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Antibacterial Activity of *Mentha piperita* (Peppermint) Oil against Wound Infections Caused by Carbapenem-Resistant Klebsiella in Rats

Fahad Mohammed Alturaiki[1-2*], Babu Joseph[1], Mohammed Asad[1]

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Saudi Arabia.
[2]Central Military Laboratory and Blood Bank Department of Microbiology Division, Prince Sultan Military Medical City, Riyadh 12233, Saudi Arabia

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***Corresponding author:** Fahad Mohammed Alturaiki
Central Military Laboratory and Blood Bank Department of Microbiology Division, Prince Sultan Military Medical City, Riyadh 12233, Saudi Arabia

Abstract

Drug resistant *Klebsiella pneumoniae* causes nosocomial infections, pneumonia, wound sepsis and infections of newborns and intensive care patients. In many cases antibiotics are no longer very effective in patients treated for *K. pneumoniae* infections. Various studies have shown antimicrobial properties of essential oils and different compounds that are part of them. This study aimed to determine the antibacterial efficacy and minimum inhibitory concentration of the *Mentha piperita* essential oil against resistant strain of *K. pneumoniae*. Efficiency of peppermint oil whenused with antibiotics in wound management as well as controlling *K. pneumoniae* infection was also investigated in this study. Well diffusion method was used to determine the antibacterial activity of peppermint oil, where its inhibitory capacity was observed along with antibiotics and compared with control. Bacterial load of *K. pneumonia OXA48 resistant* strain on wound in rat models was also studied and compared. *Klebsiella pneumoniae OXA48 and K. pneumoniae ATCC 700603* were inhibited by effectively by 40 µl/ml of peppermint oil in well diffusion assays. In ratmodels the wounds were healed, and epithelization was quicker in 10% peppermint oil treated wounds. Wounds applied with a combination of peppermint oil and Imipenem were more effectively protected from infection and showed better healing as well. Peppermint oil depicted both inhibitory capacities against *K. pneumonia OXA48* and promoted wound healing as well. Further studies regarding PMO and its efficacy in wound management as well as its associated molecular mechanism would provide better understanding regarding its potential.

Keywords: Excision wound, epithelization, essential oils, wound healing.

INTRODUCTION

Several bacteria have the ability to develop enzymatic mechanisms, to attain antibiotic resistance. In the family Enterobacteriaceae, the production of carbapenemase by *Klebsiella pneumoniae*, it is an emerging problem of high clinical importance. Carbapenemase is an enzyme that confers resistance to carbapenem antimicrobial. in addition, is capable of inactivating groups of antimicrobials that have a lactam ring in their structure (Iredell *et al.*, 2016) Research and knowledge about this enzyme are relevant in order to limit its dissemination, contributing to the reduction of morbidity and mortality rates linked to different infectious disease, during which microbiological surveillance is essential, together with the action of hospital infection prevention. The routine emergence of outbreaks in the hospital environment has been highlighted as a challenge to be considered, as it has become a difficult problem to solve, which affects both the healthcare institutions and patients who are hospitalized. In addition, there are social and economic impacts that are considerably applicable, due to the increasing the number of people, as well as the frequency of immunosuppressive conditions, with the introduction of new pathogens and increased resistance of microorganisms to antimicrobials (Avershina *et al.*, 2021). In this context, it is important to carry out additional studies that may help to development of safer clinical practices in the choice and indication of the use of antibiotics. In addition to allowing more knowledge about this resistance mechanism, in order to increase the

information required to carry out advanced research in this area.

To overcome the resistance towards conventional antimicrobials, searching for novel and cost- effective natural products has been increased (Stan *et al.,* 2021). Plant products such as extracts, oils and formulations have been used to treat a wide range of infections and alleviate symptoms (Yazarlu *et al.*, 2021). Peppermint oil extracted from the leaves of *M. piperita* is reported for anti- inflammatory, analgesic, diuretic antipyretic, antispasmodic, sedative properties and even used in aromatherapy (Sevindik, 2018) The presences of menthol, menthone, menthylacetate, limonene, pulegone, menthofuran, β-pinene, eucalyptol, isomenthone and other phytochemicals been reported in *Mentha piperita* for its medicinal properties (Mamadalieva *et al.,* 2020). The decrease in the effectiveness of conventional antibiotics, therefore, increases the need for prospecting forthe development of new compounds with antimicrobial activity. In this context, the use of essential oils appears as an alternative either to be used alone or with conventional antibiotics for the prevention as well as inhibition such drug resistant microorganisms (AlSheikh *et al.,* 2020). Essential oils are natural products synthesized by aromatic plants which are mixtures of organic compounds, present in different concentrations and usually possess bioactive characteristics (Nazzaro *et al.*, 2013). The antimicrobial and antibiofilm activity of essential oils including *M. piperita* oil, used alone or in combination with commercial antibiotics has been studied in several pathogenic bacteria (Kang *et al.*, 2019). The main constituents which are responsible for the oil's bioactivity are in most cases molecules from the class of terpenes, terpenoids and phenylpropanoids. Among these, terpenoids and phenylpropanoids are the main ones known to have antibacterial activity (Stephane *et al.*, 2020). Use of essential oil from *Mentha piperita* for inhibiting the growth of *K. pneumoniae* can provide crucial information regarding infection and biofilm formation prevention especially for drug resistant strains (Muntean *et al.*, 2019). Knowledge of KPC identification methods and strategies is of paramount importance in order to help reduce morbidity and mortality rates linked to infections, in addition to limiting its spread. Therefore, the present studies analyzed the phytochemical components of *M. piperita* by GM-MS analysis and investigate the antibacterial properties of peppermint oil. Evaluation of antimicrobial bioactivity of *M. piperita* peppermint oil against *K. pneumoniae* OXA48 was carried out.

MATERIALS AND METHODS

Bacterial culture

The bacterial cultures of *K. pneumoniae* OXA48 that showed carbapenem resistance and were available at department of Clinical Laboratory Sciences, College of Applied Medical Science was selected for this study. For the present study, *Mentha piperita* essential oil (Majestic Pure, CA, USA) was procured from scientific suppliers.

Screening of carbapenem-resistant *Klebsiella pneumonia*

The bacteria were subjected to an antibiotic sensitivity test to determine multidrug resistance using all conventional antibiotics used in treatment of *Klebsiella*. Amikacin (32), amoxicillin/K clav (16), ampicillin (16), cefepime (16), ciprofloxacin (2), colistin (2), doripenem (4), ertapenem (1), imipenem (2), levofloxacin (4), meropenem (8), minocycline (8), norfloxacin (1), tigecycline (1) Tobramycin (8). Antibiotic sensitivity was determined by the microscan system. Dilution trays were prepared according to the instruction manual. Negative combo panel containing antibiotics and biochemical agents were used for identification. Minimum inhibitory concentration was noted by the lowest or minimum concentration in which visible growth was inhibited. The trays were read by using autoSCAN-4 reader at 620, 560, 505,470, 440 and 590 nm and the results were recorded.

Antibacterial assay (well diffusion assay)

For determining the antibacterial activity of peppermint oil (PMO) well diffusion method was adopted. Bacterial cultures were grown on nutrient broth at 37°C and diluted to get 0.5 McFarland standard turbidity. These cultures were swabbed on Muller Hinton agar (MHA) using a sterile cotton swab. Wells were made on agar plate by using a cork borer and the bottom of the wells were sealed by a few drops of molten MHA. The wells were loaded with different concentrations of oil, antibiotics along with DMSO. DMSO served as a negative control and the plates kept in incubator at 37 °C for 24 hours. The zone of inhibition was measured and recorded.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Muller Hinton broth (4.5 ml) was inoculated with 0.5 ml of K. pneumonia OXA48 containing 1.5x108 CFU/ml (0.5 McFarland standard turbidity). The PMO at different concentration (1.25, 2.5, 5, 10, 20 µl/ml) in culture tubes was maintained for MIC determination. The tubes were incubated at 36±1 °C for 24 hours. The minimum concentration of PMO in which no visible growth was taken as MIC. The culture (10 µl) was inoculated into MacConkey agar and incubated at 36±1°C for 24 hours. The minimum concentration at which no bacterial growth is seen was taken as minimum bactericidal concentration.

Synergetic effect of antibiotics and extracts on MDR strains

Antagonistic and synergetic assay of the essential oil and antibiotics was evaluated by broth dilution method after determining the MIC. After serially diluting the antibiotic, and oil to its sub inhibitory concentration and the MIC were calculated. Wells with different antibiotic concentration, without oil was noted

as MIC for antibiotic. The fractional inhibitory concentration (FIC) index was calculated as follows:

FIC index = FIC of extract + FIC (antibiotic) FIC of extract = MIC of oil
FIC of antibiotic = MIC of antibiotic along with oil/ MIC of antibiotic alone

The synergetic potential was assessed as if FIC index is ≤ 0.5 the combination is synergetic; more than 0.5 and ≤ 2 the combination is indifferent and if the FIC index > 2, measured as antagonistic. Gas chromatograph mass spectrometric analysis of M. piperita oil. The GC-MS 7890A and 5975C VL MSD systems were chosen to analyze the *M. piperita* essential oil (Agilent Technologies, CA, USA). One funnel contained M. piperita essential oil (100 µl) and a water-ethyl-acetate mixture (250 µl). Forceful shaking was used to gather and concentrate the sample's top layer. Last additions of trimethylchlorosilane and trifluoroacetamide were made, followed by (BSTFA-99 µl + TMCS- µl), and finally the addition of the 10 µl of pyridine at the end, as the final step. After 30 minutes of heating at 60 degrees Celsius, the samples were transferred to GC vials. The samples were dried using liquid nitrogen before being dissolved in methanol and analyzed by GCMS. Membrane filter 0.22-m was used to remove the contaminants after dissolving 20 mg of HPLC-grade methanol in 5 ml of methanol. The sample was injected using an Agilent capillary column (DB5MS) with dimensions of 30 m, 0.25 mm internal diameter, and a film thickness of 0.25 microns (3 l). A temperature of 270 °C and a pressure of 80 kPa was maintained in the injector. Using hydrogen as a carrier gas, the GC procedure was completed in 25 minutes. The NIST mass spectral database and the collected mass spectra were used to identify the compounds.

Wound healing activity in rats (excision wound model): Albino Wistar rats of either sex weighing between 180 to 220 g were be selected for this study. Animals were provided with water and normal diet ad libitum. The ARRIVE recommendations for standard procedure were strictly adhered to. Precautionary steps were taken to avoid the spread of K. pneumoniae among test animals, and affected animals were maintained in a separate area. The Ethical Study Committee of Shaqra University (Approval number - 53/18911) examined and approved the research protocol. Researchers who weren't aware of the therapy performed all the measures. PMO formulation preparation: Using the fusion technique, a formulation was made with peppermint oil ointment with a 5 percent and 10 percent v/w concentration using soft paraffin (50 g), liquid paraffin (20 g), and emulsifying wax (30 g) (Nayeem *et al.*, 2021). Study of skin irritation: One day before the experiment, the rats had their dorsal skin shaved. On a 500 mm2 area of shaved skin, a PMO formulation (500 mg) was applied and then taped over. An untreated region was used as a reference point for the experiment. After 1, 24, 48 and 72 hours, the skin response was documented (Ekom *et al.*, 2022). Animals were divided to into groups of six animals each as follows.

Group I – infected with carbapenem-resistant K. pneumonia OXA48 control (treated with emulsifying base)
Group II- positive control (Standard drug-imipenem ointment)
Group III- peppermint oil (5% w/w in emulsifying base)
Group IV - peppermint oil (10% w/w in emulsifying base)
Group V- peppermint oil (10% w/w in emulsifying base) + imipenem ointment
Group VI – infected with carbapenem-resistant K. pneumonia OXA48

A mixture of ketamine (91 mg) and xylazine (9.1 mg) at a dosage of 1 ml/kg intraperitoneally was used to anaesthetize the rats on the day of the experiment (Al-Ghanayem *et al.*, 2022). The dorsal thoracic area of the animals was shaved and formed an imprint of 500 mm2 on the day of the experiment. The whole thickness of the skin was removed from the impressed region. An area of 500 mm2 was marked and the skin was excised. The dorsal thoracic region of the animals was shaved and made an impression of 500 mm2. The skin of the impressed area was excised to full thickness. An area of 500 mm2 will be marked and the skin will be excised (Mukherjee *et al.*, 2000). Carbapenem-resistant K. pneumoniae 1.5 x 108 CFU/ml) was inoculated into the freshly excised wound (Fayez *et al.*, 2021). Animals will be then housed individually in cages and will be given different treatments as mentioned above every day once starting two hours after inoculation of carbapenem-resistant K. pneumonia. Physicochemical, stability and diffusion properties of the formulation were evaluated and standardized (Kolhe *et al.*, 2018). Base formulated with soft paraffin, paraffin oil, and emulsifying wax was used as a control in untreated animals. The wound area was determined every 4 days till the scar formed falls off. Wound contraction (%) was determined in each group.

$$\text{Wound contraction (\%)} = \frac{\text{wound area day 0} - \text{wound area day } (n)}{\text{wound area day 0}} \times 100$$

The day of scar falling was considered as day of epithelization. The healed area of the wound was subjected to histological examination and for determination of CFU/g tissue.

Bacterial quantification assay

The tissue samples (1 g) collected on the final day of experiment and homogenized for 5 min using phosphate buffer saline (1 ml) under aseptic technique. Homogenates were serially diluted (up to 109) and plated on nutrient agar. Plates were incubated at 37 ºC and the colonies were counted accordingly.

Statistical analysis

The statistical analysis was carried out and the footnotes indicate that the values are presented as mean SEM. Using SPSS, one-way ANOVA and Tukey's post-test were used to determine statistical differences between the groups (version 20 for Windows).

RESULTS

Klebsiella pneumoniae OXA48 and K. pneumoniae ATCC 700603 was inhibited by peppermint oil in well diffusion assays on Muller Hinton agar (Table 1). Other carbapenem-resistant strains of *Klebsiella pneumoniae NDM and K. pneumoniae KPC* were not inhibited by peppermint oil. Essential oil was diluted in 10% DMSO. DMSO alone served as control that showed absence of inhibition.

Table 1: Zone of inhibition shown by peppermint oil against different strains of KBC

Bacterial strain	Zone of inhibition (mm)
Klebsiella pneumoniae NDM	0
Klebsiella pneumoniae KPC	0
Klebsiella pneumoniae (Clinical isolate)	0
Klebsiella pneumoniae OXA48	12
Klebsiella pneumoniae ATCC 700603	15
DMSO 10% (Negative control)	0

Table 2: Peppermint oil in different concentrations and combinations against carbapenem- resistant *K. pneumonia* OXA48

Antibacterial agent	Zone of inhibition (mm)
Peppermint oil (20 µl/ml)	09
Peppermint oil (40 µl/ml)	12
Vancomycin (2 mcg)	17
Vancomycin + Peppermint oil (40 µl/ml)	17
Imipenem (4 µg/ml)	18
Imipenem (4 µg/ml) + Peppermint oil (40 µl/ml)	18

Peppermint oil at different concentrations were used to determine the antibacterial activity (Table 2). Peppermint oil at 20 µl/ml and 40 µl/ml were tested for antibacterial activity against carbapenem-resistant *Klebsiella pneumonia.* Among them, the higher concentration at 40 µl/ml were more effective than lower concentrations (Figure 1). Apart from this peppermint along with antibiotics such as vancomycin and imipenem has also been studied for qualitative assay of antibacterial activity. From the results it is clear that there was no interactive activities between antibiotics and peppermint oil.

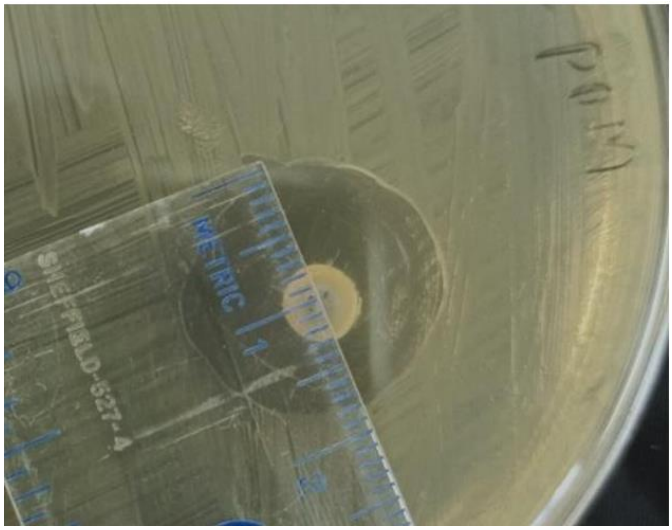


Figure 1: Zone of inhibition shown by carbapenem-resistant *K. pneumonia* OXA48 in presence of peppermint oil along with imipenem.

Table 3: Antibiotic resistance profile of carbapenem-resistant *K. pneumonia*OXA48

Antibiotic	Concentration (mcg/ml)	Result
Amikacin	32	Resistant
Amoxicillin/K Clav	16	Resistant
Ampicillin	16	Resistant
Cefepime	16	Resistant
Ciprofloxacin	2	Resistant
Colistin	2	Intermediate
Doripenem	4	Resistant
Ertapenem	1	Resistant
Imipenem	2	Intermediate
Levofloxacin	4	Resistant
Meropenem	8	Resistant
Minocycline	8	Resistant
Norfloxacin	1	Resistant
Tigecycline	1	Sensitive
Tobramycin	8	Resistant

Carbapenem-resistant *K. pneumoniaOXA48* showed high resistant pattern to all the antibiotics. In this view, the antibiotic resistance profile of the carbapenem-resistant *K. pneumoniaOXA48* was studied. The strain showed resistant pattern towards most of the antibiotics tested and intermediate towards colistin and imipenem (Table 3).

Table 4: Minimum inhibitory concentration of peppermint oil against carbapenem-*resistant K. pneumonia*OXA48

Peppermint essential oil (µl/ml)	Visible growth (MIC)	Growth in nutrient agar (MBC)
40	--	--
20	--	+
10	+	+
5	+	+
2.5	+	+
1.25	++	+
0.62	++	+

Muller Hinton broth was used to determine the MIC of peppermint oil against carbapenem- resistant *K. pneumonia*OXA48. A concentration of 20 µl/ml was found to be the MIC and 40 µl/ml was found to be the minimum bactericidal concentration of peppermint oil against carbapenem- resistant *K. pneumonia*OXA48 (Table 4).

Table 5: The fractional inhibitory concentration of peppermint oil with different concentrations of antibiotics

Bacterial pathogens	Fractional inhibitory concentration				Remarks
	MIC Oil (µl/ml)	MIC of Imipenem (mg/ml)	MIC of Imipenem +oil	FIC Index	
K. pneumonia OXA48	20	0.004	0.004	1.0002	Indifference

FIC indices were calculated using the formula: FIC index = (MIC of oil in combination/MIC of oil alone) + (MIC of antibiotics in combination/MIC of antibiotics alone). The synergetic potential will be assessed as if FIC index is ≤ 0.5 the combination is synergetic; more than 0.5 and ≤ 2 the combination is indifferent and if the FIC index > 2, it is considered as antagonistic. The fractional inhibitory concentration of the peppermint oil along with antibiotic to evaluate the synergetic and antagonistic effect was carried out. The peppermint oil showed antibacterial effect when tested separately, however there was no antagonistic or synergetic effect when combined with imipenem (Table 5).

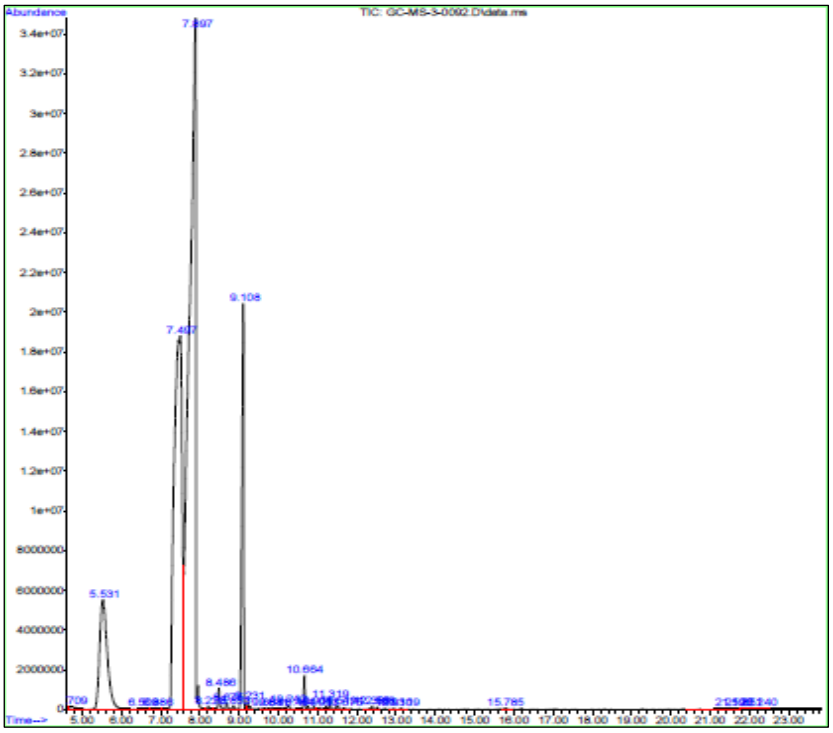


Figure 2: GCMS of peppermint oil showing major peaks

Table 6: Major components analyzed by GCMS of peppermint oil

Peak	Retention Time	Area (%)	Name
1	4.709	0.20	Bicyclo[3.1.1]heptane
2	5.531	9.20	Limonene
3	6.509	0.06	1,6-Octadien-3-ol
4	6.886	0.05	Cyclohexanol
5	7.497	33.13	Cyclohexanone
6	7.897	47.76	Menthol
7	8.220	0.05	3-Octanone
8	8.486	0.03	Pulegone
9	8.675	0.010	2-Cyclohexen-1-one
10	8.853	0.06	Bicyclo[4.1.0]heptane
11	9.108	7.55	Menthyl acetate
12	9.231	0.12	1-Methoxy-3-methyl
13	9.664	0.03	2-Propenoic acid
14	9.831	0.03	Phenol, 2-methoxy-4-(1-propenyl)
15	10.242	0.16	Cyclobuta[1,2:3,4]dicyclopentene
16	10.464	0.02	cis-1,4-Dimethyl-2-methylenecycl
17	10.664	0.44	Caryophyllene
18	10.908	0.04	1,6,10-Dodecatriene, 7,11-dimethyl
19	11.053	0.03	alpha.-Caryophyllene
20	11.319	0.13	1,6-Cyclodecadiene, 1-methyl-5-m
21	11.519	0.08	2(4H)-Benzofuranone, 5,6,7,7a-tetra hydro
22	11.675	0.03	Naphthalene
23	12.386	0.05	Caryophyllene oxide
24	12.508	0.03	Azulene
25	12.930	0.02	Cyclopentaneacetic acid
26	13.119	0.02	Cycloheptyl N.N-dipropylphospho-
27	15.785	0.03	di-(+)-menthyl-(-)-methyl salicylate
28	21.596	0.18	2-Methyl-7-phenylindole
29	21.851	0.02	2-Amino-4,4,6,6-tetramethyl-4,6
30	22.240	0.03	Silane

The gas chromatography mass spectrophotometry has been carried out to determine the components present in the oil (Figure 2). The major components were identified as limonene, cyclohexanone, menthol, menthyl acetate and caryophyllene (Table 6). There were some minor peaks on the chromatogram and the compounds present in trace amount were beta-pinene (monoterpene); 1,6-Octadien-3-ol, 3,7-dimethyl (acyclic monoterpenoids); 3-Octanone (dialkyl ketone); Bicyclo [4.1.0] heptane; 2-propenoic acid (unsaturated carboxylic acid); Eugenol (phenylpropanoid) etc.

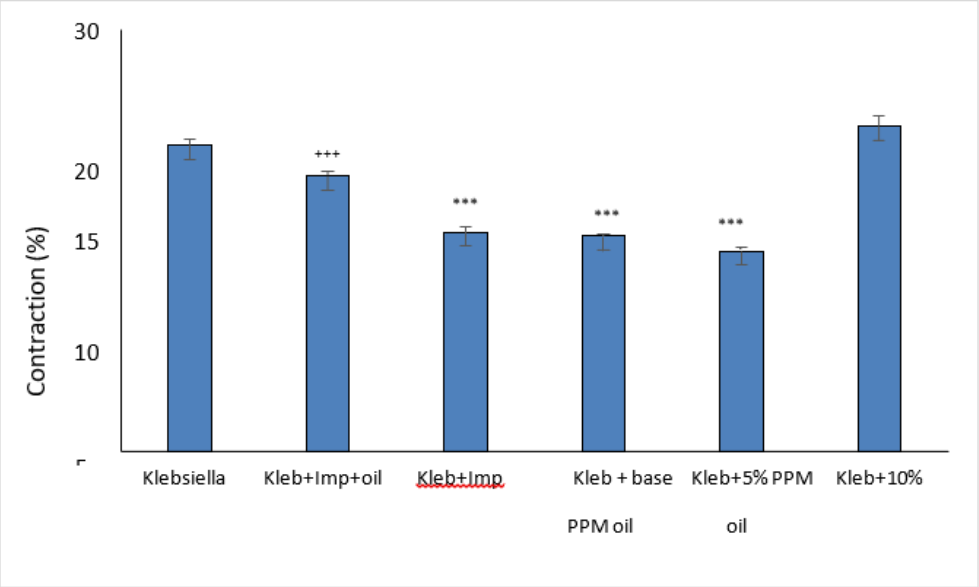


Figure 3: Effect on the period of epithelization in carbapenem-resistant *Klebsiella pneumonia* infected wounds in rats All values are mean±SEM, n=6, ***P<0.001 when compared to control (infected); +++P<0.01 when compared to base.

Erythema or inflammation was not observed till 72 h after the application of the PMO formulation on de-haired skin during skin irritation test. The period of epithelization on wounds were determined. The inoculation of wounds by carbapenem-resistant *K. pneumonia* significantly reduced the epithelization period. The wounds were healed, and epithelization were quicker in 10% peppermint oil treated wounds. However, its effect was lesser when compared to imipenem treated wounds and the wounds applied with a combination of peppermint oil and imipenem. The epithelization in combination treated wounds were little lesser (14 days) when compared to both imipenem (15 days) and peppermint oil (15 days) separately (Figure 3).

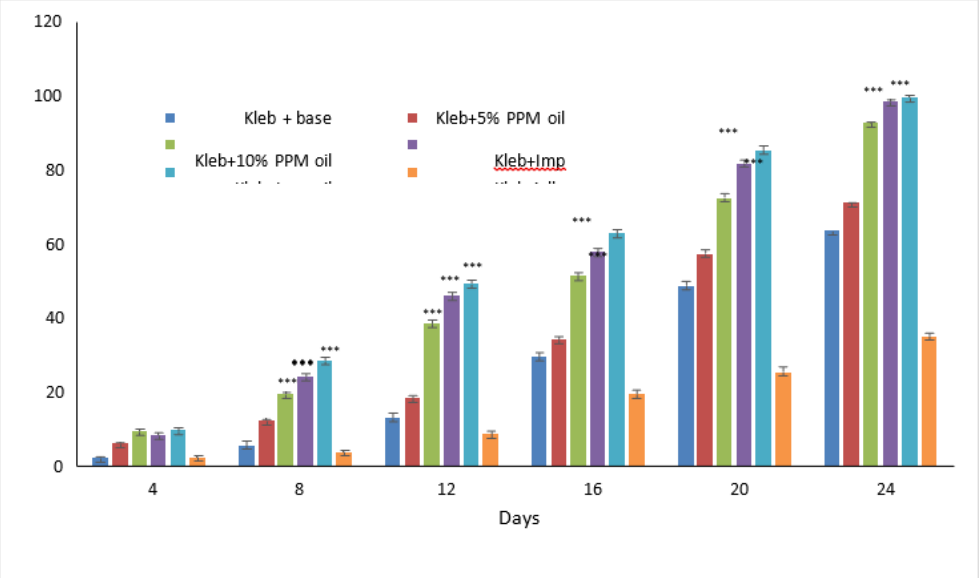
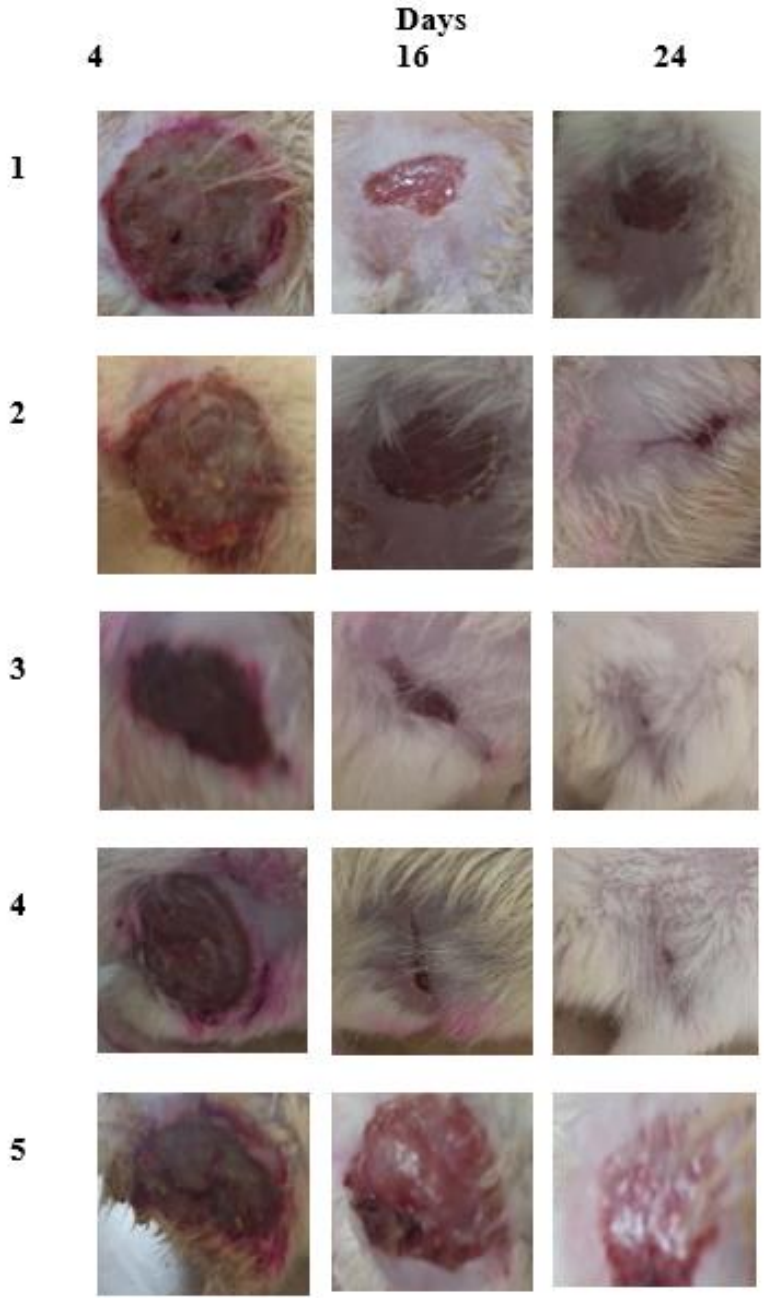


Figure 4: Peppermint oil preparation on the percentage of wound contraction in carbapenem- resistant *K. pneumonia* infected wounds. All values are mean ±SEM, n=6, *P<0.05, **P<0.01,
***P<0.001 when compared to control (infected). Imp- imipenem (4mcg/ml).

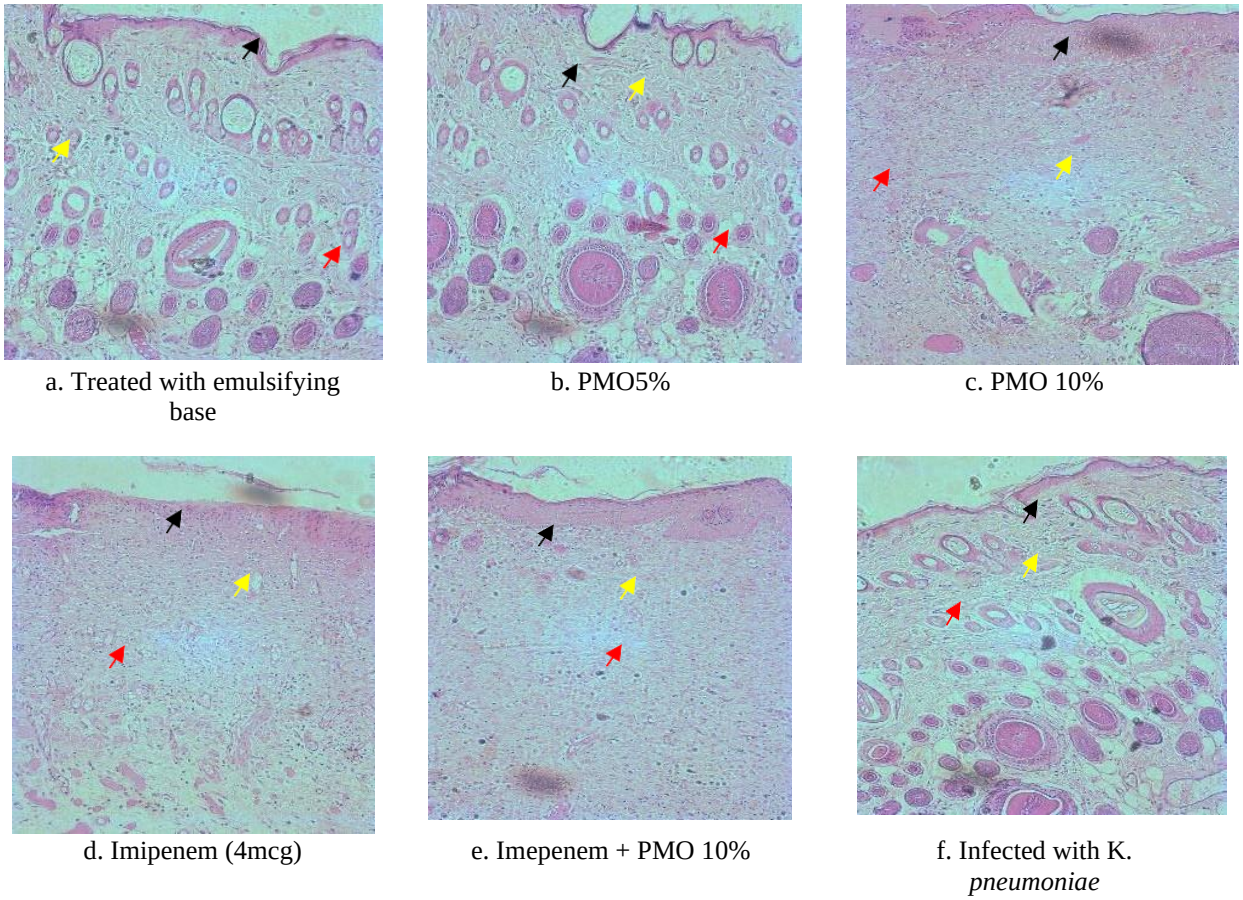
The wound contraction was measured at 4 days intervals up to 24 days. *K. pneumonia* in diced severe infection on the wounds that showed as fluid oozing out of the wounded tissues. *K. pneumonia* produced a severe infection in all animals including the control group succumbing to the infection during first three to five days. More rats had to be added to each group to bring the number to the required sample size. The lower concentration (5 % v/w) of PMO did not produce much wound healing action on infected models whereas, the higher concentration (10% v/w) showed wound healing activity after 8 days. Animals treated with imipenem showed significant wound contraction from the 4th day onwards (Figure 4). However, there was a significant increase in the healing of wound treated with PMO 10% and imipenem in combination. There was a delay in wound healing (below 50%) even after 20 days in *K. pneumonia* infected when compared with other treated models (Figure 4).



1. K. pneumonia + base;
2. K. pneumonia 10% PPO;
3. K. pneumonia +Imipenem;
4. K. pneumonia + Imipenem +PPO
5. K. pneumonia infected

Figure 5: Images of wounds infected and treated with different concentrations of PMO along with control

In the histological examination worn-out epidermal layer was observed in the tissues from *K. pneumonia* infected control animals compared to the PMO and antibiotic treated animals. all the treatments, imipenem and PMO combination showed better healing followed by higher dose of PMO (10% v/w) with least effect observed with PMO lower dose (5% v/w). As expected, the epidermal layer was broken in control animals infected and not subjected for treatments, with a high density of inflammatory cells and less number of capillaries. Imipenem and higher concentration of PMO (10% w/w) and also in combination increased epidermal regeneration, capillary formation (Figure 4.6).



a. Treated with emulsifying base | b. PMO5% | c. PMO 10%

d. Imipenem (4mcg) | e. Imepenem + PMO 10% | f. Infected with K. *pneumoniae*

Figure 6: Photomicrographs of regenerated epithelium in several groups. The epidermis is represented by black arrows, capillaries by yellow arrows, and inflammatory cells by red arrows. Normal animals have a high epidermal height with many capillaries.

Table 7: The bacterial load (CFU/g) in the skin tissue after treatment

Treatment	Bacterial load (CFU/g of tissue)
Carbapenem-resistant *K. pneumonia* infected wounds +base	89 X 10^9
Carbapenem-resistant *K. pneumonia* infected wounds +5% PMO	17 X10^3
Carbapenem-resistant *K. pneumonia* infected wounds +10% PMO	13 X 10^2
Carbapenem-resistant *K. pneumonia* infected wounds +Imipenem	2.7 X 10^2
Carbapenem-resistant *K. pneumonia* infected wounds +imipenem +10% PMO	1.9 X 10^2
Carbapenem-resistant *K. pneumonia* infected wounds	102 X 10^9

The bacterial count in the infected wounds was more in control groups compared to higher dose of the PMO (10% w/w) and imipenem. However, no such effect was seen with lower dose of the PMO (5% v/w) when compared with high concentration. The microbial load in *K. pneumonia* infected control was around 102 x 10^9 CFU/g while in the 5% PMO treated group; it was 174x10^3 CFU/g. The effect was more in the 10% PMO treated group, where it was reduced to about 13x10^2 CFU/g and in the Imipenem treated animals, it was around 2.7x10^2 CFU/g tissue. The pathogen load was significantly reduced to 1.9 x 10^2 in wounds treated with a combination of imipenem and PMO 10% v/w. However, the bacterial count was almost similar to the untreated wounds; 89 x 10^9 CFU/g. the reduction in bacterial count shows that the PMO was effective in

controlling the growth and spread of *K. pneumonia* in wounds (Table 7).

DISCUSSION

In the present research, wound healing was examined in relation to the formulation of PMO. In-depth research of the antibacterial effect showed *K. pneumonia* that are known to infect the wounds. We have investigated the effect of PMO on different strains of *K. pneumoniae* that are showing carbapenem resistance. Antibacterial activity and histological studies were done to support its wound healing potential and infection control ability. Essential oils enriched in phytochemicals have been reportedly effective for improving wound healing as well as reducing the chances of further infections. Tea tree oil, oregano oil, clove oil and lavender oil are more commonly investigated for their efficacy in preventing the infection along with wound healing (Kennewell *et al.*, 2019). The usual mainstay of wound management involves antibiotics which combat only one aspect to prevent any further infection; however this has been impacted due to strong emergence of resistant bacterial strains. Biological effects of essential oils are mediated by innate immune response; studies have shown better healing in human patients as well where dressings are done using water miscible oils which effectively prevent resistant bacterial infection (Samuelson *et al.*, 2020). PMO has also been studied before but its effect only as an antimicrobial are mostly reported in previous studies. PMO's antibacterial, anti-inflammatory, analgesic, antioxidant, fungal, and anticancer capabilities are only a few of the numerous documented pharmacological effects (Chaemsanit *et al.*, 2019). Analysis using GC-MS enabled to identify several of the oil components. Evaporation occurs above 60°C for the majority of essential oil components. Because organic volatile ingredients are identified by GC-MS, the oil's vast majority of chemicals were also discovered using this method.

For the formulation of ointments, there are a variety of options. The British Pharmacopoeia's emulsifying ointment recipe was employed (Al-Ghanayem *et al.*, 2022). The prepared ointment was tested on an agar medium to validate the diffusion of active ingredients. The ointment's active elements were able to diffuse across Muller Hinton agar, showing that the ointment contains active ingredients (data not shown). A literature review was conducted to identify the most often encountered and relevant microorganisms for in vitro antibacterial and wound infection.Carbapenem-resistant Enterobacteriaceae (CRE) like *K. pneumoniae* (CRKP) are a growing source of antibiotic-resistant infections in healthcare settings. With regard to *K. pneumoniae*, peppermint oil has been found to have antibacterial properties (Mohamed *et al.*, 2018). The range of antimicrobial effects in antibiotic resistant strains may be identified by studying the impact of oil on these bacteria. Gram-negative bacteria *Listeria monocytogenes* Scott A and *Staphylococcus aureus* ATCC 25923 were successfully treated with PMO (Liang *et al.*, 2012). PMO helped in healing wounds quickly while inflicting little pain on the skin. Histological testing and wound contraction, for example, both indicate a healing stage. Healing progress is shown by wound contraction, but complete wound healing is signified by wound epithelization (Sardari *et al.*, 2007). When used in greater concentrations and in conjunction with antibiotics on infected wounds, PMO formulation improved wound healing. For infected wounds without antibiotics, its effects were not as strong as for healthy ones, indicating that it may be less effective without them. Gram-negative bacteria have cell walls that are resistant to chemicals and natural compounds, according to previous study.

The lipopolysaccharide layer in the cell wall and the periplasmic gap make Gram negative bacteria more resistant to antimicrobial treatments than Gram positive bacteria. In order to validate the macroscopic results, histological microscopic exams determined the epidermal height, capillary density, and presence of inflammatory cells. The cytoplasm, nucleus, and extracellular matrix are all present in the H&E stained samples (A. H. Fischer *et al.*, 2008). An increase in capillary density suggests angiogenesis, which is critical for healing blood flow, whereas an increase in epidermal height denotes epithelial regeneration. Wound healing is delayed and incomplete beyond 20 days because of the presence of inflammatory cells in the wound such as macrophages, neutrophils and plasma cells (Schultz *et al.*, 2011). Animals administered with a PMO formulation had wounds that healed more quickly. After topical administration of peppermint oil, wound healing was shown to be enhanced by a variety of processes. The findings also imply that PMO may be less effective at low concentrations in treating *K. pneumoniae*-infected wounds than previously thought. An unknown chemical component is responsible for each of these side effects. Although no one molecular entity may be responsible for all of the claimed effects, it is possible that essential oils include a varied array of chemical compounds. It is possible to learn more about wound healing by experimenting with a variety of chemical substances for each of these functions. Recent studies have also investigated essential oil like lavender oil in clinical context of wound healing where its role has been noted not only in infection prevention but its possible involvement with fibroblast differentiation and collagen synthesis as well in rat models (Mori *et al.*, 2016). Our findings shows clearly enhanced healing with topical application of PMO along with Imipenem, however further studies to understand its possible molecular mechanisms which might be aiding immune response or cellular differentiation will bring better insights on PMO’s efficacy.

CONCLUSION

Our finding depicts showed antibacterial activity of peppermint oil against carbapenem-resistant

K. pneumonia OXA48. A clear zone of inhibition was observed at the site of bacterial growth where peppermint oil was added along with the Imipenem. The results also showed higher efficiency with increased concentration of peppermint oil suggesting its potential as dosage compensated approach for known site of infection. This study also assessed its interaction with Imipenem when applied topically at the wound site. However, peppermint oil does depicted any interaction with Imipenem either *in-vitro* or *in-vivo conditions.* Experiments performed in rat model also showed positive results, topical application of peppermint oil aided in healing the wound and contraction rate of wound was also higher at the wound sites infected with bacteria in rat skin where peppermint oil was added. The application of PMO reduced both the period of epithelization and the bacterial load in wounds compared to control.Essential oil extracted from Mentha Piperita might have components which functions as active ingredient against bacterial infection. Further studies are needed to specifically identify this component/s for evaluating its therapeutic option against frequent infection caused by carbapenem-resistant *K. pneumonia OXA48*. Components of PMO responsible for growth inhibition of carbapenem-resistant *K. pneumonia OXA48* can provide better insight in its possible clinical usage. Recent developments in wound management strategies has shown essential oils containing biopolymer dressings are becoming promising options for better treatment in chronic wounds (Pérez-Recalde *et al.*, 2018). Further studies regarding PMO and its efficacy in wound management as well as its associated molecular mechanism would provide better understanding regarding its potential.

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