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Isolation, identification and characterization of skin microbiome and effect of commercial essential oils

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The study of human skin microflora provides insight into host-associated microbes. Skin microflora varies by site on human body. This research involved isolating and characterizing microbes from different body regions using microscopic, morphological, biochemical, and antibiotic sensitivity tests to identify any association patterns with the sites. Statistical analysis revealed a site-specific association of bacteria.The microflora isolated from skin included Coagulase Negative *Staphylococcus* spp. *Aspergillus* sp. and *Curvularia* sp. known opportunistic pathogens that can cause infections in individuals with compromised immunity or disrupted skin barriers. The study included examination of the antimicrobial effects of commercial essential oils (Lemongrass, Lemon, and Peppermint) on these opportunistic pathogens using agar well diffusion method. Lemongrass proved effective against most isolates, while Lemon and Peppermint showed limited efficacy. *Curvularia* sp. was sensitive to Lemon, Peppermint and resistant to Lemongrass. Consequently, Lemon grass essential oil is recommended for treatments targeting these pathogens. However, the combination of Lemon, Lemongrass, and Peppermint exhibited antagonistic effects, suggesting these combinations should not be included in skin ointments as therapeutic agents aimed at reducing opportunistic pathogens. Further investigation is needed to confirm the molecular identification of isolates and to examine the components of essential oils that have antagonistic effect against the isolated opportunistic pathogens.

Keywords : Agar well diffusion method, Antagonistic effect, Antibiotic sensitivity, Essential oils, Skin Microflora

INTRODUCTION

The skin is the largest organ of the body, serving as a physical barrier that protects against foreign organisms and toxic substances. It acts as an interface with the external environment and hosts a diverse collection of microorganisms, including bacteria, fungi, and viruses. This diverse microbial community, known as the skin microbiota, varies significantly across different body sites due to anatomical and physiological differences (Grice and Segre, 2011). The skin normal microflora must adapt to challenging conditions, including the presence of lysozyme and antimicrobial peptides produced by sweat glands, which leads to different body sites hosting specific microbes suited to these environments.

Various studies on skin microflora have been conducted, comparing the microbiome differences across different genders, ages, body sites, and health conditions, including infections (Gao *et al.* 2008; Kong *et al.* 2012; Perez *et al.* 2016; Gautam *et al.* 2017; Lam *et al.* 2018; Hamdy *et al.* 2024).

There exists an intricate interplay between skin microflora which helps in preventing the colonization of pathogenic bacteria and maintaining homeostasis. While Coagulase-Negative *Staphylococci* (CoNS) like *S. hominis* and *S. lugdunensis* can prevent colonization of opportunistic pathogen, *Staphylococcus aureus*, they can also become opportunistic pathogens themselves, particularly in immunocompromised individuals, patients with catheters, medical implants, devices and dialysis (Bieber and Kahlmeter, 2010; Widerström *et al.* 2011; Kilic and Basustaoglu, 2011; Findley *et al.* 2013; Heldt

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Manica *et al.* 2017; Gowda *et al.* 2018; Mani and Chandrasekharan, 2022).

There is a growing interest in using natural antimicrobial compounds to combat bacterial infections, especially given the rise in antimicrobial resistance (Antimicrobial Resistance Collaborators, 2022). Plant extracts and essential oils have been used historically for their antimicrobial properties with minimal side effects (Baser and Buchbauer, 2009). Essential oils show different biological activities like antibacterial, antiviral, antifungal, antioxidant, insecticidal, natural therapies and pharmaceuticals (Irshad *et al.* 2019). Lemon (*Citrus limon*), Lemongrass (*Cymbopogon flexuosus*) and Peppermint (*Mentha piperita*) essential oils are proven to be antimicrobial in nature (Mahboubi and Kazempour, 2014; Boukhatem *et al.* 2014; Desam *et al.,* 2017; Orchard and Vuuren, 2017; Hamdan *et al.,* 2024). Studies conducted so far have not tried a combined antimicrobial activity of the above-mentioned essential oils.

Despite their potential, only a few studies have examined the impact of essential oils on skin microflora, particularly regarding opportunistic pathogens (Imelouane *et al.* 2009; Ebani *et al.* 2020; Kaèániová *et al.* 2020; Abers *et al.* 2021). This study aims to isolate, identify, and characterize skin microflora from different body sites, look for site specific association and assess the antimicrobial activity of essential oils against these microbes. Interestingly, while one of the essential oils showed maximum antimicrobial activity, their combination exhibited an antagonistic effect by reducing the antimicrobial activity, suggesting a complex interaction between the components, indicating a need for further investigation.The present study addresses the gap in understanding the antimicrobial activity of specific essential oils on particular skin microflora isolates and also summarizes the site-specific patterns of the bacteria. The study suggests the development of new antimicrobial treatments targeting opportunistic pathogens on the skin to avoid this combination of essential oils.

MATERIALSANDMETHODS

Sample collection and isolation of microbes

The two healthy subjects were selected for the study. The four sites on the body were chosen (Armpit, Behind the Earlobe crease, Foot Web and Toenail) for the sample collection. Sterile cotton swabs were damped with sterile water and brushed against the surface of the skin of the subjects. The swab was streaked onto the surface of the Nutrient agar (NA, HiMedia) plate incubated at 37 ^{0}C for 24 hr and Potato Dextrose Agar with streptomycin (PDA, Hi Media) plate incubated at room temperature for 5 days. After respective incubation period, the isolated colony was selected and sub-cultured on a fresh NA (bacteria) plate and PDA (fungi) plate and maintained as pure cultures at 4°C in a refrigerator and used for further studies.

Bacterial isolates - characterization studies

The study was performed according to Bergey's Manual of Systematic Bacteriology (De Vos *et al.*, 2010) and methods described by Cunha *et al.* (2004) and Kawamura *et al.*(1998). Colony morphology was noted based on size, shape, elevation, margin, texture and color. Gram staining was performed to observe the size, shape, arrangement and gram characteristic. Characterization was done based on biochemical tests like Catalase test, Oxidase test, Sugar fermentation test, Nitrate reduction test, Voges Proskauer test, Urease test and Novobiocin susceptibility test.

Biochemical tests

Catalase Test

A loopful of the overnight pure culture was mixed with a drop of Hydrogen Peroxide to observe formation of bubbles (Reiner, 2010).

Oxidase Test

The overnight pure culture was streak on the oxidase disc (HiMedia) and color change was observed within 8 seconds (Shields and Cathcart, 2010).

Sugar fermentation test

Sugars selected were Glucose, Mannitol, Maltose, Sucrose and Fructose. To alkaline peptone water (HiMedia), these sugars were added individually

along with inverted Durham's tubes and phenol red in the test tubes and autoclaved at 10 lbs. The overnight pure culture of 0.5 McFarland units was inoculated and incubated for 24 hr (Reiner, 2012).

Nitrate reduction test

The overnight pure culture of 0.5 McFarland units was inoculated in Nitrate Broth (HiMedia) and incubated for 24 hrs. Sulfanilic acid (Reagent A) and α–naphthylamine (Reagent B) was added. Red color was considered positive. Zinc powder was also added at the end to confirm true negatives (MacFaddin, 1980).

Voges Proskauer test

The overnight pure culture was inoculated in sterile MRVP Broth (HiMedia) and incubated for 24 hrs. Potassium Hydroxide and α naphthol were added. Cherry Red was considered positive (McDevitt, 2009).

Urease test

On to sterile Urea Agar (HiMedia), containing 40% Urea was inoculated with overnight pure culture and incubated for 24 hrs (Brink, 2012).

Novobiocin sensitivity test

The overnight culture of 100 μl was plated on Mueller Hinton Agar (HiMedia) using spread plate technique and Novobiocin disc (HiMedia) 5 g was placed on the surface of the agar medium, incubated for 24 hrs and the zone of inhibition of above 16 mm was considered sensitive (Bauer *et al.*, 1966).

Statistical Tests

To find the significant association between the sites and bacteria, Simulated Chi square test was performed due to low expected frequencies. The data was organized into a contingency table with rows representing sites and columns representing bacterial species. This table was constructed using R's matrix function. chisq.test() function from R's base package which was used along with simulate.p.value. Similar approach was used for identifying the association between the type of essential oil and sensitivity of all isolates. One sample T test was performed for combined antimicrobial activity of essential oils using t.test() function with the hypothesized mean value as 17 mm and 18 mm for bacterial and fungal isolates, respectively. Sample mean and p values were generated and compared with hypothesized mean to conclude the resistance or sensitivity of isolates to the combination of oils. Stacked bar plot, heatmap and bar graph were used for visualization and were plotted using R software (v4.3.1) (R Core Team, 2023).

Fungal Characterization studies

The identification of fungal isolates was done on the basis of colony morphology and Lactophenol Blue staining.

Morphological study on SDA, YES, PDA, Czapek Dox agar and Malt extract agar

Morphology of the G Fungal colony was studied on Sabouraud's Dextrose agar (SDA), Malt Extract Agar, Yeast Extract Sucrose (YES) Agar and Czapekdox Agar (HiMedia). Color on the Front and reverse side of the plate and duration was studied by inoculating the spores on the media and incubating for 5 days at room temperature (Kadhim and Faleh, 2020). Morphology of B isolate was studied on PDA.

Lactophenol Blue staining

The small quantity of mycelium along with spores was scraped off, placed on a clean grease-free slide and teased with a drop of distilled water; stained with a drop of lactophenol blue stain, HiMedia (Leck, 1999).

Aflatoxicity test

The spores were inoculated on YES medium with the help of sterile needle and incubated for 5 days at room temperature. A few drops of 10% Ammonium Hydroxide were added on the lid of the petri dish and incubated for 2 hr (Saito and Machida *et al.*1999).

Antibacterial activity with Essential Oils by Agar well diffusion method

This test was performed using Mueller Hinton Agar (HiMedia), 100 μl of overnight culture was plated; among three agar wells, each well was filled with

50 μl of essential oil (Peppermint, Lemon and Lemongrass) respectively; one agar well was filled with 50 μl of mixed essential oil (16.66 μl of each Peppermint, Lemon and Lemongrass); Novobiocin (30 μg) and Vancomycin (30 μg) discs, (HiMedia) were used as positive controls,the zone of inhibition above 16 mm was considered sensitive (Costa Júnior *et al.* 2017).

Antifungal activity with Essential Oils by Agar well diffusion method

This test was performed on Potato Dextrose Agar (HiMedia), 100 μl of pure culture was plated; agar well diffusion assay was performed. Itraconazole (10 μg) (HiMedia) was used as positive control and the zone of inhibition above 17 mm was considered sensitive. (Abu El- Hamd *et al.* 2020).

RESULTS AND DISCUSSION

Sample collection and isolation of microbes

The four bacterial colonies from each medium plate labelled; Armpit site (A1, A2, A3, A4)); Behind the earlobe crease (B1, B2, B3, B4); Toenail (T1,T2,T3, T4); Foot web (F1, F2, F3, F4);Foot web (G, B) fungal colonies were selected for the study.

Bacterial isolates - characterization studies

The colony morphological features, Gram nature (Fig. 1), Biochemical tests and Novobiocin sensitivity test (Table 1) with reference to Bergey's manual,Cunha *et al.* 2004 and Tektook *et al.,* 2016, the isolates were identified (Table 2) as *Staphylococcus hominis* (A1, A2, A3, A4); *Staphylococcus lugdunensis* (B1, B2, B3, B4, F1, F2, F3, T1, T2); *Staphylococcus caprae*(F4), *Staphylococcus haemolyticus* (T3), *Staphylococcus saprophyticus* (T4).

Statistical Test

Simulation-based chi-square test generated a p-value of 0.0014. This p-value is much smaller than the common significance level of 0.05. This indicates strong evidence against the null hypothesis which states that there was a uniform distribution. The results suggest that there was a significant association between the sites and bacteria and that certain bacteria were more prevalent in specific sites than others.

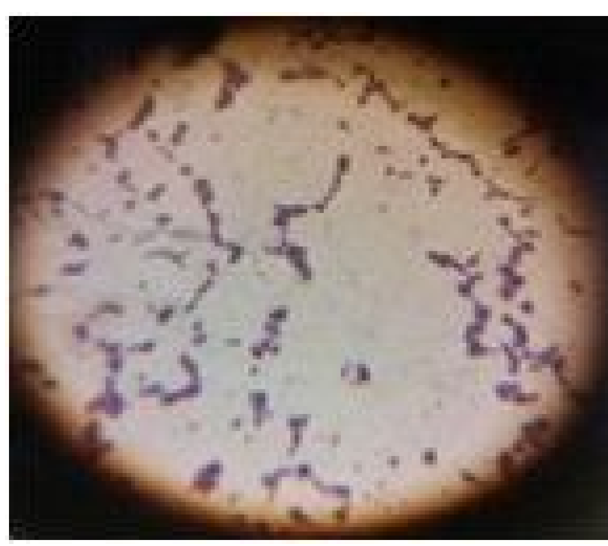


Fig .1 : Gram positive cocci in clusters

The results of, observed versus expected frequencies are indicated in Figure 2. The stacked bar plot highlights that the observed frequencies of certain microbes, such as *S. lugdunesis* in "Behind the Earlobe crease" and "Foot Web", and *S. hominis* in "Armpit", deviate significantly from the expected uniform distribution, reinforcing the statistical finding that the bacterial distribution is non-uniform across different skin sites, could be due to different conditions across the sites, indicating site specific association of bacteria.

The studies indicate that *Staphylococcus hominis* is highly abundant in the armpit, which aligns with our findings where all isolates from the armpit were *S. hominis* (Callewaert, 2013; Troccaz *et al.* 2015). *S. lugdunensis*, typically found in lower extremities such as toes and foot webs, was isolated from behind the ear, possibly due to the similar warm and moist environment (Bieber and Kahlmeter, 2010). A study on foot microbiome showed that 90% of bacteria were *S. haemolyticus* which explains our isolation *of S. haemolyticus* from the toenail (Stegliñska *et al.* 2019). *S. saprophyticus*, usually found in high humidity and nutrient-rich areas like the gastrointestinal tract, vagina, and perineum, was also isolated from the toenail, likely due to the humid, nutrient-rich environment provided by the toenail and surrounding skin, especially while

Table 1: Biochemical tests

Test	Isolates															
	A1	A2	A3	A4	B1	B2	B3	B4	T1	T2	T3	T4	F1	F2	F3	F4
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sugar fermentation																
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Maltose	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Voges- Proskauer	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+
Novobiocin sensitivity (mm)	29	27	30	28	29	27	24	24	24	28	26	12	20	20	20	19

Table 2 : Identification of isolates

Isolates	Identification	No. of isolates
A1, A2, A3, A4	*Staphylococcus hominis*	4
B1, B2, B3, B4, F1, F2, F3, T1, T2	*Staphylococcus lugdunensis*	9
F4	*Staphylococcus caprae*	1
T3	*Staphylococcus haemolyticus*	1
T4	*Staphylococcus saprophyticus*	1

wearing shoes during monsoon (Ehlers and Merrill, 2023). *S. caprae*, originally isolated from goat milk and later found on human skin and nails, was found to be isolated from the toenail in the study by Gowda *et al.* (2018).

Fungal Isolates- Characterization studies

Morphological study on SDA, YES, PDA, Czapek Dox agar and Malt extract agar

The morphological features were indicated in Table 3. Similar observations were observed by Kadhim and Faleh (2020). The morphology on the Yeast Extract Sucrose Agar doesn't correlate with the investigations of Diba *et al.*(2007); and Mamo *et al.*(2017). Morphological features of B isolate were similar to that observed by Pan *et al.* (2018) and Shivakumar *et al.*(2023).

Lactophenol Blue staining

According to the staining, microscopic and morphological results of fungal isolates, G and B

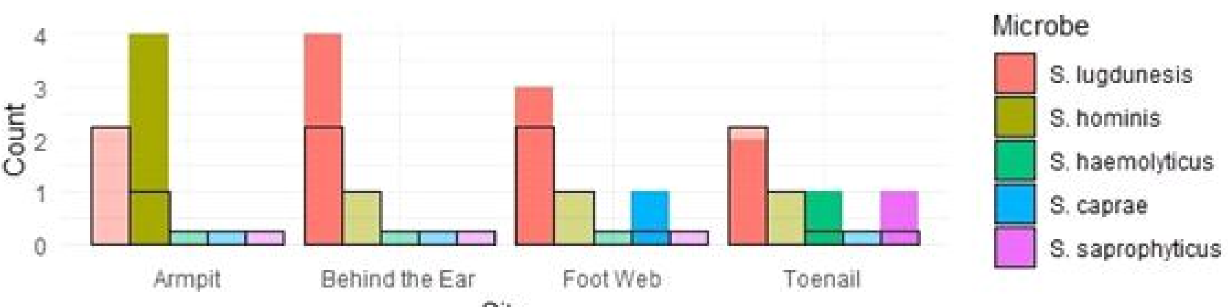


Fig. 2: Stacked bar plot - Comparison between observed and expected frequencies of five different bacterial isolates from four different skin sites. The Black border ones represent the expected frequencies.

Table 3: Morphological Characteristics of G and B fungal isolates

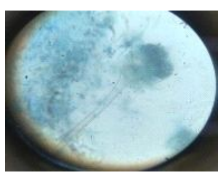
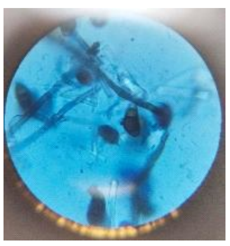
Isolate	Media	Morphology and Duration	Pictures
G	Sabouraud's Dextrose Agar	Front view: White edges, yellow in center and green velvety. Reverse side: Yellow color. 5 days at 27 ^{0}C	
	Malt Extract Agar	Front view: Dark green cobr on the top surface. Reverse side: Yellow. 5 days 27 ^{0}C	
	Czapekdox Agar	Front side: White edges Reverse side: White colonies 5 days 27 ^{0}C	
	Yeast Extract Sucrose Agar	Front side: Yellowish colonies. Reverse side: Yellow coloration. 5 days 27 ^{0}C	
B	Potato Dextrose Agar	Initially, fast growing white cottony growth is seen which turns into black, suedelike upon further incubation. 5 days 27 ^{0}C	

were identified as *Aspergillus* sp. and *Curvularia* sp. (Table 3 and 4). Similar observations were reported by Kadhim and Faleh (2020), Okayo *et al.* (2020) and Sanon *et al.* (2022).

Aflatoxicity test

The yellow color was observed on the reverse side of the plate, indicating low to no Aflatoxin produced by the colony upon exposure to Ammonium Hydroxide. There are two types of strains prevalent among *Aspergillus flavus*, non-toxigenic strains being one of them. Non-toxigenic strains will show yellow color for this test (Norlia *et al.* 2019**).** The morphological characteristics of isolate G and B (*Aspergillus* sp. and *Curvularia* sp.) were indicated in Table 4. Fungi are known to be a part of the foot

Table 4: Microscopic observation of G and B isolates

Isolate	Microscopic observation	Microscopic view
G	Conidiophore aseptate, unbranched, with vesicle bearing metulae and phialides radiating in all directions, producing conidia	
B	Conidiophore septate, conidia are straight or pyriform, brown, multiseptated, and have dark basal protuberant hila. The transverse septa divide each conidium into multiple cells. The central cell is typically darker and enlarged compared to the end. The central septum appears darker	

microbiome, as proven by a study conducted by Shivaji *et al.* (2019). Additionally, a study by Stegliñska *et al*. (2019) found that *Aspergillus* sp. were dominant in both men's and women's feet. Another study by Anagor *et al.* (2023) showed that *Aspergillus* sp. and *Curvularia lunata* were isolated from Foot web, which matches with the observation of our study.

Antibacterial activity with Essential Oils by Agar well diffusion method

Most of the bacterial isolates were sensitive to Lemongrass while resistance was show towards Lemon and Peppermint (Fig.3).

Antifungal activity with Essential Oils by Agar well diffusion method

The *Curvularia* sp. was sensitive to Lemon and Peppermint and resistant to Lemongrass whereas the *Aspergillus* sp. was sensitive to Lemongrass and resistant to Lemon and Peppermint (Fig.3).

Statistical Analysis - Individual essential oil activity against bacterial and fungal isolates

The Simulated Chi-square test confirms that there was a statistically significant association between the type of essential oil and sensitivity, p- value generated was 0.001. The heatmap visually represents this relationship, highlighting the effectiveness of Lemongrass compared to Lemon and Peppermint.Recently, some researchers have reported that monoterpene or sesquiterpene hydrocarbons and their oxygenated derivatives, which are the major components of essential oils, exhibit potential antimicrobial activities. In the present study, these components might be in less concentration in the lemon and peppermint essential oils to be effective against most isolates. Other reasons could be that the strains isolated could be resistant or form biofilm which confer resistance (Ben Hsouna *et al.,* 2017).

Combined essential oil antagonistic activity against bacterial and fungal isolates

The seven isolates were resistant and rest of the isolates showed less sensitivity to combined than individual essential oil demonstrating antagonistic effect (Fig. 3). The less zone of inhibition compared to individual essential oil could be due to reaction between components of essential oils (Van Zyla *et al.,* 2010).

A one-sample T test was conducted with hypothesized values of 17 mm for bacteria and 18 mm for fungi, representing the minimum inhibition to be considered sensitive to essential oils. The sample means were found to be 10 mm and 19 mm, respectively. The p-value for bacteria was 0.02, indicating a statistically significant difference between the sample mean and the hypothesized mean of 17 mm. The sample mean of 10 mm suggests resistance of bacterial

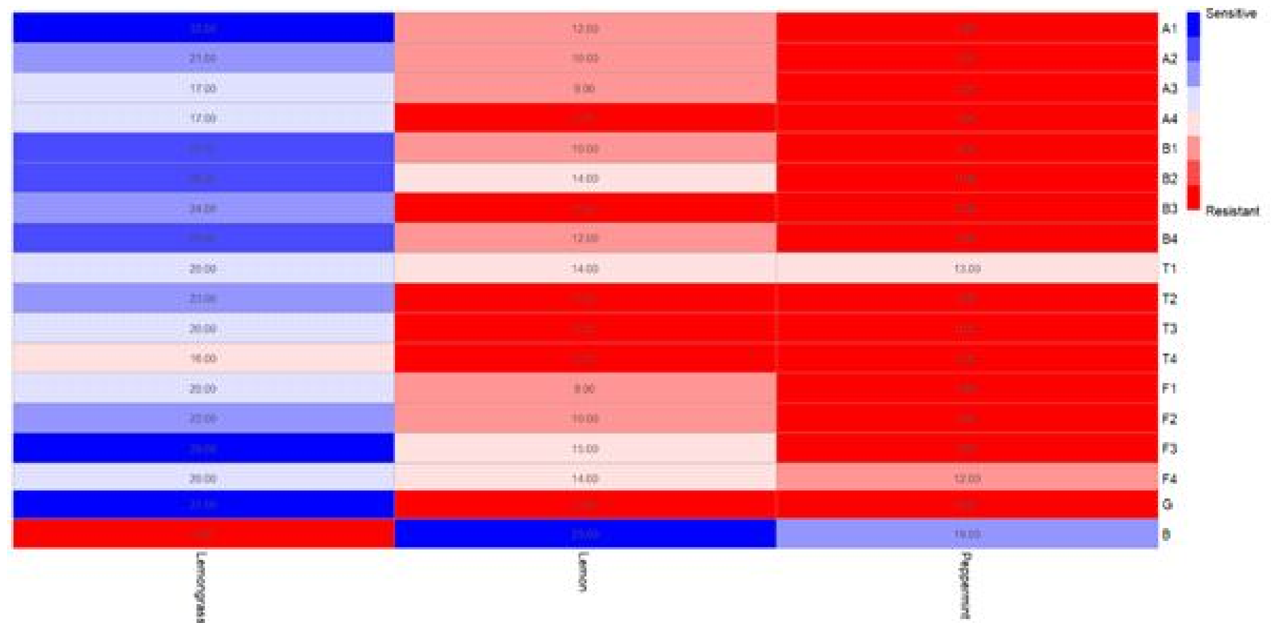


Fig. 3: Heatmap showing most isolates being sensitive to Lemongrass; Resistant to Lemon and Peppermint Essential Oils; Zone of inhibition (mm) of isolates, represented in the heat map

isolates to the antimicrobial agent. For fungi, the p-value was 0.6, indicating no significant difference from the hypothesized mean of 18 mm, which could be due to the small sample size.

The sample mean of 19 mm suggests sensitivity. To understand antagonistic activity, more data is needed to prove reduction in antimicrobial activity statistically. Further investigation is required to confirm the results of identification of isolates, also composition and interactions between essential oils.

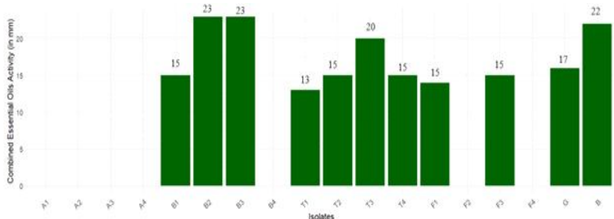


Fig. 4: Combined essential oil antagonistic activity against bacterial and fungal isolates; Zone of inhibition (mm) of isolates, represented at the top of bar.

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DECLARATION

Conflict of interest. Authors declare no conflict of interest

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