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Original Article

Anti-bacterial activity of essential oils against multidrug-resistant foodborne pathogens isolated from raw milk

Atividade antibacteriana de óleos essenciais contra patógenos alimentares multirresistentes isolados de leite cru

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Abstract

The presence of pathogenic bacteria in food is considered as a primary cause of food-borne illness and food quality deterioration worldwide. The present study aimed to determine the effectiveness of five essential oils (EOs) against multidrug-resistant foodborne pathogens. In the current study Gram-negative bacteria (*Escherichia, Enterobacter, Citrobacter, Proteus, Pseudomonas, and Klebsiella*) and the Gram-positive bacteria *Staphylococcus* were isolated from raw milk and biochemically characterized. The anti-bacterial effect of different antibiotics and EOs (thyme, oregano, lemongrass, mint, and rosemary) was determined using the standard disc diffusion method. The antibiogram study revealed that Gram-negative bacteria were highly resistant to penicillin while *Staphylococcus* was resistant to streptomycin, amoxicillin, and lincomycin. Moderate resistance was observed to doxycycline, amikacin, enrofloxacin, kanamycin and cefixime. Isolates were found less resistant to gentamycin, chloramphenicol, and ciprofloxacin. EOs showed a broad range of antimicrobial activity against all bacteria except *P. aeruginosa*. Of these, thyme was more effective against most of the multi-drug resistant bacterial strains and formed the largest zone of inhibition (26 mm) against *Escherichia* followed by oregano oil (18 mm) against *Staphylococcus* ($p<0.05$). *Klebsiella* spp and *Citrobacter* spp showed resistance to mint and lemongrass oil respectively. The EOs such as lemongrass, mint and rosemary were less active against all the bacteria. The findings of the recent study suggest the use of EOs as natural antibacterial agents for food preservation.

Keywords: pathogenic bacteria, zone of inhibition, antibacterial agents, lemongrass oil, aldehydes.

Resumo

A presença de bactérias patogênicas em alimentos é considerada a principal causa de doenças transmitidas por alimentos e deterioração da qualidade dos alimentos em todo o mundo. O presente estudo teve como objetivo determinar a eficácia de cinco óleos essenciais (OEs) contra patógenos de origem alimentar multirresistentes. No presente estudo, bactérias Gram-negativas (*Escherichia*, *Enterobacter*, *Citrobacter*, *Proteus*, *Pseudomonas* e *Klebsiella*) e as bactérias Gram-positivas *Staphylococcus* foram isoladas do leite cru e caracterizadas bioquimicamente. O efeito antibacteriano de diferentes antibióticos e OEs (tomilho, orégano, capim-limão, hortelã e alecrim) foi determinado usando o método padrão de difusão em disco. O estudo do antibiograma revelou que as bactérias Gram-negativas eram altamente resistentes à penicilina, enquanto o *Staphylococcus* era resistente à estreptomicina, amoxicilina e

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lincomicina. Foi observada resistência moderada à doxiciclina, amicacina, enrofloxacina, canamicina e cefixima. Os isolados foram encontrados menos resistentes à gentamicina, cloranfenicol e ciprofloxacina. Os OEs mostraram uma ampla gama de atividade antimicrobiana contra todas as bactérias, exceto *P. aeruginosa*. Destes, o tomilho foi mais eficaz contra a maioria das cepas bacterianas multirresistentes e formou a maior zona de inibição (26 mm) contra *Escherichia* seguido de óleo de orégano (18 mm) contra *Staphylococcus* ($p<0,05$). *Klebsiella* spp e *Citrobacter* spp apresentaram resistência ao óleo de menta e capim-limão, respectivamente. Os OEs como capim-limão, hortelã e alecrim foram menos ativos contra todas as bactérias. Os resultados do estudo recente sugerem o uso de OEs como agentes antibacterianos naturais para conservação de alimentos.

Palavras-chave: bactérias patogênicas, zona de inibição, agentes antibacterianos, óleo de capim-limão, aldeídos.

1. Introduction

Milk and milk-related products are good sources of nutrition for humans. However, its high water, protein and vitamin contents, and neutral pH provide excellent conditions for microbial growth which can happen in raw, pasteurized, or refrigerated milk. Bacteria significantly affect milk quality and its quantity by releasing toxins which increases the risk of food poisoning and infections (Bytyqi et al., 2013; Hernández-Cortez et al., 2017; Mankai et al., 2012). Many of these illnesses are caused by *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* (toxin-producing) (Addis and Sisay, 2015; Bintsis, 2017; Havelaar et al., 2015). These bacteria interact with antibiotics which can be present in the food system and develop resistance. Antibiotic resistance among foodborne pathogens is a worldwide problem. This problem arises due to the extensive use of antimicrobial feed additives for therapeutic purposes and as a growth enhancer for animal production (Baynes et al., 2016; Manyi-Loh et al., 2018; Moyane et al., 2013). With the increase of drug resistance, the efficacy of several drugs and antimicrobial agents has been reduced significantly. Therefore, regulatory authorities which monitor the food and beverage industries urge the use of natural food preservatives. Essential oils (EOs) are aromatic oily materials that can be extracted from different parts of the plant. Their chemical composition often includes terpenoids (specifically monoterpenes and sesquiterpenes) and low molecular weight compounds (alcohols, aldehydes, ketones, lactones, acetyls, oxides, esters and phenols). EOs are known to have antibacterial, antifungal, antiviral, anticancer and antioxidant effects (Fitsiou and Pappa, 2019; Helal et al., 2019; Nadjib, 2020; Wińska et al., 2019). Moreover, they are non-toxic to humans and the environment and are believed to have limited chances for the development of resistance in bacteria (Chouhan et al., 2017; Yap et al., 2014). The antibacterial activity (in vitro and food assays) of EOs have been reported against several food borne pathogens such as *L. monocytogenes*, *Staphylococcus*, *S. enterica*, *E. coil*. *Pseudomonas. aeruginosa* and *Candida albicans* (Mittal et al., 2019; Puškárová et al., 2017; Santos et al., 2017). Some EOs can control food fermentation by controlling *Lactobacillus plantarum* and *Saccharomyces cerevisiae* growth (Liu et al., 2017; Mith et al., 2014). Their bactericidal mechanisms include hydrophobic interactions with bacterial cell envelopes, coagulating of membrane protein and destruction of membrane potential which result in cellular death (Chouhan et al., 2017). Although the antibacterial activity of EOs has been reported against large numbers of foodborne pathogens, there are limited reports on the activity of EOs against multi drug-resistant (MDR) foodborne pathogens. The present study aimed to determine the activity of five EOs against MDR bacteria isolated from raw milk from Quetta valley of Baluchistan.

2. Materials and Methods

2.1. Study area and sampling

The study was carried out at Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan, Quetta, Pakistan. Geographically representative milk samples (n=48) from different markets of north, south, east and west regions of Quetta city were collected during January 10 to January 18, 2022. Samples (12 each from the four regions) were collected into sterile glass bottles and transported to the Microbiology laboratory at the Center for Advanced Study in Vaccinology and Biotechnology (CASVAB) via a cold chain for bacterial analysis.

2.2. Isolation and purification of bacteria

For isolation of the Gram-negative bacterial strains, milk samples (0.01 mL) were spread aseptically on different selective and differential media i.e., Eosine Methylene blue agar (LAB, Heywood, UK) for Escherichia, MacConkey agar (LAB, Lancashire, UK) for *coliform* and *Proteus* spp, and Cetrimide agar (Oxoid, Basingstoke, UK) for Pseudomonas spp. They were further identified through biochemical tests including indole production (Oxoid, Basingstoke, UK), glucose metabolism with the methyl red (Merck, Darmstadt, Germany), acetone production using *Voges proskauer* (Merck, Darmstadt Germany), the simmon citrate utilization (BBL, Sparks, MD, USA), triple sugar iron (LAB, Heywood, UK), catalase, oxidase, urease and motility tests. Moreover, coagulase test, catalase test, indole production, methyl red test, Voges-proskauer reaction, urease production, citrate utilization were also utilized to identify *Staphylococcus*. In addition, for isolating of *Staphylococcus*, the loop full (0.01 mL) of the milk sample was spread on mannitol salt agar (MSA) (Oxoid, Basingstoke, UK) and incubated aerobically at 37°C for 24 h. The isolated bacteria were preserved in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) with the addition of 30% glycerol at -70°C for further experiments.

2.3. Antibiotic sensitivity assay

The antibiogram assay used the disc diffusion method. Bacterial strains were dispensed in tubes containing 5 mL normal saline and the turbidity was adjusted to a McFarland turbidity standard of 0.5 yielding 1.5 × "108" CFU/mL. Cotton

swabs were soaked and spread onto the Muller Hinton Agar ((MHA), Oxoid, UK). The antibiotic discs (Oxoid, Basingstoke, UK) amikacin (30µg), amoxicillin (30µg), cefixime (5µg), chloramphenicol (5µg), ciprofloxacin (5µg), doxycycline (30µg), enrofloxacin (5µg), gentamycin (10µg), lincomycin (10µg), kanamycin (30µg), methicillin (10µg), penicillin G (10 Unit), streptomycin (10µg), sulphamethoxazole trimethoprim (25µg), tetracycline (30µg), and vancomycin (30µg) were used and aerobically incubated at 37 °C for 24 hours in an inverted position. After incubation, the antibiotic sensitivity and resistance pattern of each strain was studied. The clear zone around each disc was measured. The results were recorded according to the Clinical and Laboratory Standards Institute (Weinstein and Lewis II, 2020).

2.4. Selection of plants

Five herbal plants including Thyme (*Thymus vulgaris*), Oregano (*Oregano vulgare*), Rosemary (*Rosemarinus officinalis*), Mint (*Mentha spicata*), and Lemongrass (*Cymbopogon citratus*) were selected to investigate their antimicrobial potential. All these plants were collected from Arid Zone Research Center (AZRC) Quetta, Pakistan.

2.5. Extraction of essential oils

Fresh ariel leaves were collected from plants and chopped into pieces. Approximately 250 g leaves of each plant were used for steam distillation in a Clevenger type instrument for three (3) hours. A light denser layer of oil developed in a burette at the surface of the water and was carefully separated and the yield was calculated. Extracted EOs were stored at 4°C in a sealed vial covered with aluminium foil till further use (Baj et al., 2015).

2.6. Antibacterial assay of essential oils

Three antibiotic-resistant strains of each bacterial species were selected for this assay. The antibacterial effect of EOs in the liquid phase was analyzed through the disc diffusion method 25. As described above 100 µL of 0.5 McFarland (yielding "108" CFU/mL) were applied to the Muller Hinton Agar (Oxoid) plates. A pre-sterilized Whatman filter paper (6 mm diameter) was then placed on the plates. Afterwards, 10 µL of each EO was added to the Whatman filter paper. The plates were allowed to dry for 30 to 60 minutes at room temperature under aseptic conditions and then incubated in an inverted position at 37 °C for 24 h. After incubation, the antibacterial activity of EOs in the form of the clear zone (in millimetre) around the disc was measured (Fernandez-Lopez et al., 2005).

2.7. Statistical analysis

Data with three repeated measurements (antimicrobial activity of oils) was analyzed and calculated using the one-way ANOVA test setting the $p < 0.05$.

3. Results

3.1. Incidence of pathogenic bacteria in raw milk

Bacterial species including *Escherichia, Enterobacter, Citrobacter, Proteus, Pseudomonas, and Klebsiella* and *Staphylococcus* were identified in the raw milk samples. Among all the isolates, Escherichia was the most abundant contaminant of raw milk with a 27% prevalence. The prevalence of *Escherichia, Enterobacter, Citrobacter, Proteus, Pseudomonas, and Klebsiella* and *Staphylococcus* species were approximately 18%, 15%, 13%, 13%, 13%, 10%, and 4% respectively (Table 1).

3.2. Antibiotic resistance pattern

Variable trends of antibiotic resistance were shown by both types of (Gram-negative and Gram-positive) bacteria identified from raw milk (Figure 1). For example, *Escherichia* was resistant to amoxicillin, penicillin G, streptomycin (100%), lincomycin (62%), kanamycin (46%), doxycycline and sulfamethoxazole-trimethoprim (31%). While, less resistance of *Escherichia* was also observed to cefixime (23%), chloramphenicol, gentamycin, and tetracycline (15% each), and enrofloxacin (8%). Moreover, all the strains of *Escherichia* were sensitive to ciprofloxacin (Figure 1). It is previously acknowledged that in Gram-negative bacteria, different factors like a change in hydrophobic properties or mutations in porins can create the MDR (Breijyeh et al., 2020), therefore, might be due to this reason, here in our study, the Gram-negative bacteria showed the maximum MDR.

3.3. Antibacterial efficacies of essential oils against drug-resistant strains

The highest yield of rosemary (*Rosemarinus officinalis*, 0.89%) followed by lemongrass (*Cymbopogon citratus*, 0.80%), oregano (*Oregano vulgare*, 0.78%), mint (*Mentha spicata*, 0.71%) and thyme (*Thymus vulgaris*, 0.67%) plants were determined (Table 2).

EOs were categorized as being effective based on previously published study by Celikel and Kavas (2008). According to which, if the total diameter was <8 mm the organism was resistant, inhibition zones of 9 to 14 mm sensitive (+), and 15 to 19 mm very sensitive (++), for diameter > 20 mm extremely sensitive (+++).

Thymus vulgaris (thyme) was most active among all tested EOs and formed the largest zone of inhibition of 26 mm against *Escherichia* that was significantly higher ($p<0.05$) than any other EOs zones of inhibition. The zone of inhibition against the other bacteria *Enterobacter* spp, *Staphylococcus, Citrobacter* spp, *Proteus* spp, and *Klebsiella*

Table 1. The incidence of pathogens in milk samples.

Pathogens	Number of bacterial isolates	Percentage incidence
Escherichia	13	27%
Enterobacter spp.	9	18%
Staphylococcus	7	15%
Citrobacter spp	6	13%
Proteus spp	6	13%
Pseudomonas spp	5	10%
Klebsiella spp	2	4%

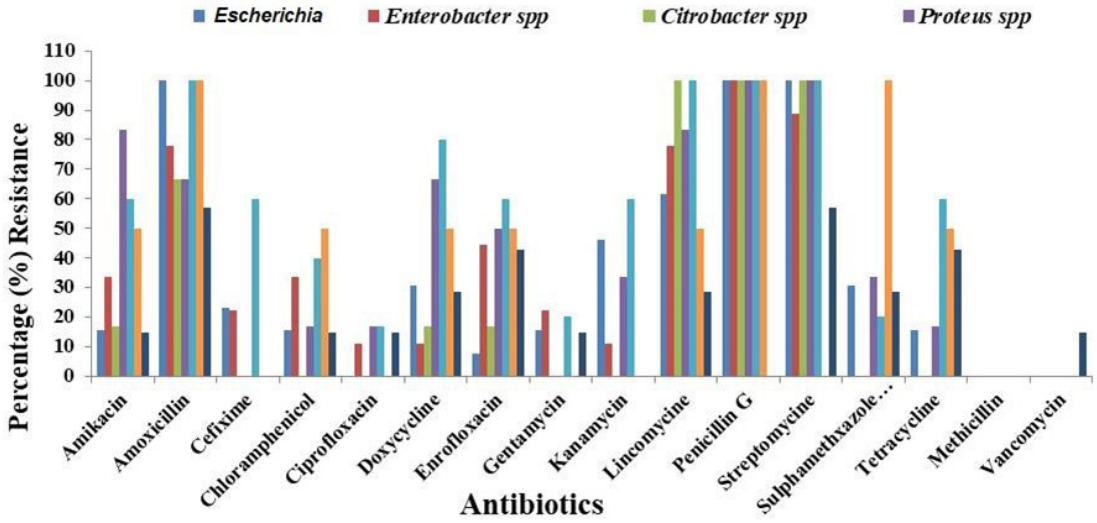

Figure 1. This graph depicts the percentage antibiotics resistance of bacterial isolateed from raw milk samples. X-axis = percentage of antibiotic resistence, and Y-axis = names of antibiotics.

Table 2. The yields of essential oils (v/m) from different plants using steam distillation process.

Plant name	Part used	Essential oils (% Yield)
Rosemary	Leaves	0.89
Lemon grass	Leaves	0.80
Oregano	Leaves	0.78
Mint	Leaves	0.71
Thyme	Leaves	0.67

spp, were 21, 20, 20, 18 and 13 mm respectively (Figure 2). The oregano oil formed the largest zone of inhibition against *Staphylococcus* (18 mm) and smallest against *Klebsiella* spp (12 mm). Oregano oil gave a significantly ($p<0.05$) larger zone of inhibition against *Enterobacter* spp, compared to lemon, mint, or rosemary oils. The *Cymbopogon citratus* (lemongrass) oil showed moderate activity. Lemongrass oil was more effective against Staphylococcus (15.1 mm zone of inhibition) than the Gram-negative bacteria. Figure 3 represent the zone of inhibition formed by lemongrass, thyme, and mint oils against Staphylococcus. The *Rosemary officinalis* (rosemary) and *Mentha spicata* (mint) oils gave the lowest antibacterial activity. The highest zone of inhibition for *Rosemary officinalis* (rosemary) oil was 10.4 mm against *Enterobacter* and *Proteus spp*, and then reduced to 9 mm against *Citrobacter* spp. Mint oil had the largest zone of inhibition (14 mm) against *Enterobacter* spp and the smallest zone of inhibition (10.4 mm) against *Escherichia*.

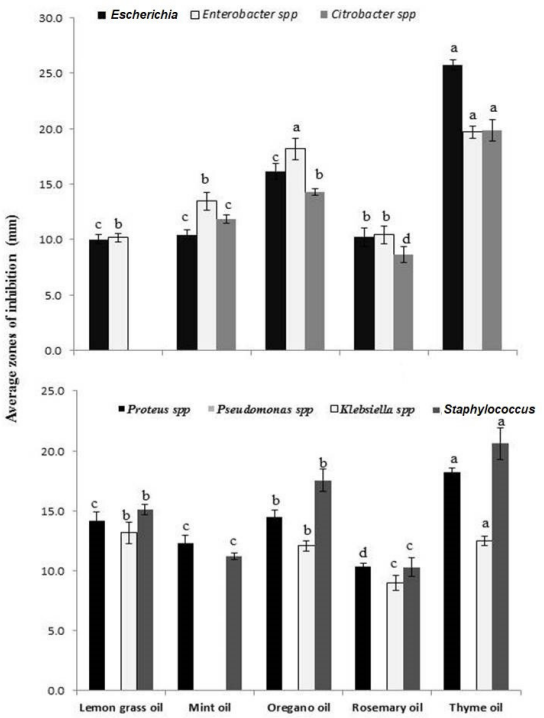

Figure 2. Mean inhibition zones of different essential oils against different strains of *Escherichia*, *Enterobacter* spp, *Citrobacter* spp, *Proteus* spp, *Pseudomonas* spp and *Staphylococcus*, and *Klebsiella* spp through disc diffusion method (Results are mean of three independent experiments). X-axis = zone of inhibition in mm, and Y-axis = names of pathogens.

4. Discussion

Milk is known to be an excellent medium for microbial growth (Abdul Khalil et al., 2014). Bacteria may gain access to the milk from different sources including, workers, the animal's normal microbiota, the farm environment, and utensils and cause a variety of milk-borne diseases (Ahmedsham et al., 2018; Berhe et al., 2020). In the

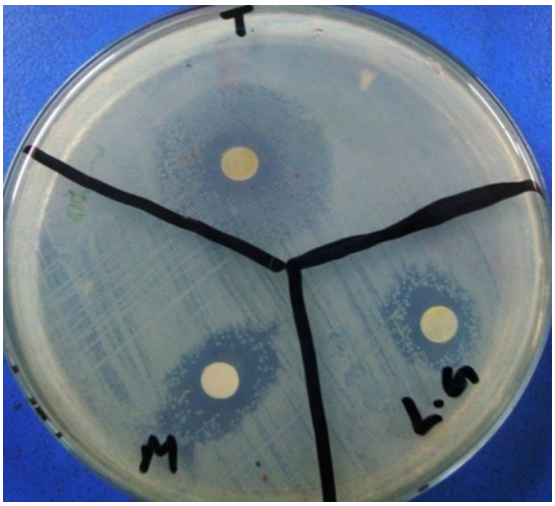

Figure 3. This figure depicts the inhibition of *Staphylococcus* by thyme, mint and lemongrass oils through disc diffusion method. Footnote: where T, thyme; M, mint; and L.G, lemongrass.

current study, several human pathogens were detected in raw milk. Some strains of these bacteria were found resistant to commercially antibiotics. In the present study, Escherichia was the most abundant contaminant of raw milk (27%), but other bacteria were also present. Similar bacteria with slightly different rates such as *Escherichia* 32% *Enterobacter* spp 29%, *Klebsiella* spp 19%, and *Citrobacter* spp 1% were also detected in raw milk in an earlier study (Salman and Hamad, 2011). A high rate of isolation of *Escherichia* is a sign of the poor quality of milk, unhygienic conditions in the farm, and milk collection and processing (Iqbal et al., 2004). *Escherichia* in the milk is the major sign of faecal contamination, which may enter the milk through animal faeces or by the unwashed hands of milkmen (Islam et al., 2018). Similarly, the high incidence of Gram-negative bacteria like *Enterobacter*, *Citrobacter*, *Proteus* and *Pseudomonas* spp may also point out the poor sanitation and hygiene (Drzewiecka, 2016; Garedew et al., 2012). Similar to other bacteria, *Staphylococcus* in raw milk may hurt public health as enterotoxins produced by *Staphylococcus* are heat stable and can survive at pasteurization (McMillan et al., 2016). Milk can be contaminated with *Staphylococcus* directly from the animal's udder during causes of mastitis (Cobirka et al., 2020; Petersson-Wolfe et al., 2010).

The indiscriminate use of antimicrobials leads to drug resistance which threatens the health of both animals and humans. Antimicrobial resistance in food animals has a significant impact on animal health and may be associated with resistant infections in humans (Ma et al., 2021). In our study, several isolates have shown a multi-drug resistant (MDR) pattern. Gram-negative bacteria had higher resistance to penicillin G 100%, streptomycin 91%, amoxicillin 85%, lincomycin 72%, sulfamethoxazole-trimethoprim 43%, and enrofloxacin 39%. Multidrug-resistant bacteria such as *Escherichia, Klebsiella, Enterobacter, Proteus vulgaris* and *Staphylococcus* species have been detected in raw milk previously (Mahami et al., 2011). According to Koluman and Dikici (Koluman and Dikici, 2013) several food born human pathogens were resistant to tetracycline and chloramphenicol but in the present work tetracycline and chloramphenicol were highly effective antibiotics and generally low level of resistance was observed. The low resistance towards chloramphenicol might be because its use is rare at local dairy farms. The isolated bacteria including *Escherichia, Enterobacter, Citrobacter, Proteus, Pseudomonas, and Klebsiella* and *Staphylococcus have also shown MDR against different antibiotics*. Moreover, the antibacterial activity of five EOs were evaluated against these bacteria. All the EOs were found effective against Gram-positive as well as Gram-negative bacteria. EOs are hydrophobic in nature so can easily make partition in the lipids of the bacterial cell membrane, resulting disturbing the structures and rendering cell membrane more permeable to leak out K+ and ATP (Lopez-Romero et al., 2015; Sikkema et al., 1994). Probably, their mechanism of action is therefore be similar to other phenolic compounds which involves disturbance of the cytoplasmic membrane, the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Chouhan et al., 2017). Resistance among *Pseudomonas spp* to the various EOs has been reported earlier (El-Hosseiny et al., 2014). *Pseudomonas spp* poses a serious resistance to EOs could be due to possession of restrictive outer membrane barrier (Mann et al., 2000) and the presence of specific lipopolysaccharides in their cell wall (Nostro et al., 2000). But how *P. aeruginosa* resist EOs to work against it, needs to be explored in future.

Thyme had strong antimicrobial activity against *Escherichia* followed by oregano oil. Such effect of both the EOs was also investigated previously (Mith et al., 2014; Puškárová et al., 2017). Our finding did not match with earlier reports where thyme was effective against P. aeruginosa but remained ineffective towards Escherichia (Nascimento et al., 2000). High antimicrobial action of thyme and oregano species have been attributed to the presence of phenolic compounds e.g., thymol and carvacrol (Gavanji et al., 2015; Swamy et al., 2016) and p-cymene or γ-terpinene (Simirgiotis et al., 2020). Individual inhibitory effects of thymol and carvacrol against Gram-positive e.g., *Staphylococcus* and Gram-negative bacteria e.g., *P. aeruginosa*, *Escherichia*, *K. pneumonia* was also reported previously (Fadli et al., 2011; Gavanji et al., 2015; Rosato et al., 2010). There is a possibility that these constitutes work together against bacteria. There were different active compounds in the oil extracts as different sizes of zones were seen. It may indicate that when they are together, as likely near the filter paper, they act in synergy to inhibit the growth (Figure 3) which needs to be tested in future studies.

Rosemary oil produced the smallest zone of inhibition compared to other EOs when tested at the same concentration. Variation in zones of inhibition might be due to the presence of different active compounds such as α-pinene, boranyl acetate, camphor, 1,8 cineole in rosemary EO (Tomi et al., 2016). Lemongrass oil showed strong antibacterial activity against *Staphylococcus* than Gram-negative bacteria. All the tested isolates were susceptible to lemongrass oil except *Citrobacter* and *Pseudomonas*

spp. A relatively higher zone of inhibition (15 mm) was observed against *Staphylococcus* followed by *Proteus* spp. Moderate inhibition was observed with all the other experimental strains. These findings are supported by previous study (Wannissorn et al., 2005). A larger zone of inhibition (28 mm) of lemongrass oil was reported against *Staphylococcus* in a previous study (Silveira et al., 2012) but the inhibition areas to all the other isolates were similar to recent findings. A larger zone of inhibition may be due to the presence of several active compounds in lemongrass oil such as citral, limonene, neral, geranial, citronellal, and neryl acetate (Ugbabe et al., 2016). Spearmint oil was found active against both Gram-positive and Gram-negative bacteria except *Pseudomonas* and *Klebsiella* spp which were resistant. In similar studies, significant inhibition (15 mm) against *Escherichia* was noted with spearmint oil (Lixandru et al., 2010; Sulieman et al., 2011). In another study, Gram-negative bacteria e.g., *Escherichia*, *Pseudomonas* spp and *Enterobacter* spp were resistant but *Staphylococcus* and *Proteus* spp were susceptible to spearmint oil (Silveira et al., 2012). These findings differ from a recent study as *Escherichia*, and *Enterobacter spp* were sensitive to spearmint oil in the current study. Giving the differences in the activity of spearmint oil might be due to differences in bioactive constituents such as β-myrcene, limonene, 1,8-cineole and menthone (Benabdallah et al., 2018; Snoussi et al., 2015).

The diameter of inhibition zones formed by EOs varied in different studies. This could be due to many factors, firstly, the climatic and environmental conditions which bring changes in the composition of EOs (Janssen et al., 1987; Sivropoulou et al., 1995). This suggested that there may be a difference in the susceptibility of strains isolated from different sites or a difference in active compounds of EOs extracted from different geographical regions. Secondly, the technique used to evaluate the antimicrobial potential of EOs, and the selection of test organisms differs from previous experiments. Also tests oils with the same common name may be derived from different plant species.

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