Research article

Inhibitory activity of *Mentha spicata* oils on biofilms of *Proteus mirabilis* isolated from burns

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ABSTRACT

Introduction and Aim: *Proteus mirabilis* is an opportunistic pathogen, infecting humans, through the release of endotoxins and enzymes such as urease, hemolysin, protease, DNase etc. One of the factors contributing to its virulence is its unusual ability to form crystalline biofilms. This study aimed to investigate the effect of *Mentha spicata* volatile oil on *P. mirabilis* biofilm formation.

Materials and Methods: *P. mirabilis* was isolated from infected wound of burns of patients using conventional biochemical tests. Vitek 2-Compact System was used to confirm the diagnosis of bacterial isolates. The isolates were tested for their susceptibility to 11 antibiotics. Aqueous and alcoholic extracts as well as Volatile Oil and natural Menthol extracted from *M. spicata* were tested for their ability to inhibit biofilm formation by *P. mirabilis.*

Results: In this study 16 out of the 45 burn injury samples were tested positive for *P. mirabilis*. Bacterial isolates were found to be resistant to the drugs Levofloxacin and Norfloxacin, with percentages of 10.2% and 11.8%, respectively. Majority of these isolates had the capacity to produce several virulence factors, including biofilm in variable amounts and the enzymes protease, hemolysin, DNase, and gelatinase. The volatile oil and natural component menthol extracted from *M. spicata* inhibited the formation of biofilm at increasing concentrations.

Conclusion: The volatile oil and the natural menthol compound of *M. spicata* are effective in inhibiting biofilm formation by *P. mirabilis*.

Keywords: *P. mirabilis***;** biofilm; virulence; *M.spicata*; burns**.**

INTRODUCTION

Proteus mirabilis is a small, gram-negative, motile, non-lactose fermenter, facultatively anaerobic, dimorphic member of the Enterobacteriaceae family. *P. mirabilis* is characterized by its swarming motility and in their ability to produce the enzyme urease, which is one of the most important factors of their virulence. *P. mirabilis* an opportunistic pathogen, as it can settle in the urinary tract, it is one of the causes of urinary tract infection in hospitalized patients. A characteristic feature of *P. mirabilis* is its unique efficiency to form crystalline biofilms, which is also considered as one of the virulence factors of *P. mirabilis*. The biofilm formed can withstand external factors and external stress, as it protects the bacteria from antibiotics, as well as the inability of the body's defenses to eliminate bacteria (1). Biofilm layers are composed of extracellular polymeric materials that include polysaccharides, extracellular DNA, lipids, and proteins. Biofilms membranes can form on various surfaces, whether natural or on medical devices and catheters, which leads to the development of chronic diseases that are difficult to treat. It is found that biofilms are the main cause of chronic infection in 80% or more of all microbial infections that cause the release and secretion of toxins harmful to the body. *P. mirabilis* can cause infection in various anatomical sites of the human body through the secretion of endotoxin and enzymes such as Urease, hemolysin, protease, DNase, and others (2).

Antimicrobial resistance (AMR) is a major public problem in all countries of the world, which arises due to their overuse and overexposure of microbes to antibiotic compounds, recently during and after the outbreak of COVID-19 the rate of antimicrobial resistance increased due to overuse of therapies (3). As a result, Orientation of researchers to search for new methods and technologies to process cases of increased drug tolerance associated with various pathogenic bacterial and fungal organisms. There are many plant varieties, which are a source of natural metabolic products with an inhibitory effect on different types of microorganisms, as their extracts are used in the treatment of many diseases caused by bacteria and fungi (4). *Mentha spicata* is an aromatic plant that belongs to the *lamiaceae* family, having several biological properties. Hence, it is used in the manufacture of cosmetics, chewing gum, sweets, toothpaste, and some pharmaceutical products, It is used an, antibacterial, antifungal, and antioxidant agent, as well as for the treatment of colds, flu, respiratory problems, hemorrhoids, and stomach pain (5). In this study, we aimed to study the effect of volatile oils of *M. spicata* against biofilm formation.

MATERIALS AND METHODS

Sample collection

Forty-five swab samples were collected from burn injury infected patients of both sexes visiting the Baquba Teaching Hospital and few outpatient clinics in Diyala Province, Iraq, between November 2022 and February 2023.

Cultural and identification of bacterial isolates

Samples were cultured onto blood agar media, MacConkey agar and Enteric Hekton Agar and incubated at 37°C for 24 h. Preliminary identification of *P. mirabilis* was based on their culture characteristics such as growth on different culture media, the shape and colour of the bacterial colony, and their characteristic swarming motility and unpleasant odour (like rotten eggs) on Blood agar medium. The isolates were picked and subjected to Gram staining and several biochemical tests such as oxidase, catalase, indole, citrate reduction, methyl red and Voges Proskaur, fermentation of glucose and production of H_2S on Klingler Iron Agar (6). The isolated identified as *P. mirabilis* were further confirmed using the Vitek 2-Compact System kit (BioMérieux, France) as per the manufacturer's instructions.

Antibiotic susceptibility test

P. mirabilis isolates were checked for their antibiotic sensitivity based on Kirby -Bauer Diffusion Method (7) against 11 antibiotics which included Amoxicillin (20 µg), Cefepime (30 µg), Cefotaxime (30 µg), Imipenem (10 µg), Azeteronam (30 µg), Amikacin (30 µg), Gentamicin(10µg), Doxycycline (30µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Levofloxacin 5µg). All antibiotic discs were procured from Mast Group, United Kingdom.

Virulence factors test

The isolates were tested for few virulence factors and enzyme production such as Hemolysin, Protease, DNase, Gelatinase, and biofilm formation which was carried out as previously described (8). The ability of the bacteria to form biofilms was detected by the microtiter plate method (9).

Preparation of *Mentha spicata* extract

M. spicata samples were collected from the local markets in Iraq. The samples were sent to the National Herbarium for its identification and confirmation. Preparation of aqueous and alcoholic concentrations of *M. spicata* was carried out according to a previous protocol (10). Briefly, the plants were dried and powdered. To obtain the alcoholic extract, after sifting the *M. spicata* powder, 100 gm of it was weighed and placed in a glass beaker to which 1000 ml of 96% ethyl alcohol was added and left for 24 hours with occasional shaking. After 24 h, the mixture was filtered by using filter paper, followed by concentration of the total filtrate by using a rotary evaporator. The extract obtained was further evaporated in an oven maintained at 40°C. The resulting extract was stored in a refrigerator until further use. To obtain the aqueous extract, the same process was followed, using distilled water instead of ethyl alcohol. Various concentrations (50, 100, 200, 250 mg /ml) of the crude extract were obtained, by serially diluting 0.5 gm of the aqueous and alcoholic extract separately, using distilled water.

Preparation of volatile oil concentrations to *Mentha spicata*

Volatile oils are complex natural compounds formed in aromatic plants as secondary metabolites that dissolve in fats and organic solvents. The volatile oil of *M. spicata* was extracted by the method of Hydro-distillation (11) using the Cleavenger apparatus. 50 gm of dry *M. spicata* powder was weighed and mixed with 250 ml of distilled water in a beaker and left to boil for 3 hours. The oil that separated out was collected and dried using anhydrous sodium sulfate (12). The oil thus obtained was stored in a glass bottle and stored at 18°C until use. Serial concentrations of *M. spicata* volatile oil were prepared by mixing 50,150, 300 microliters of the crude oil concentrate with 1 ml of the solvent dimethyl-sulfoxide (DMSO), to obtain concentrations of 5%, 15%, 30% of the volatile oil respectively.

Preparation of menthol concentrations to *Mentha spicata*

Various menthol concentrations (100,150,300µg/ml of *M. spicata* was prepared by dissolving 0.1 of the aqueous and alcoholic extract to *M. spicata* in 10 ml of the solvent dimethyl-sulfoxide (DMSO), followed by filter sterilization using a 0.22 µm Millipore filter. The *M. spicata* concentrations were stored in the refrigerator until use.

Effect of inhibitors on *P. mirabilis* biofilm formation

Five bacterial isolates were selected based on the severity of their biofilm formation, resistance to antibiotics and its production of some virulence factors to study of the inhibitor effect of the aqueous and alcoholic extract and the effect of the volatile oil and natural compound menthol for *M. spicata*. Biofilm inhibition studies were carried out in 96 well plates by spectrophotometric assay (9). Biofilm formation was measured by using ELISA reader at an optical density (O.D) of 630 nm. The percentage of biofilm inhibition was calculated using the formula,

$$\% \text{ inhibition} = \frac{\text{O.D in control} - \text{O.D in treatment}}{\text{O.D in control}} \times 100$$

RESULTS

All isolates showed growth on blood agar media and nutrient agar media, which caused of the swarms, and

colorless colonies appeared on the medium of MacConkey because it does not ferment lactose sugar, due this medium contains bile salts that inhibit the fermentation process (13). As for the selective medium Hekton Enteric, the bacterial colonies were single, non-fermentative to lactose and black in color due to their production and precipitation of H_2S hydrogen sulfide. This medium is a differential that distinguishes between Gram-negative bacteria that ferment lactose and those that do not ferment lactose (8).

Biochemical identification

The minimal biochemical tests used in the identification of *P. mirabilis* in this study is given in Table 1. The tests were performed based on standard protocols (14).

Table 1: Biochemical characterization of *P. mirabilis*

Number of isolates	**20**
Shape	Rods
Motility	+
Oxidase	-
Catalase	+
Indole	-
Voges- Proskauer	-
Urease	+
Citrate utilization	+/-
H_2S product	+
lactose fermentation	-

Positive (+), Negative (-), Variable (+/-)

Vitek 2-compact system (GN-Card)

The Vitek 2-Compact System was used to confirm the diagnosis of bacterial isolates isolated from burns. This system provides 64 biochemical tests needed for the diagnosis of bacterial isolates. The test revealed 16 burn samples to be positive for *P. mirabilis.*

Table 2: *P. mirabilis* antibiotic susceptibility

Antibiotic	**Concentration µg/disk**	**Family**	**Resistance percentage**
Amoxicillin	20	Penicillin	70.5%
Cefepime	30	Cephalosporin	91.6%
Cefotaxime	30		82%
Imipenem	10	Carbapenem	14.3%
Aztreonam	30	Monobactam	43.3%
Amikacin	30	Aminoglycoside	72.4%
Gentamicin	10		88.1%
Doxycycline	30	Tetracycline	90.2%
Norfloxacin	10	Quinolone	11.8%
Ciprofloxacin	5		25.3%
Levofloxacin	5		10.2%

Susceptibility for antibiotics

P. mirabils burn isolates (n=16) were tested for their drug sensitivity to different antibiotics belonging to four different groups (beta-lactams, aminoglycosides, quinolines, tetracyclines). The results for antibiotic susceptibility are shown in Table 2. It was found that most of the bacterial isolates were multidrug resistant. *P. mirabilis* was also found to be least resistant to the quinolone antibiotics Levofloxacin (10.2%) and Norfloxacin (11.8%) (Table 2).

Detection of virulence factors

Protease production

The capacity of *P. mirabilis* to produce the protease enzyme varies depending on the source of the isolate as well as the kind of bacterial strain (15). The test findings showed that 88.3% of the *P. mirabilis* isolates cultivated on the medium of Skim Milk agar were able to create the protease.

Hemolysin production

The bacterial isolates were cultured on blood agar to determine whether *P. mirabilis* bacteria have the ability to produce the hemolysin enzyme. The results showed that all the bacterial isolates produced hemolysin, but their ability to analyze blood varied. It was discovered that 15 (91.6%) of its isolates were partially analyzed by blood of the hemolysin type, while only 1 (8.3%) were fully β-hemolysin analyzed, the difference in the rate of ability of *P. mirabilis* isolates to produce hemolysin and the type of degradation may be due to the isolates owning or not possessing responsible coding genes (16).

DNase enzyme production

The ability of bacterial isolates belonging to *P. mirabilis* to produce DNA degrading enzymes was investigated according to Forbes *et al.,* (13). The bacterial isolates were grown on DNase Media Agar, the results showed that *P. mirabilis* 13 (75%) isolates produced this enzyme, while in 3 (25 %), it was not produced.

Gelatinase enzyme production

To reveal the ability of *P. mirabilis* isolates to produce gelatinase enzyme, as it is one of the most important factors of its virulence and a major reason for its

pathogenicity through its work on analyzing and destroying enzymes such as Insulin and protein substances such as Hemoglobin, Casein, Fibrinogen, Collagen and Gelatin, as the results showed all isolates of *P. mirabilis* produced this enzyme (17).

Biofilm production

All isolates of *P. mirabilis* in this study produced biofilm in varying degrees. Different concentrations of aqueous and alcoholic solutions of *M. spicata* were studied for the ability to inhibit biofilm formation. Results showed the ineffectiveness of all the aqueous and alcoholic extracts in inhibiting bacterial growth and biofilm formation. The effect of *M. spicata* volatile oil on *P. mirabilis* biofilm formation was also studied. Results showed volatile oil of *M. spicata* to be significantly effective in inhibiting biofilm formation. It was also observed that the biofilm formation by *P. mirabilis* decreased with increased concentration of the volatile oil. At a concentration of 30%, the biofilm inhibition percentage for *P. mirabilis* isolates P1, P2, P3, P4 ,P5 was 89,87,90,82 ,91% respectively for (Fig.1). Similarly, at 15% concentration, the inhibition percentage calculated was 62, 70, 75, 69, 65% respectively, and at 5% concentration, the percentage of inhibition was 47, 45, 51, 52 , 55% respectively for the 5 isolates studied (Fig.1).

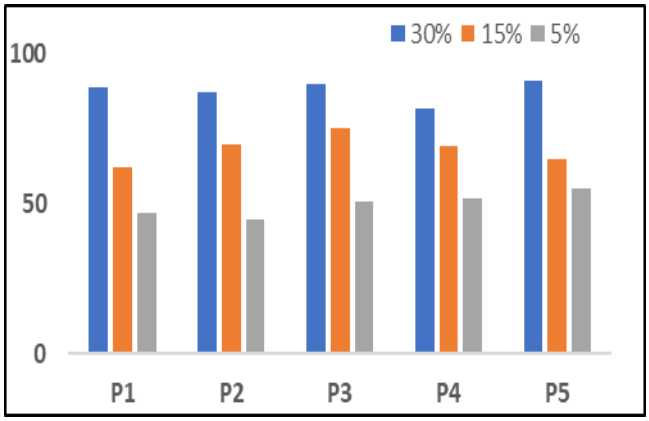

Fig. 1: Biofilm inhibition rates of *P. mirabilis* isolates using the volatile oil of *M. spicata*

Effect of natural menthol for *M. spicata* on biofilm formation in *P. mirabilis*

The natural compound menthol It has great inhibitory effect on the formation of the biofilm, as its production decreases with increase in the concentration of the natural compound menthol. The results indicate that the menthol compound has the ability to inhibit the biofilm of *P. mirabilis* at a concentration of 300 µg/ml, the inhibition percentage was (67, 72, 65,70, 64%) respectively, and at a concentration of 150 µg/ml the percentage of inhibition was (44, 40, 33, 36, 27%), respectively, but at a concentration of 100 µg/ml the inhibition percentage was (24, 20,15,18,13%) respectively and as shown in Fig. 2.

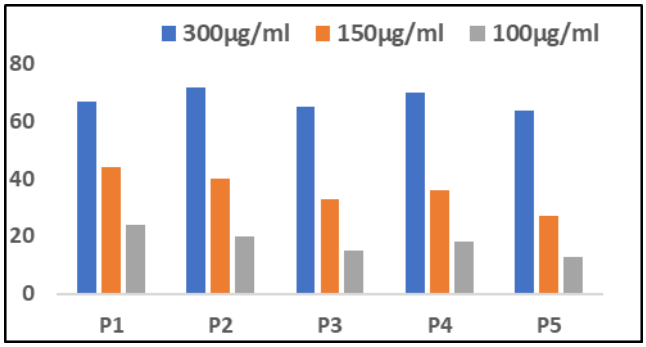

Fig. 2: Biofilm inhibition rates of *P. mirabilis* isolates using the natural menthol of *M. spicata*

DISCUSSION

Many diseases are caused by *P. mirabilis*, which is an opportunistic organism that moves to burn sites in the body and grows there because of the favorable conditions. Bacterial cells are protected from their surroundings by the layers of materials that make up biofilm. These materials include polysaccharides, fats, proteins, etc., and they are responsible for the development of multidrug resistance in bacteria (18). Bacterial resistance to antibiotics can be innate or acquired through mutations in the genes of bacterial cells (19), and the excessive use of these antibiotics is a major factor in the continuous increase in antibiotic resistance by bacterial strains that the world experiences. Although all *P. mirabilis* isolates were able to form biofilm, the amounts produced varied greatly between strains and sources (20). Isolates were collected from burns suffered by people of all ages and both sexes.

Oils of *M. spicata* contain biologically active compounds like gallocatechin, luteolin, catechins, hexadecanoic, caffeic acid, epigallocatechin gallate, rosmarinic acid, and isomenthone, which exhibit effective antimicrobial activity against many Gram-positive and Gram-negative bacteria like *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa* (22). Phenolic compounds in *M. spicata* have been to have antiviral properties, while *M. piperita* has been shown to contain luteolin, menthol, and rosmarinic acid which can act as an anti-herpes simplex virus agent (23). There was a significant decrease in the ability of the bacterial isolates to form the biofilm, which is consistent with the findings of a previous study (21) which demonstrated the effect of some volatile oils on the formation of biofilm by a group of positive and negative bacterial species. Our study also demonstrated that *M. spicata* menthol extract influences biofilm formation, which agrees with a previous study, which showed the effect of a menthol compound to inhibited biofilm formation by *Escherichia coli* (24).

CONCLUSION

P. mirabilis isolated from burn patients produce biofilms. *P. mirabilis* strains were resistant to most of the antimicrobial drugs used. While the aqueous and

alcoholic extracts of *M. spicata* did not inhibit biofilm formation by *P. mirabilis*, the volatile oil and natural menthol components of this plant species could inhibit biofilm formation by this bacterium.

CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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