatography has the R_f value of 0.68 using n-hexane: ethyl acetate: methanol: water as solvent system. The purified menthol as well as its crude extract when subjected to biofilm inhibition assay shows a reasonable inhibition of biofilm, whereas the maximum inhibition of 99.5% was achieved by crude menthol extract. This result illustrates that menthol is a medicinal plant and can be used to alter the antibiotic resistance of many disease-causing microbes.

Conflict of interest

The authors declared absence of any conflict of interest.

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