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Harnessing Nature's Arsenal: Investigating the Antibacterial Efficacy of Commercial Essential Oils against *Staphylococcus* Strains Isolated from Poultry Meat.

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ABSTRACT

Pathogens prevalent in the food supply chain provide a significant worldwide risk to both human health and the economy. Poultry meat, a staple in global diets, serves as a reservoir for bacterial contamination. *Staphylococcus*, a gram-positive bacterium belonging to family *Staphylococcaceae* has been identified as a potential causative agent of food borne illnesses. The presence of antibiotic-resistant *Staphylococcus* strains in poultry products raises concerns about the transmission of resistance genes through the food chain, necessitating thorough investigations into alternative antimicrobial agents for effective bacterial control. Essential oils (EOs) hold profound importance in terms of their known and potential application. This study focuses on the evaluation of commercial essential oils and their effectiveness against *Staphylococcus* strains isolated from poultry meat. Briefly, 150 raw chicken meat samples were collected, and *Staphylococcus spp.* was identified based on morphological and cultural characteristics. Antibiogram analysis and essential oils activity was determined by disc diffusion and agar well diffusion assay respectively. Results showed that 27 (18%) samples tested positive for *Staphylococcus spp*, out of which *S. aureus* was identified in 25 isolates (16.66%). The antibiogram profile reveals that three antibiotics namely, gentamicin, vancomycin and ciprofloxacin were the most effective antibiotics showing sensitivity against 74.07%, 70.37% and 62.96% of the isolates respectively. Moreover, amongst the tested essential oils cinnamon oil and clove oil exhibited the highest antimicrobial activities ZOI ranges from 19-41mm and 19-33 mm respectively. While focusing on the evaluation of antimicrobial activity of essential oils, the study endeavors to aid in development of sustainable strategies for mitigating bacterial contamination in the food industry.

Keywords: *Staphylococcus*, Essential oil, Antibiotic resistance, Poultry meat, Mannitol salt agar

1. INTRODUCTION

During the last three decades the emergence of resistance in bacterial strains to conventional antimicrobials has become a global health concern, prompting scientists to explore alternative treatment modalities (El Abed et al., 2014). One such alternative modality is the utilization of essential oils (EOs) derived from various plant sources (Pinto et al., 2021). Essential oils have been recognized to contain diverse and complex volatile low molecular weight compounds such as aldehydes, terpenes, and phenols etc. which have been cited in the literature with promising antimicrobial activities (Stojković et al., 2013, Nazzaro et al., 2013, Sadgrove et al., 2022). The plethora of aromatic volatile compounds have antimicrobial potential against a diverse group of microorganisms such as *E. coli, P. aeruginosa, S. pneumonia, E. faecalis, S. typhimurium, L. monocytogens* and the list is non-exhaustive (Burt et al., 2004, Oussalah et al., 2007). The effectiveness of EOs varies with the nature and composition of essential oil subject to various factors. The method of extraction is one of them. Various methods are employed to derive EO such as steam distillation, solvent extraction, cold pressing, and supercritical CO_2 extraction (Ashraf et al., 2020, Moreira et al 2023). Amongst them steam distillation is the most common and widely used method in which the steam is passed through plant material to evaporate the aromatic compounds, which are then condensed and collected (Tisserand & Young, 2014).

The EOs are effective against microorganisms via targeting different cellular mechanisms such as peptidoglycan synthesis or alteration of the membrane hydrophobicity etc. (Budri et al., 2015). They contain phytochemicals which are mainly responsible for antioxidant and antimicrobial activity (Ambreen et al., 2022). Compounds such as terpenes and phenolics present in EOs can interact with the lipid bilayer of bacterial cell membranes, resulting in increased permeability and leakage of cellular contents. However, some components such as thymol and carvacrol, can inhibit enzymes like DNA gyrase and RNA polymerase, disrupting bacterial replication and protein synthesis; respectively. Additionally, compounds like eugenol and cinnamaldehyde, found in clove and cinnamon oils are known for their ROS-inducing properties which are bactericidal (Pepeljnjak et al., 2005, Adams et al.,

2011, Badsha et al., 2021). Numerous studies have highlighted the efficacy of various EOs in combating Staphylococcal strains due to their complex chemical compositions and diverse mechanisms of action (Zhang et al., 2016, Lopez et al 2015, Wang et al., 2020).

Meat serves as an essential source of protein and other vital nutrients in people's diet. Global meat consumption in 2014 resulted in the slaughter of more than 62 billion chickens, 545 million sheep, 444 million goats, and 301 million cattle. Consumption of meat is most prevalent in high-income regions whereas least prevalent in low-income nations. Most meat varieties have a substantial water content, leading to a water activity level of around 0.99. This level is conducive to the proliferation of microbes. The proliferation of microorganisms can cause food to decay and contribute to foodborne illnesses in people, resulting in both financial and health-related negative effects. Poultry meat can be contaminated by both pathogenic and non-pathogenic bacteria with notable culprits including *Staphylococcus, Salmonella, Campylobacter, C. perfringens, E. coli, and L. monocytogenes*. According to the various studies, the prevalence of *Staphylococcus aureus* in poultry meat can range from 10% to 80%, depending on factors such as production practices, processing methods, and storage conditions (Khoshbakht et al., 2020, Ribeiro et al., 2021).

Staphylococci are gram-positive cocci which have grape like arrangements under the microscope. They have widespread niches such as upper respiratory tract and skin in living organisms and on natural biomes such as soil, sewage, water, and arable land. However, *S. aureus* can become an opportunistic organism under certain conditions. This is mainly influenced by multiple factors such as environmental conditions, host immunity and bacterial virulence mechanisms. *They can cause* significant mortality and a diverse range of invasive infections in animals and humans (Aneela et al., 2021, Kadariya et al., 2014, Onyango et al., 2018). This is attributed to its toxin production and effective ability to escape host immune system (RM et al., 2007, Chen et al., 2014). It is also noteworthy that this pathogen is known for its significant association with foodborne illnesses.

Meat can be contaminated by *Staphylococcus spp* through various pathways for example processing, handling, and storage. The widely consumed protein sources globally such as chicken may serve as a potential reservoir for *Staphylococcus* contamination (Halpin et al., 1989). The mere presence of antibiotic-resistant *Staphylococcus* strains in poultry raises concerns about food safety (Sergelidis et al., 2017). They are notorious for their ability to develop resistance to multiple antibiotics, making traditional antibiotic treatments less effective. The study aims to characterize the antibiotic resistance mechanism of *Staphylococcus* strains isolated from poultry samples. The study also addresses the prevalence and distribution of antibiotic resistant strains. In addition, it screens the antimicrobial efficacy of commercial essential oils (EOs) (derived from steam distillation method) against antibiotic-resistant *Staphylococcus* strains.

2. MATERIALS AND METHODS

2.1 Sample Collection.

A sum of 150 fresh raw poultry meat samples were collected from different retail shops in Karachi, Pakistan from March 2021 to November 2021. They were kept in icebox to maintain the temperature (0°C) for sample preservation until they were transported to the laboratory for further analysis.

2.2 Identification and Isolation of Staphylococcus species

The 25grams of meat from each sample were cut and minced followed by homogenization in 225 ml of peptone water (w/v). Homogenate (0.1 ml) was spread on mannitol salt agar (MSA) plates and incubated at 37°C overnight. In addition to the examined samples, a positive control of S. aureus (ATCC 25923) was also run in parallel. Next day colonial characteristics were observed and the isolates exhibiting yellow color via mannitol fermentation were further subjected to biochemical test for confirmation.

2.3 Antibiotic Susceptibility Test

The antibiotics susceptibility pattern of the confirmed Staphylococcus isolates was determined by using the Kirby-Bauer disk diffusion method according to the standard protocol (CLSI, 2017). Antibiotic discs purchased from Thermo Fischer Scientific Oxoid were used. In this test, refreshed cultures of Staphylococcus isolates (matched with 0.5 McFarland standards) were inoculated on MHA plates. Antibiotic discs such as vancomycin, tetracycline, gentamicin, ciprofloxacin, amoxycillin, levofloxacin and erythromycin were placed aseptically, and the plates were incubated at 37°C over-nightly. Following the incubation time, the ZOI around the disc were measured in millimeters (mm). Subsequently, the results were interpreted according to established guidelines (CLSI, 2017) and were considered as sensitive, resistant, and intermediate based on the size (mm) of ZOIs.

2.4 Essential Oil Activity by Agar Well Diffusion Method

The Agar well diffusion method was employed to assess the EOs antimicrobial activity. Briefly, the grown bacterial suspension was evenly spread on Mueller Hinton Agar (MHA) plates, then the equally spaced wells were made with a sterile borer (6mm diameter). The wells were labeled and filled with 20µl of undiluted essential oil

and 20µl of 40% dimethyl sulfoxide (DMSO) was used as a negative control. All experiments were performed thrice their average and standard deviation were calculated.

3. RESULTS & DISCUSSION

A total of 150 samples of collected raw chicken meat were explored for the presence of *Staphylococcus* spp. The results showed that 27 (18%) samples were positive for *Staphylococcus* species based on their cultural characteristics, out of which 25 (16.66%) were *S. aureus* as shown by the MSA, catalase and coagulase test which were further confirmed by methyl red and voges proskauer. The results were compared with the positive control of *S. aureus* (ATCC 25923) ran concomitantly with the isolated samples. There were biochemical similarities between MSA positive (golden-yellow colonies) isolates and isolates reported by Konuku et al further confirming the identification of *Staphylococcus aureus*. (Table 1). Out of the 27 strains, 2 strains (A6A and A45A) exhibited characteristic features other than *S. aureus* as both strains were coagulase negative.

Varying patterns of drug resistance were observed among the isolates. 70.37% of the isolates were found resistant to amoxycillin followed by levofloxacin and tetracycline with 55.55% resistance each. Moreover, antibiotics gentamicin, vancomycin and ciprofloxacin were the most effective antibiotics showing sensitivity against 74.07%, 70.37% and 62.96% of the isolates; respectively (Table 3). However, previously ciprofloxacin resistant strains isolated from meat origin have been reported (Waters et al 2011). Furthermore, most of our isolates (48.14%) were also resistant to erythromycin but 37.03% of strains showed intermediate level of susceptibility. Whereas the *Staphylococcus* spp. from study reported by Tassew et al. were observed to be resistant to erythromycin (65%), amoxicillin (60%), and vancomycin (20%) (Tassew et al. 2010). Furthermore, it has also been observed that all the isolates were resistant to at least one antibiotic (7.4%) while most of them showed multi drug resistant to three antibiotics (44.44%) (Figure 1).

To combat antibiotic resistance, essential oils have been extensively studied as they offer a sustainable and multifaceted approach in this regard (Núñez et al., 2018), (Ooi, et al., 2006). The analysis of antimicrobial activity showed that all five essential oils; clove, cinnamon, rosemary, eucalyptus, and palm rose are active against most of the isolated strains. The results indicate significant variations in the effectiveness of these essential oils, emphasizing more on their potential as alternative antibacterial agents. The findings obtained in this study are more encouraging to the practical use of EOs in inhibiting the growth and activity of *Staphylococci*. The results are shown as the triplicates of the mean of zone of inhibitions (ZOI). Cinnamon oil in its pure form showed the highest antibacterial activity against the isolated strains with ZOI ranging from 19-41mm (Figure 2). Parallel to this the isolated strains also showed a greater susceptibility from clove oil, its ZOI ranges from 19-33mm (Figure 3). Compared to this, rosemary, eucalyptus, and palm rose oil were less effective showing resistance to 11 (40.74%), 6 (22.22%) and 3 (11.11%) strains respectively (Figure 4, 5 & 6). Palm rose oil and eucalyptus oil were active against 88.88% and 70.73% of isolated Staphylococcal strains with ZOI ranging from 15-34 mm and 13-21 mm; respectively (Figure 5 & 6). No ZOI was observed in the well containing negative control.

These findings of our study are in line with the studies conducted by Ali et al., 2013 and Wang et al., 2018 who observed 18.18% and 11.5% prevalence of *S. aureus* in poultry meat: respectively. Some authors, however, reported varying prevalence rates such as Kitai and co-workers reported 57.1% (Kitai et al., 2005, De Boer et al., 2009, Szafraniec et al., 2020). The two strains (A6A and A45A) were observed as *S. warneri* strains which is one of the coagulase negative strains isolated from meat samples. They are known to be a part of the skin flora of animals (Xiao et al., 2022).

A significant drug resistance was observed by the antibiogram analysis of *Staphylococcus* spp. (Table 2). The use of antibiotics beyond their sub-lethal concentrations and inappropriate prescription for antibiotics in poultry farms contribute to high resistance levels of isolates found in poultry products. Like other findings cited in the literature, our results also revealed a varying pattern of resistance and sensitivity of isolates ranging from 25% to 73.3% against the tested antibiotics (Jaja et al., 2020, Yucel et al., 2011).

Globally, due to the pathogenicity and multidrug resistant phenomenon, *Staphylococcus* has garnered substantial public attention. The findings of this study reflect the potential prevalence of MDR Staphylococcal strains in poultry meat. The presence of MDR strains can be a serious threat to the community through the food chain. Not only is the mere presence of the *Staphylococcus* rather the strains having MDR can also create a therapeutic dilemma.

The study findings strongly support the research done by Reham et al., 2013 showing the maximum activity of cinnamon oil followed by clove oil whereas rosemary was less effective. These results are aligned with previous research highlighting the potent antimicrobial properties of palm rose and eucalyptus EOs. Our findings revealed that both cinnamon and clove oils exhibit substantial effectiveness against *Staphylococcus spp*. This is because cinnamon oil contains compounds like cinnamaldehyde which has been known for its broad-spectrum activity against bacteria (Huang et al., 2021). Likewise, the major component in clove oil is eugenol and has been well documented for its diverse antimicrobial properties. In addition, the research observed lower effectiveness of palm

rose oil and rosemary oil against *Staphylococcus spp*. This might be due to the composition of these oils which have reduced antibacterial efficacy.

4. CONCLUSION

This study investigates the diverse activity of EOs against *Staphylococcus spp*. The increased and promising efficacy of cinnamon and clove oil underscores the importance antimicrobial potential of EOs for practical/ industrial applications. This study can be used as a template for relatively expanded research against the pathogens of the same or other genera as it may lead to provide insights with the eco-friendly approach into their pharmaceutical and nutraceutical applications.

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Table 1. Identification and confirmation of *Staphylococcus aureus* by biochemical tests.

Strains	Catalase	Coagulase	Sulfur	Indole	Motility	Citrate	MR	VP
A1A	Positive	Positive	Negative	Positive	Non-Motile	Positive	Positive	Positive
A4A	Positive	Positive	Negative	Negative	Non-Motile	Negative	Positive	Positive
A5A	Positive	Positive	Negative	Negative	Non-Motile	Negative	Positive	Positive
A6A	Positive	Negative	Negative	Negative	Motile	Positive	Positive	Positive
A7A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A8A	Positive	Positive	Positive	Positive	Motile	Positive	Positive	Positive
A9A	Positive	Positive	Positive	Negative	Motile	Negative	Positive	Positive
A17A	Positive	Positive	Negative	Negative	Non-Motile	Negative	Positive	Positive
A21A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A35A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A45A	Positive	Negative	Negative	Negative	Non-Motile	Positive	Positive	Positive
A46A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A47A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A48A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
A57A	Positive	Positive	Negative	Negative	Non-Motile	Negative	Positive	Positive
A58A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y5A	Positive	Positive	Positive	Negative	Motile	Positive	Positive	Positive
Y6A	Positive	Positive	Negative	Positive	Motile	Positive	Positive	Positive
Y9A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y18A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y19A	Positive	Positive	Negative	Positive	Non-Motile	Positive	Positive	Positive
Y22A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y23A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y24A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y29A	Positive	Positive	Negative	Negative	Non-Motile	Negative	Positive	Positive
Y32A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Y35A	Positive	Positive	Negative	Negative	Non-Motile	Positive	Positive	Positive
Positive (%)	27 (100%)	25 (92.5%)	3 (11.1%)	4 (14.8%)	5 (18.5%)	6 (22.2%)	27 (100%)	27 (100%)
Negative (%)	0	2 (7.4%)	24 (88.8%)	23 (85.1%)	22 (81.4%)	21 (77.7%)	0	0

Table 2. Antibiotic susceptibility pattern of isolated *Staphylococcus* strains.

STRAINS	VA	TE	CN	AMC	LEV	E	CIP
A1A	0	9±0.047	19±0.081	8±0.047	17±0.081	0	17±0.047
A4A	0	16±0.081	12±0.094	15±0.081	12±0.047	0	8±0.047
A5A	0	10±0.047	15±0.081	0	23±0.081	0	21±0.081
A6A	9±0.047	11±0.081	21±0.047	13±0.094	16±0.081	11±0.047	13±0.047
A7A	13±0.094	30±0.047	21±0.047	17±0.081	17±0.081	24±0.081	26±0.081
A8A	15±0.081	10±0.047	15±0.081	19±0.047	11±0.047	0	0
A9A	18±0.047	20±0.081	16±0.081	19±0.047	11±0.047	21±0.081	11±0.081
A17A	13±0.081	31±0.047	19±0.081	15±0.081	12±0.047	25±0.081	29±0.081
A21A	16±0.081	20±0.081	19±0.081	14±0.047	14±0.081	18±0.094	31±0.047
A35A	16±0.081	33±0.081	20±0.047	14±0.047	13±0.081	20±0.081	32±0.047
A45A	16±0.081	36±0.047	0	16±0.081	0	0	34±0.081
A46A	19±0.081	28±0.081	17±0.094	13±0.094	17±0.081	24±0.081	35±0.047
A47A	18±0.081	32±0.081	14±0.081	0	15±0.094	17±0.081	30±0.081
A48A	15±0.081	32±0.081	20±0.047	13±0.081	14±0.047	21±0.081	32±0.047
A57A	19±0.081	10±0.047	20±0.047	13±0.081	13±0.081	18±0.047	32±0.081
A8A	18±0.081	10±0.047	23±0.081	13±0.081	14±0.047	0	39±0.047
Y5A	17±0.081	11±0.081	19±0.081	23±0.047	9±0.081	0	20±0.047
Y6A	17±0.081	13±0.047	0	37±0.081	0	0	29±0.081
Y9A	15±0.081	10±0.047	23±0.081	20±0.081	31±0.047	0	27±0.094
Y18A	19±0.081	15±0.047	25±0.047	16±0.081	35±0.081	25±0.047	0
Y19A	15±0.081	11±0.094	20±0.047	9±0.081	26±0.047	21±0.081	27±0.081
Y22A	22±0.047	11±0.081	12±0.094	10±0.047	19±0.081	18±0.047	32±0.047
Y23A	12±0.094	12±0.081	29±0.047	13±0.081	19±0.047	19±0.081	29±0.081
Y24A	15±0.081	9±0.047	0	30±0.047	11±0.047	20±0.081	22±0.047
Y29A	22±0.047	18±0.081	9±0.081	10±0.047	23±0.081	0	17±0.081
Y32A	0	0	26±0.047	21±0.081	0	0	0
Y35A	19±0.081	12±0.081	29±0.047	33±0.047	25±0.047	10±0.081	9±0.081

Table 3. Percentage of Resistance and Sensitivity.

Percentage of Resistant, Sensitive & Intermediate	VA	TE	CN	AMC	LEV	E	CIP
Resistant	29.60%	55.55%	18.51%	70.37%	55.55%	48.14%	25.92%
Sensitive	70.37%	33.33%	74.07%	22.22%	29.62%	14.81%	62.96%
Intermediate	0%	11.11%	7.40%	7.40%	14.81%	37.03%	11.11%

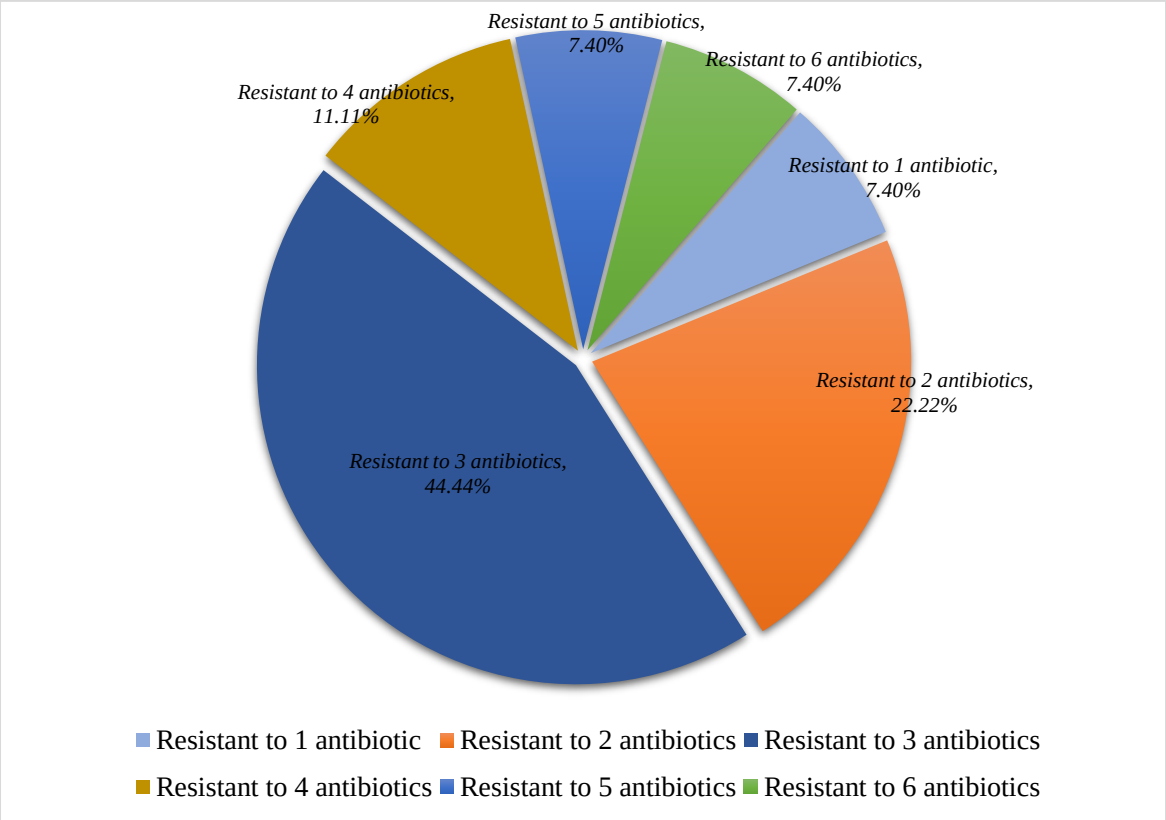

Figure 1: Pie chart depicting antibiotic resistance profile of *Staphylococcus* strains.

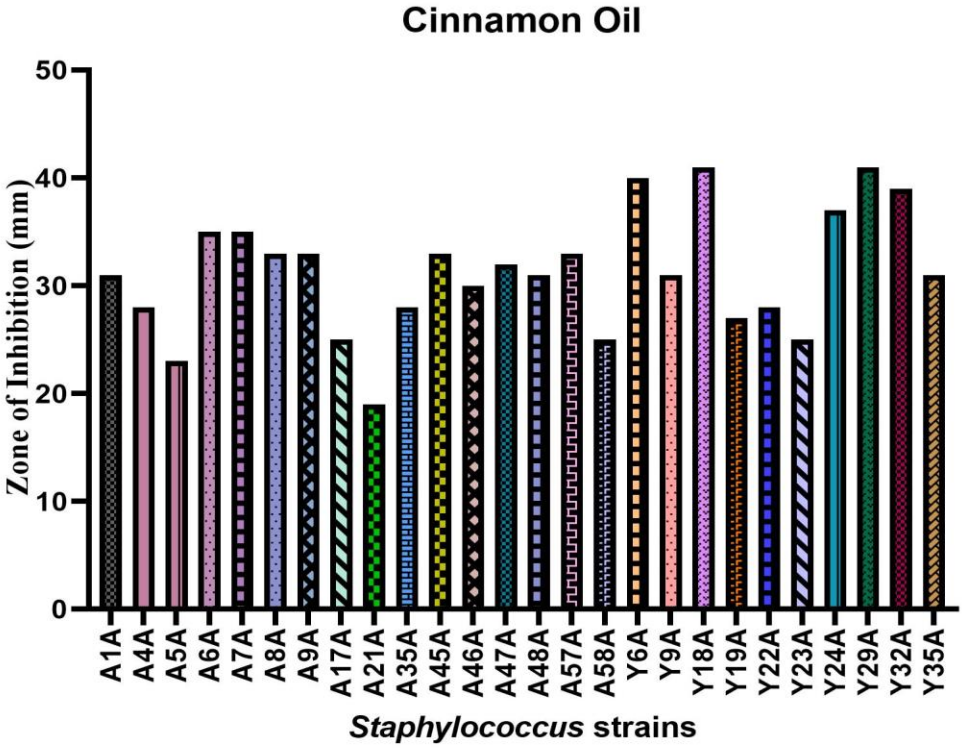

Figure 2: Antibacterial activity of cinnamon essential oil (undiluted) against isolated *Staphylococcus* strains. *Staphylococcus* strains with cinnamon oil and control via agar well diffusion method was incubated for 24 h and 37 ºC. Data represents mean values of triplicate measurements of zone diameters ±SD.

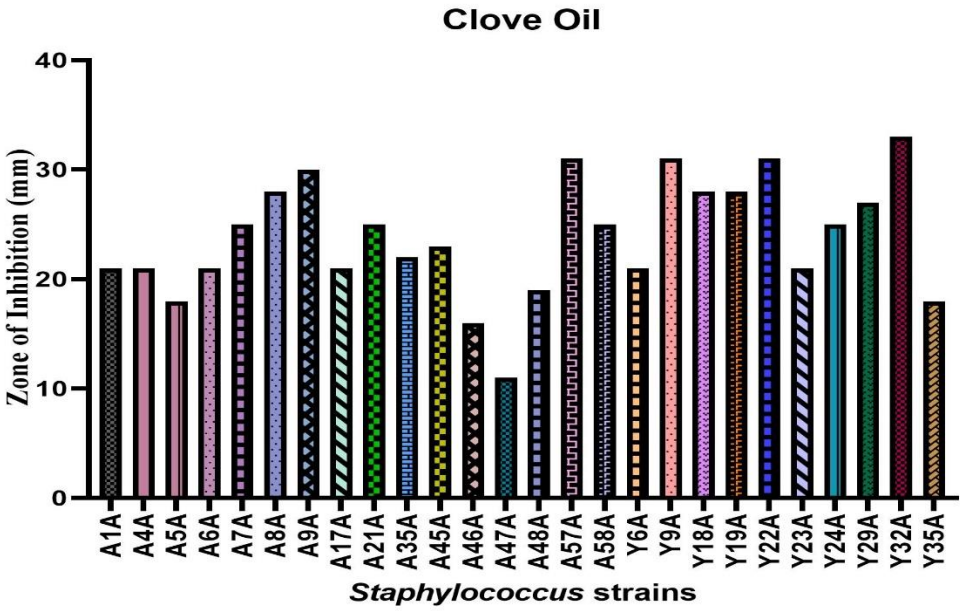

Figure 3: Antibacterial activity of clove essential oil (undiluted) against isolated *Staphylococcus* strains. *Staphylococcus* strains with clove oil and control via agar well diffusion method was incubated for 24 h and 37 ºC. Data represents mean values of triplicate measurements of zone diameters ±SD.

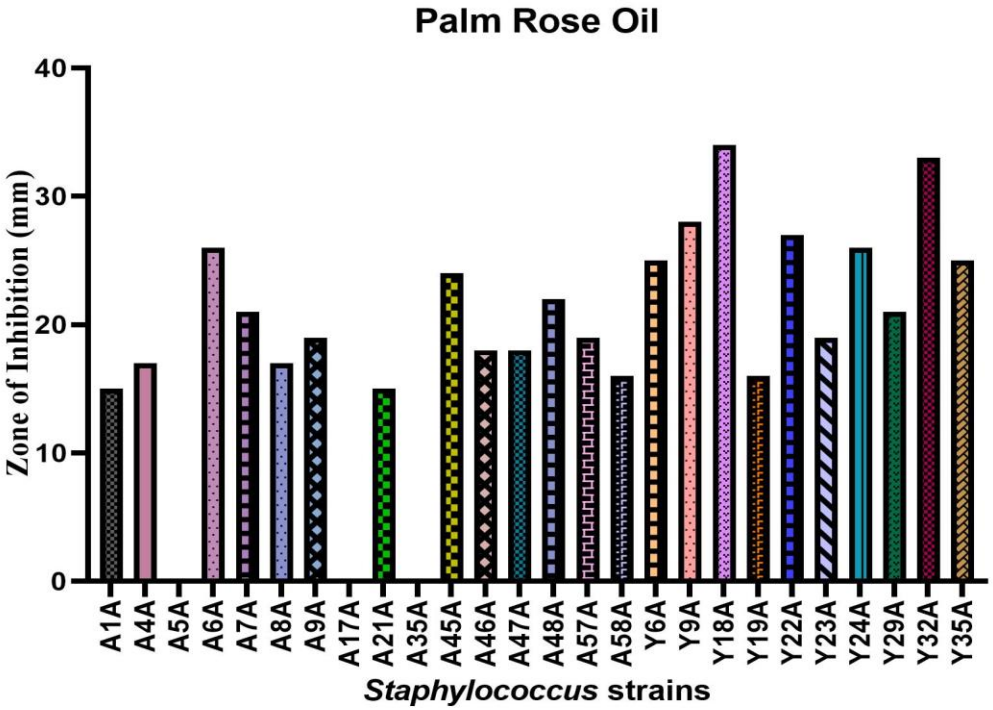

Figure 4: Antibacterial activity of palm rose essential oil (undiluted) against isolated *Staphylococcus* strains. *Staphylococcus* strains with palm rose oil and control via agar well diffusion method was incubated for 24 h and 37 ºC. Data represents mean values of triplicate measurements of zone diameters ±SD.

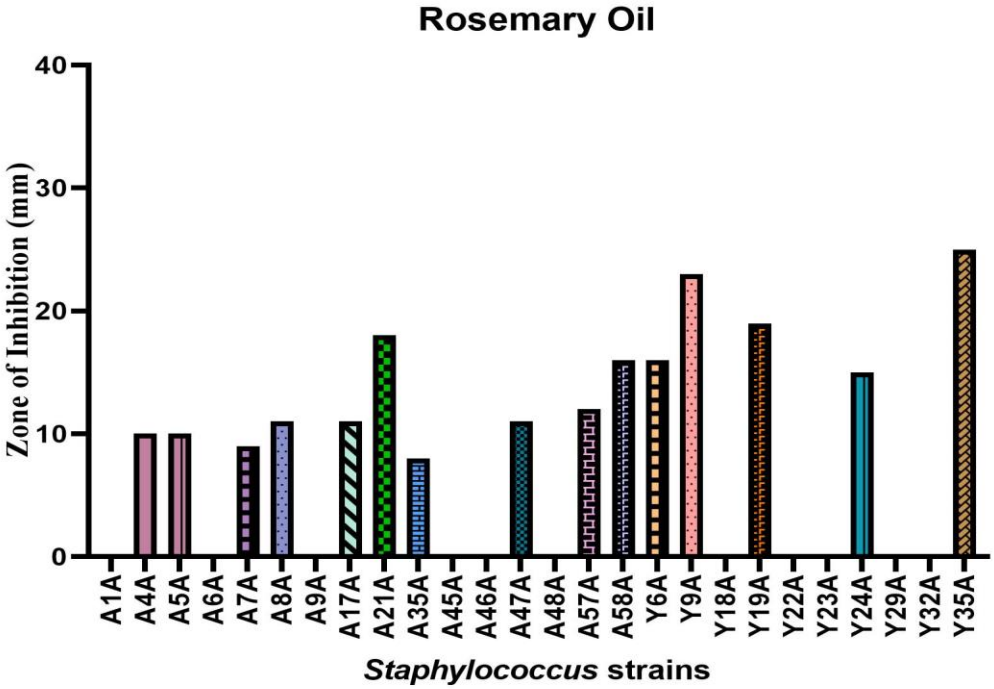

Figure 5: Antibacterial activity of rosemary essential oil (undiluted) against isolated *Staphylococcus* strains. *Staphylococcus* strains with rosemary oil and control via agar well diffusion method was incubated for 24 h and 37 ºC. Data represents mean values of triplicate measurements of zone diameters ±SD.

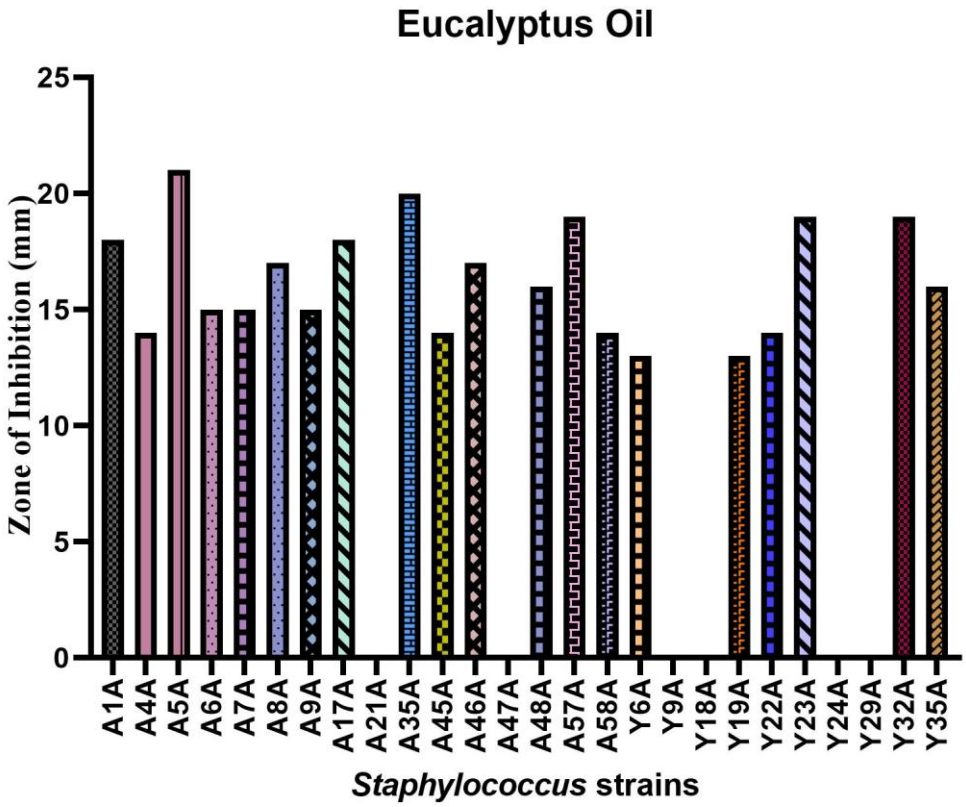

Figure 6: Antibacterial activity of eucalyptus essential oil (undiluted) against isolated *Staphylococcus* strains. *Staphylococcus* strains with eucalyptus oil and control via agar well diffusion method was incubated for 24 h and 37ºC. Data represents mean values of triplicate measurements of zone diameters ±SD.

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