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Evaluation of the Antimicrobial Property of Combined Fractionated Extract of Peppermint Leaves with Eucalyptus Oil

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ABSTRACT

Antimicrobial resistance (AMR) poses a significant global challenge, impacting both public health and economies worldwide. Efforts to address this issue have focused on developing innovative solutions within the limitations of existing medications, such as combining different drugs to combat it more effectively. This study investigated the combined antimicrobial effects of peppermint and eucalyptus oil extracts on three harmful bacterial strains: *Escherichia coli* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853), and *Staphylococcus aureus* (ATCC28213). The researchers extracted aerial components from *Mentha piperita* using ethanol and further separated them into fractions with hexane, ethyl acetate, and water. These fractions were then combined in various ratios and tested for antimicrobial activity using Kirby Bauer Disk Diffusion Assay and MIC by microbroth dilution technique. The results showed that combinations of the ethyl acetate fraction effectively inhibited *E. coli* growth, particularly at a 1:1 ratio. Other combinations did not meet susceptibility criteria outlined in CLSI guidelines. Ethyl acetate fractions in the 1:1 and 1:3 combinations showed synergistic effects according to CI analysis against *E. coli* and *S. aureus*, respectively. The study concluded that combining *Mentha piperita* and *Eucalyptus globulus* extracts as antibacterial agents did not enhance the bioactive compound's efficacy. And it exhibited antagonism, necessitating further investigation into their in vitro activities.

Keywords: *antimicrobial activity; eucalyptus oil; Kirby Bauer disc diffusion assay; minimum inhibitory concentration; peppermint leaves*

INTRODUCTION

Antimicrobial resistance (AMR) is an urgent need to resolve global public health and socioeconomic problems. This resistance happens when microorganisms, which cause millions of infections worldwide, have developed a resistance or a means to protect themselves against the antimicrobial effect of antibiotics or antimicrobials (WHO, 2020). Hence, it makes the drugs ineffective and the infections more challenging to treat. In 2017, the World Health Organization (WHO) released a list of priority pathogens that require the development of new antibiotics to fight antimicrobial resistance worldwide. The ESKAPE pathogen has three levels: the first priority, or "critical," are *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*. The second priority or "high" are *Enterococcus faecium*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter spp.*, *Salmonellae*, and *Neisseria gonorrhoeae*. Lastly, the third priority or "medium" are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Shigella spp*. This notification and cautionary message pertain to the existence of AMR (WHO, 2017).

An antimicrobial agent exhibits specific inhibitory effects and mechanisms tailored to each microorganism. Thus, choosing an antimicrobial agent relies on its effectiveness against the intended target. There isn't a universal "magic bullet" that can effectively combat all spoilage and pathogenic microorganisms, as each agent operates differently on various microorganisms (Han et l., 2013). Recently, numerous studies have aimed at finding effective solutions to address these challenges, resulting in successful alternative methods for controlling antibiotic-resistant pathogens. Combining antimicrobial agents can yield synergistic effects when each drug targets a different pathway or mechanism of action (Basavegowda and Baek, 2022).

In the study by Jeong et al. (2023), the researchers explored the combined inhibitory effects of several medicinal plants—*Caesalpinia sappan L.* (CS), *Glycyrrhiza uralensis Fisch*. (GU), *Sanguisorba officinalis L.* (SO), and *Uncaria gambir Roxb.* (UG) on the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and its clinical isolates. Most combinations demonstrated enhanced inhibitory effects, except for the GU + SO pairing. The minimum inhibitory concentrations (MICs) for each extract were reduced by fourfold when tested individually. Similarly, Delaquis et al. (2002) examined the individual and mixed fractions of dill, cilantro, coriander, and eucalyptus essential oils, finding that while some combinations displayed synergistic activity, others resulted in additive or antagonistic effects against specific microorganisms.

This discussion can be narrowed down to the use of natural products which could serve as a viable alternative to conventional therapeutic agents. It underscores how natural products can effectively combat multidrug-resistant infections. Using natural products, the discovery of new sources of antibiotics can address the rising threat of antibiotic resistance and enhance overall health outcomes (Moloney, 2016).

Peppermint oil (*Mentha piperita)* is proven to control a broad range of Gram-positive and Gram-negative bacteria, as well as fungi, and yeasts; the oils' antimicrobial effects involve direct interaction with the bacterial cell wall structure by altering the cytoplasmic membrane integrity with the use of hydrogen (H^+) and potassium (K^+) ions leading to the microbial cell death (Benzaid et al., 2019). Eucalyptus oil (*Eucalyptus globulus*) is found to be another natural substance that has an antibacterial activity due to a monoterpenoid cyclic ether, specifically 1,8-cineole, that is found in the eucalyptus plant. This ether has a mechanism of action to affect the cytoplasmic membrane of the bacteria (Asiaei et al., 2018).

Although both have been found to have antibacterial properties, the use of this can be challenging due to the rise of resistant strains (Asiaei et al., 2018) leading to the use of combination extracts. Plants contain various bioactive substances with different mechanisms of action, which, when

combined with another plant, can form synergistically, and boost their antibacterial effects (Stefanović, 2017). A study used ethanolic peppermint extract as a combination with a drug. amikacin has enhanced its antibacterial potency due to its synergistic action, thus, surpassing the action of amikacin alone (Indrayudha, 2021). This current study aims to combine two natural plant sources and determine their antimicrobial activity.

METHODS

Materials and Equipment. This study employed an experimental research design to assess the synergistic effects of the ethanolic extracts from peppermint leaves and commercial grade eucalyptus oil in inhibiting the growth of selected pathogenic strains. The reagents used for the study are technical grade ethanol, hexane, and ethyl acetate obtained from Bellman Laboratories. Lastly, the peppermint leaves (*Mentha Piperita*) were collected from Farm2Metro in Manila and were authenticated by the University of the Philippines, Institute of Biology, College of Science.

Preparation of Peppermint Extract. Four kilograms of fresh peppermint leaves were washed with running water and shade-dried at room temperature for seven days. Once dried, they were pulverized into a fine powder using a grinder machine for 2 min. Then 200 grams of this powder were incorporated in 2,000 mL of 95% undiluted technical grade ethanol using maceration extraction. The ensuing extract was decanted using Whatman filter paper (No. 1) and concentrated in a rotary evaporator to get dry residue with a pressure of 160 millibars (mbar) at 40°C and with a speed of 70 revolutions per minute (rpm) (Hidayati, et al. 2023)

Fractionation of Peppermint Extract. As shown in Figure 1, after extracting the Peppermint extract, the researchers further divided it into three fractions: hexane, ethyl acetate, and water fractions to isolate the compound of interest. The ethanolic extract of peppermint was suspended in water (250 mL). Researchers sequentially extracted the compound using various organic solvents such as hexane, ethyl acetate, and water. This process led to the isolation of 13.68 g of hexane extract, 1.00 g of ethyl acetate extract, and 19.26 g of aqueous extract. All the crude extracts were separated from the separatory funnel. The particle-free crude extract underwent complete evaporation using a rotary evaporator (Shimadzu, Rotary Evaporator, model-QR 2005-S) under reduced pressure to obtain dry crude extracts. The pressure required for hexane to be evaporated was 335 mbar at 40°C, followed by ethyl acetate for approximately 240 mbar at 40°C, and lastly, water, within 72 mbar at 40°C. (Abubakar & Haque, 2020).

Preparation of Fractionated Extract and Eucalyptus Oil. Each fractionated extract was prepared into three different ratios of mixtures of extract and commercial-grade eucalyptus oil by weight (1:1, 1:2, and 1:3) to determine the synergistic effect. To ensure complete homogenization of the mixture, 0.15 - 0.25 mL of tween-80 surfactants were added dropwise, taking care not to overpower the mixture's antibacterial properties (Micheline et al., 2018).

***Combination Index*.** A statistical analysis tool was used to evaluate the synergistic interaction of each treatment group. Using the Zone of Inhibition of both the single and combined extracts, they were categorized as follows: if it exceeds 1, then it is antagonistic; if it is less than 1, then it is synergistic; and if it is equal to 1, then it is additive (Chou, 2010).

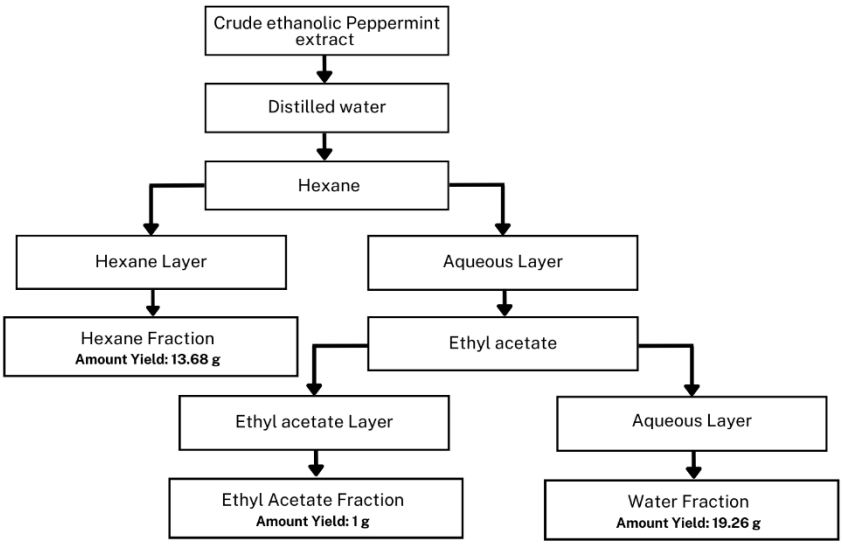

Figure 1. The experimental flow diagram for the fractionation of crude ethanolic Peppermint extract

Kirby Bauer Disk Diffusion Assay. All bacterial inoculums were standardized to a 0.5 McFarland standard and then uniformly spread on a 100 mm Mueller Hinton Agar (MHA) plate. Sterile water served as the negative control, while the antibiotic gentamicin, azithromycin, and ceftazidime were used as positive controls. Subsequently, five paper discs (Whatman No. 3) with a diameter of 6 mm were placed in the petri dish which contained the negative control, positive control, 1:1, 1:2, 1:3 ratios of the combined extract. The assay was conducted in triplicate. The plates were incubated overnight at 37°C. A clear halo indicated the zone of inhibition, which was measured and interpreted (Wayne, 2020)

Microdilution Assay. In this process, each well holds the triplicate of each fraction per bacteria. The first three columns contain serially diluted extract. The process of serial dilution began with placing 200 µL of the extract into the first well of a microwell plate. Following this initial step, 100 µL was transferred from the first well to the second well. This is done each time moving 100 µL to the next well, until the final well is reached. Once the dilutions were complete, a bacterial suspension was evenly distributed, with 100 µL added to each well. This process was replicated in two additional columns, ensuring consistency across the experiment. The entire procedure was repeated with different extracts for another six columns, effectively establishing triplicates for comparison.

After all preparations were complete, the microwell plate was incubated at a stable temperature of 37°C for 24 hours (Wayne, 2020). After the incubation period, 10 µL of a 0.01% resazurin solution was introduced into each well. The resazurin solution had been prepared by dissolving 0.01 g of resazurin powder in 100 mL of sterile distilled water, followed by filtration through Whatman filter paper to ensure purity. Following the addition of the resazurin solution, the plate was incubated for an additional 2 hours. The color reaction of resazurin serves as the basis for determining the minimum inhibitory concentration (MIC) of the fractionated extracts. A blue or purple color indicates inhibition in the growth of the microorganism, while a pink color or a colorless change indicates active cells. The lowest concentration without a color change (blue or purple) is considered as the MIC (Kebede & Shibeshi, 2022).

RESULTS AND DISCUSSION

Three fractions of the peppermint extracts were tested: hexane, ethyl acetate, and water. Among these, the 1:1 ethyl acetate fraction exhibited the highest mean Zone of Inhibition (ZOI) at 20.10 mm in *E. coli*. The 1:2 fraction exhibited 17.77 mm, and the 1:3 fraction had a 15.67 mm diameter zone of inhibition (Figure 2). According to Clinical & Laboratory Standard Institute (CLSI) guidelines, these are categorized as susceptible. Likewise, when tested against *S. aureus*, the 1:1 and 1:3 ratios of the ethyl acetate fraction showed the highest ZOI with a zone diameter of 13.73 mm and 15.07 mm, respectively. This is considered intermediate in line with the CLSI guideline. However, the others show inhibition but do not conform to the CLSI guideline and are therefore considered resistant.

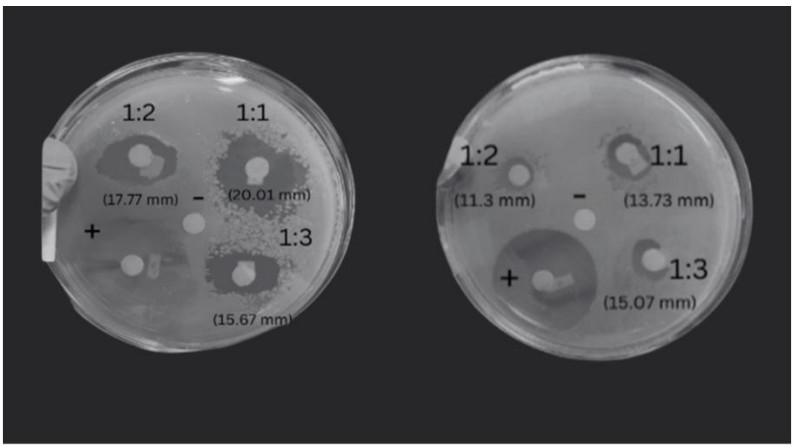

Figure 2. Antimicrobial activity of Ethyl Acetate Fraction in different ratios (1:1, 1:2, 1:3) against *E. coli ATCC25923* and *Staphylococcus aureus ATCC28213*.

Table 1. Two-way analysis of variance (ANOVA) of the fractionated extract with eucalyptus oil (1:1, 1:2, 1:3) in Gram-Positive and Gram-Negative Bacteria.

	Effects	***SS***	***Df***	***MS***	***F***	***P***
E. coli	Kirby	1200.71	4	300.18	11.09	0.0024
	Fraction	274.82	2	137.41	5.08	0.0377
	Residuals	216.53	8	27.10	-	-
	Total	1692.10	14	120.86	-	-
P. aeruginosa	Kirby	743.33	4	185.83	71.25	<0.001
	Fraction	30.10	2	15.03	5.76	0.0282
	Residuals	20.87	8	2.61	-	-
	Total	794.25	14	56.73	-	-
S. aureus	Kirby	1584.40	4	396.10	86.79	<0.001
	Fraction	49.86	2	24.93	5.46	0.0319
	Residuals	36.51	8	4.56	-	-
	Total	1570.76	14	119.34	-	-

Note: SS: Sum of squares, MS: Mean of the sum of squares, Df: Degrees of freedom, and P: Probability

To ensure the accuracy of the Kirby-Bauer disk diffusion assay results, statistical analyses were conducted, specifically a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Differences between each ratio and fraction were observed. In Table 1, the data for *E. coli* bacteria revealed a *p*-value of 0.0024, which is less than the alpha level, indicating a significant difference among the three ratios (1:1, 1:2, 1:3) and the positive control. This prompted further analysis through Tukey's post-hoc test, which showed that the ethyl acetate fraction exhibited the

highest activity compared to the other two fractions. Additionally, when compared with the positive control, the positive control demonstrated higher efficacy than the mixture. In summary, both *P. aeruginosa* and *S. aureus* results also indicated that the ethyl acetate fraction had the highest activity among the three fractions, while the positive control remained the most effective overall.

Upon comparing peppermint leaves and eucalyptus oil, the oil has a higher accumulated ZOI with 11.53 mm in *E. coli* explaining that potent inhibitors with limited solubility in water yielded unfavorable or even adverse outcomes in the agar diffusion test. Additionally, the non-polar nature of these components hinders their diffusion through the agar medium. Therefore, the effectiveness of essential oils is more significant when assessed using the minimum inhibitory concentration (MIC) method than the disc diffusion method. The agar diffusion technique is not considered optimal for evaluating the antimicrobial activity of essential oils due to the likelihood of the active volatile components evaporating along with the dispersing solvent. (Goñi et al., 2009 cited by Bel Hadj Salah-Fatnassi et al., 2017).

Thus, minimum inhibitory concentration was further used to ensure antimicrobial property of the combination of the extract and oil. After assessing the absorbance values of the three tested fraction combinations, the result in Table 2 shows that in *E. coli*, all data falls into the 'resistant' category, following the projected CLSI guidelines, with a MIC value of (≥16). However, the concentrated hexane fraction of peppermint demonstrates susceptibility, being seven dilutions away from the breakpoint, and has a MIC value of (≤4), while the ethyl acetate and water concentrated fractions are resistant. With regards to *P. aeruginosa*, the absorbance value of three tested fraction combinations of mixtures obtains a value of (≥32), which is considered resistant; meanwhile, the concentrated ethyl acetate and water fractions shown to be susceptible (≤8), which is both seven dilutions away from the breakpoint while the hexane fraction is resistant. Lastly, in *S. aureus*, one of the 1:1 fraction combinations of mixtures fall under the intermediate category, having a MIC value of (4-7), and it is the 1:1 water fraction that reveals to be six dilutions away from the breakpoint while the rest appears to be resistant. Among the 1:2 fraction combinations of mixtures, the water fraction also falls into the intermediate category and is six dilutions away from the breakpoint. Finally, from 1:3, the water fraction was recorded as intermediate (4-7), while the hexane and ethyl acetate showed resistance. Compared to the concentrated fraction of peppermint, the concentrated hexane fraction is susceptible (≤4), which is seven dilutions away from the breakpoint, while the pure ethyl acetate and water fractions are resistant.

The MIC of the concentrated hexane fraction against *E. coli* and *S. aureus* strains ranged from 20 to 3.125 mg/mL suggests a strong antibacterial effect against the studied strains. These findings align with the study conducted by Dhifi et al., 2016, which explored various factors in the extraction process of peppermint's active compound, primarily menthone, responsible for the plant's antibacterial properties. The study determined that using a non-polar solvent like hexane resulted in greater yields of its active compound, which explains why the concentrated hexane fraction exhibits higher antimicrobial activity, comparable to the assigned positive controls.

Table 2. Minimum Inhibitory Concentration (MIC) of the fractionated extract with eucalyptus oil (1:1, 1:2, 1:3) in Gram-Positive and Gram-Negative Bacteria.

Bacterial Strain	Fraction	Ratio	MIC						
			A	B	C	D	E	F	G
E. coli	Ethyl Acetate	1:1	3.50	2.23	1.81	1.77	2.49	2.49	2.74
		1:2	3.50	1.48	2.02	2.20	2.03	2.10	2.19
		1:3	3.50	1.92	1.95	2.10	2.02	2.05	2.17
	Hexane	1:1	2.75	2.48	2.26	2.11	2.61	2.41	1.97
		1:2	2.82	2.55	2.55	2.50	2.55	2.22	1.79
		1:3	3.18	2.67	2.36	2.48	2.24	2.21	2.15
	Water	1:1	3.50	3.29	3.50	2.60	1.03	2.26	1.93
		1:2	3.50	3.19	3.30	3.30	2.18	1.78	1.66
		1:3	3.50	3.50	3.50	3.01	2.04	2.31	2.10
P. aeruginosa	Ethyl Acetate	1:1	3.50	3.44	3.50	2.04	2.56	2.31	2.31
		1:2	3.50	3.32	2.55	1.96	2.45	2.45	2.28
		1:3	3.50	3.02	1.56	2.55	2.63	2.83	2.24
	Hexane	1:1	3.50	3.44	1.06	1.65	1.42	2.04	2.25
		1:2	3.50	3.50	1.20	1.51	1.77	2.05	2.25
		1:3	3.50	3.50	1.18	1.41	1.67	1.97	2.20
	Water	1:1	3.50	3.43	1.53	1.80	2.01	2.15	2.01
		1:2	3.50	2.98	1.93	2.39	2.41	2.54	2.48
		1:3	3.50	2.02	1.89	2.19	2.45	2.27	2.40
S. aureus	Ethyl Acetate	1:1	3.50	3.14	3.10	3.09	3.17	2.53	2.47
		1:2	3.50	3.48	3.40	3.49	2.35	2.26	2.28
		1:3	3.50	3.23	3.50	3.50	2.53	2.38	1.97
	Hexane	1:1	3.38	1.61	2.83	2.93	2.28	2.16	1.82
		1:2	3.37	2.74	2.51	2.55	2.56	2.49	1.89
		1:3	3.50	2.45	3.08	2.86	2.69	2.45	1.86
	Water	1:1	3.50	3.39	3.44	3.35	3.49	3.50	2.47
		1:2	3.50	3.43	3.50	3.50	3.50	3.42	2.87
		1:3	3.5	3.37	3.48	3.50	3.49	3.50	3.08

Note: A (200µg/mL), B (100µg/mL), C (50µg/mL), D (25µg/mL), E (12.5µg/mL), F (µg/mL)

The MIC of the concentrated ethyl acetate fraction demonstrated susceptibility against *P. aeruginosa*, ranging from 20 to 3.125 mg/mL. The rest of the fraction combinations of peppermint and eucalyptus oil mixture showed resistance to the studied strains except for the 1:1, 1:2, and 1:3 water fractions, which demonstrated a moderate antibacterial effect against *S. aureus*. It has difficulty inhibiting Gram-negative bacteria, *P. aeruginosa* since typically, Gram-negative bacteria show higher resistance to essential oils than Gram-positive bacteria. Gram-negative bacteria possess a tough outer membrane that is abundant in lipopolysaccharides and has a more intricate structure, making it difficult for hydrophobic compounds like essential oils to penetrate. On the other hand, Gram-positive bacteria have a peptidoglycan wall, which is not as compact, and therefore, it cannot effectively retain small antibacterial molecules and prevent them from crossing the cell membrane (Yunilawati et al., 2021)

Table 3. Combination Index of the three fractions against the Gram- Negative and Gram-Positive Bacteria.

Ratio	*E. coli*			*P. aeruginosa*			*S. aureus*		
	Hexane	Ethyl Acetate	Water	Hexane	Ethyl Acetate	Water	Hexane	Ethyl Acetate	Water
1:1 [A]	1.89	0.94	0	1.59	2.70	2.22	1.85	1.09	2.04
1:2 [B]	2.25	1.06	0	1.70	3.14	2.53	1.85	1.32	2.18
1:3 [C]	1.75	1.20	0	1.47	3.44	2.51	1.79	0.99	1.99

[A] The 1:1 ratio is composed of 1 part of Peppermint Leaves Extract and 1 part of Eucalyptus Oil.
[B] The 1:2 ratio is composed of 1 part of Peppermint Leaves Extract and 2 parts of Eucalyptus Oil.
[C] The 1:3 ration is composed of 1 part of Peppermint Leaves Extract and 3 parts of Eucalyptus Oil.

For the minimum inhibitory concentration, the combination index (CI) was utilized to determine whether the ratio of the combination of peppermint extract and eucalyptus oil exhibited synergy, additivity, or antagonism. Upon analyzing the values of three fractions in *E. coli*, it became evident that hexane and ethyl acetate values exceeded 1.0, categorizing them as antagonistic for this bacterium. Additionally, the water combination did not inhibit this bacterium. However, they found synergistic at the 1:1 ratio of the ethyl acetate fraction, with a value of 0.94. When testing the three fractions in *P. aeruginosa*, all fractionated combinations of mixtures were antagonistic, CI values exceeded 1.0. In *S. aureus*, the CI values were more significant than 1.0, also categorizing them as antagonistic. Nonetheless, the 1:3 ratio of the ethyl acetate fraction obtained a value of 0.99, classifying it as synergistic.

Therefore, all three bacterial pathogens used with the combination mixture of hexane and water were found to have 1.0 values resulting in antagonism. While the ethyl acetate fraction was found to be synergistic. The antagonism could have arisen from the oxidation of the primary antimicrobial by the synergist. Combining the two may have practically lost any effective antimicrobial compounds. Non-oxygenated terpenes in peppermint extract may hint at a poor interaction with eucalyptus oil, specifically of terpinen-4-ol, thus reducing the effect and lowering its aqueous solubility (Cox et al., 2001 as cited in Iacovelli et al., 2023).

Another factor that may have resulted in this is the use of eucalyptus oil as the primary ingredient, resulting in an essential oil blend. The mechanisms by which different essential oils can damage bacteria depend on their composition. Their composition determines the ability of various essential oils to harm bacteria. Typically, the antimicrobial effects of essential oils result from a combination of mechanisms rather than a single mode of action. These mechanisms involve multiple reactions that target different aspects of the bacterial cell. Essential oils contain diverse chemical structures and possess several functional groups, contributing to their antimicrobial activity (Mancuso, 2020).

The results lead to an outcome that even though they possess an antagonistic relationship, both are still proven to have antibacterial properties. Where, *Eucalyptus globulus* contains various chemical compounds; the most active one is typically 1,8 cineole. However, it is essential to note that the compounds present in larger quantities may not always be the primary contributors to the overall activity. Researchers often attribute the activity to lesser-known components. However, it also maintains other notable compounds. Among the minor compounds commonly found with 1,8 cineole, terpinen-4-ol stood out as particularly noteworthy. Terpinen-4-ol demonstrated a notable ability to inhibit DNA and RNA synthesis in methicillin-resistant *Staphylococcus aureus* (MRSA) two hours after treatment. This effect was primarily achieved by influencing the genes and metabolites of the metabolic pathways responsible for purine and pyrimidine production (Cheng et al., 2021).

Peppermint on the other hand, harbors menthol, a compound responsible for its antimicrobial properties, which was abundant in this plant. The menthol structure reveals a hydroxyl group

with delocalized electrons, contributing to its antimicrobial properties. These similar compounds destabilize the cytoplasmic membrane and act as a proton exchanger, reducing the cytoplasmic membrane's pH gradient. The collapse of the proton motive force and depletion of the ATP pool eventually led to cell death (Ultee et al., 2002 cited by Dhifi et al., 2016).

CONCLUSIONS

The combined use of fractionated extracts from peppermint and eucalyptus oil shows varying effectiveness against three bacterial strains. However, the effectiveness against *E. coli* is solely based on the Zone of Inhibition (ZOI). To fully understand the extent of their antimicrobial activity, improving the dispersion system for these combined extracts is necessary. The mixture of extracts of the two plant samples shows antimicrobial activity. However, this activity is only minimal. Hence, they do not show synergistic but rather antagonistic effects. Utilizing plant extracts as an alternative antimicrobial agent is a strategic method to combat AMR due to their natural composition and difference in mechanism of action that can produce a synergistical relationship. As a result, they have the potential to be a promising substitute for synthetic materials in the fight against increasingly common bacterial infections.

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