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Antibacterial activity of chloroxylenol and thymol against pathogenic bacteria isolated from under long nails

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Abstract. – OBJECTIVE: Fingernails are a perfect area for harboring bacteria such as *Staphylococcus aureus, Salmonella sp., Shigella sp.,* and *Escherichia coli*. These bacteria under the long nails may cause diseases due to the contact of nails with food or while biting the nails. Our study aimed to compare the antibacterial activity of chloroxylenol and thymol, two different detergent ingredients, on microorganisms isolated from long fingernails. This study was performed to raise awareness of the dangers of long nails and the importance of good nail hygiene.

PATIENTS AND METHODS: The present study was performed on female students at the Faculty of Science, King Abdelaziz University. Bacteria were isolated from under one finger nails and cultured on both McConkey agar and mannitol salt agar. After incubation, we isolated bacteria on nutrient agar. After that, we conducted several tests to determine the isolate type. Finally, we prepared three different concentrations of chloroxylenol and thymol to compare their effect on the isolated bacteria using antibacterial activity on Mueller-Hinton agar.

RESULTS: Two types of bacteria were isolated, pathogenic bacteria called *Staphylococcus aureus* and non-pathogenic bacteria called *Staphylococcus epidermidis*. *Staphylococci* have more sensitivity to chloroxylenol than thymol. In addition, chloroxylenol, at high concentrations had a more powerful antibacterial effect.

CONCLUSIONS: The results emphasized that fingernails could harbor pathogenic bacteria which are difficult to remove. Perfect hand hygiene is essential to prevent the spread of diseases.

Key Words:
Long nails, Pathogens, Chloroxylenol, Thymol, Detergents, Hand hygiene, Diseases.

Introduction

Fingernails are considered suitable habitats for different kinds of microbes. Therefore, they spread diseases, especially foodborne diseases[1]. Unexpectedly, human fingernails contain the highest concentration of microorganisms[2]. The nail comprises several components: nail root, nail bed, nail plate, eponychium, and hyponychium. Nails mainly comprise keratin protein and encapsulated layers[3]. The area between the skin and nails is an ideal environment for the growth and reproduction of microorganisms. Therefore, nails play an essential role in the spread of many pathogens, even drug-resistant ones[4].

Staphylococcus aureus from under the fingernails is one of the most prevalent pathogenic bacteria that commonly causes infection and produces antibiotic resistance[5]. In addition, many bacteria under the nails indicate that antimicrobial agents do not reach this area during routine handwashing procedures[1,3,6].

The transmission of nail bacteria occurs through food and water or nails and fingers contaminated with infected feces, proving bacteria's role in transmitting the disease from one person to another[3]. *Staphylococcus aureus spp.* presents a high risk because it produces enterotoxins and causes food poisoning[7]. Good handwashing is the first defense against disease-causing microorganisms. It is the best way to prevent the transmission and spread of germs[8]. Many active ingredients of antiseptic detergents are on the market, such as chloroxylenol and thymol.

Chloroxylenol (para-chloro-meta-xylenol) is a popular disinfectant used in personal care products. It has been reported that chloroxylenol has antimicrobial properties against some pathogenic bacteria. It is used to treat cuts, bites, and sting. It is also used as a hand sanitizer[9]. Chloroxylenol is a broad-spectrum antimicrobial agent used extensively in industrial, consumer, and healthcare products, including cosmetics, household chemicals, and disinfection products such as preservatives or disinfectants[10]. Chloroxylenol is a solid antimicrobial capable of reducing popu-

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lations of bacteria, such as *Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris,* and *Salmonella typhi*; and fungi, such as *Aspergillus niger, Aspergillus favus, Candida albicans,* and *Candida parapsilosis*. Moreover, the virucidal activity of chloroxylenol towards Ebola virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been confirmed, making it widely used for disinfection during the SARS-CoV-2 pandemic[11-16]. Generally, it is assumed that the mechanism of action of chloroxylenol is similar to that of other phenolic and halo phenolic antibacterial agents, particularly those perturbing cell membranes and causing cell leakage[10]. In addition, chloroxylenol strongly affects Gram-positive bacteria, as it strongly affects *Staphylococcus aureus spp*[17]. Furthermore, chloroxylenol acts on the cytoplasmic membrane of bacteria, causing protein denaturation. Another study[18] also shown that the increase in the concentration of the active ingredient, chloroxylenol, resulted in coagulation of the functional proteins and nucleic acids in the cell which then stopped working, leading to rapid cell death.

Thymol, known chemically as 2-isopropyl-5-methyl phenol, has medicinal properties[19]. Thymol is the main component of thyme essential oil from the *Thymus*, Lamiaceae family. This monoterpene derivative has a wide range of uses in many industries including medical, food, pesticide, and perfume[20,21]. It has antioxidant, anti-inflammatory, anticancer, and immunomodulatory effects. This makes thymol effective in treating several diseases[21,22]. In addition, thymol has a potent inhibitory activity on both Gram-positive and Gram-negative pathogens, including *Salmonella Typhimurium[23], Aeromonas hydrophila*[24], *Streptococcus pneumoniae*[25] and *Staphylococcus aureus*[26]. The primary mechanism of action of thymol is membrane dysfunction. It can be used as a naturally occurring drug against *S. Typhimurium* instead of synthetic drugs[27]. Hydroxyl groups that found in thymol are highly reactive and form hydrogen bonds with active sites of target enzymes, inactivating them[28,29] leading to dysfunction or rupture of the cell membrane[30-32].

The objective of the present study was to isolate and identify the bacterial species associated with long fingernails using morphological and biochemical approaches. In addition, the study also aims to compare the antibacterial activity of two antiseptic detergent active ingredients, chloroxylenol and thymol, against the bacterial isolates from the under nails of female students at the Faculty of Science, King Abdelaziz University, Saudi Arabia.

Patients and Methods

The present study was performed on female students at the Faculty of Science, King Abdelaziz University. Chloroxylenol and thymol were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA) with compound purities at ~98%. Each ingredient was dissolved in 95% alcohol solution to prepare three different concentrations, 1%, 3%, and 5%.

Sampling

Samples for this study were isolated from six female students who had long nails at King Abdelaziz University from the Faculty of Science.

Ethical Considerations

Sample collection was explained to participants undergoing the study. All students were informed about the research objectives and procedures. Written valid consent was obtained from all participants.

Isolation

Twelve cotton swabs were collected, and twelve samples were rubbed all over the area under the nail and inoculated in two types of agars, McConkey agar, and mannitol salt agar. MacConkey agar is selective for only gram-negative bacteria based on lactose metabolism. Mannitol salt agar was used as a selective and differential media for *Staphylococcus aureus.*

Purification of Bacteria

After the emergence of different colonies, six cotton swabs were used to streak the colonies on Nutrient agar to make a pure culture[33].

Identification

Gram staining was used to determine the morphological characteristics and whether the bacteria were Gram-positive or Gram-negative[34,35]. The slides were examined using an oil immersion technique bright field microscope at different powers.

Coagulase Test

We used the coagulase test to determine the ability of bacteria to produce the coagulase enzyme. We took a sample of the plate and put a black

wallpaper for white clusters to appear clearly. Then we put a drop of latex on the black paper, then took colony from the plate and put it on the drop of latex and mixed it to see if there were clumps[36].

Catalase Test

The catalase test is a rapid test to see if the bacteria possess the catalyst enzyme. We put a small amount of H_2O_2 solution on the slide, then we took the colony from the plate and waited for the bubbles to appear[35].

Antimicrobial Activity on Muller-Hinton Agar

Using six cotton swabs, one colony of bacteria was taken from the N.A. plate and streaking on six Mueller-Hinton agars all over the plate to test the sensitivity for chloroxylenol and thymol. We used Cork Borer (size 8 mm) to make three holes to put 100 µl of the different concentrations of chloroxylenol and thymol, 1%, 3% and 5% in the holes. We are changing the tips after every use. After incubation at 37°C for 24-48 hours, the results were monitored and recorded[37,38].

Statistical Analysis

The obtained data from this study were statistically analyzed using Analysis of Variance (ANOVA). The statistical calculations were performed using SPSS (version 20; IBM Corp., Armonk, NY, USA). A p-value of < 0.05 was considered an indicator of statistical significance.

Results

Samples Isolation

After isolation of the samples on McConkey and mannitol salt agars and after incubation for 24 hours at 37°C, it was observed a growth on the mannitol salt agar plates, which means that it is *Staphylococcus* bacteria. However, in some samples, the bacteria fermented the mannitol salt agar, which means that it is Staphylococcus aureus. No growth appeared in McConkey agar, meaning there are no Gram-negative bacteria (Table I).

Purification of Bacteria

After streaking on a nutrient agar plate and incubating for 24 hours at 37°C, it was observed that there was growth in all plates.

Gram Stain

On Gram staining, we observe that the isolated bacteria are Gram-positive and appear as *Staphylococci*, as shown in Figure 1. The bacteria were large, spherical, creamy, swampy, dried, and raised.

Table I. The growth results of bacteria on different agars.

Plate number	Mannitol salt agar	McConkey
1	+ve (fermented)	-ve
2	+ve	-ve
3	+ve	-ve
4	+ve (fermented)	-ve
5	+ve) fermented)	-ve
6	+ve	-ve

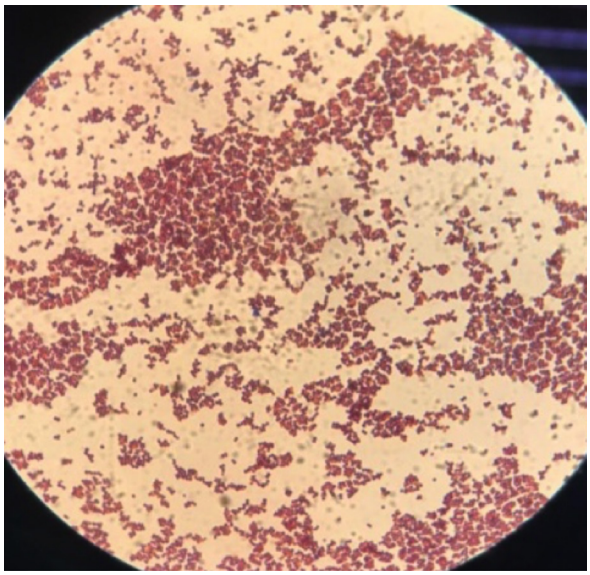

Figure 1. Positive Gram stain of *Staphylococcus spp.* (x100).

Table II. The results of the biochemical tests.

Plate number	Coagulase test	Catalase test	Oxidase test
1	+ve	+ve	-ve
2	-ve	+ve	-ve
3	-ve	+ve	-ve
4	+ve	+ve	-ve
5	+ve	+ve	-ve
6	-ve	+ve	-ve

Table III. The antimicrobial activities on Mueller-Hinton agar. Measurement of the inhibition zone (mm) created by chloroxylenol and thymol.

Plate	Chloroxylenol 1%	3%	5%	Thymol 1%	3%	5%
1	1.3	1.1	1.0	1.4	1.3	0.0
2	1.3	1.5	2.0	0.0	0.0	0.0
3	1.5	1.4	1.8	0.0	1.2	1.0
4	1.2	1.5	1.7	0.0	0.0	0.0
5	1.5	1.3	1.6	0.0	0.0	1.8
6	1.2	1.5	1.7	1.0	0.0	0.0

Coagulase Test

A clumping was observed in both strains that were taken from plates No. 1, 4, and 5. That means it is coagulase-positive, but the result was negative in the rest of the plates (Table II).

Catalase Test

Bubbles were observed in all slides taken from the six plates, meaning they are catalase-positive (Table II).

Oxidase Test

None of the strains taken had any violet color. Instead, they are oxidase-negative (Table II). The biochemical tests revealed that the bacteria in plates 1, 4, and 5 are *Staphylococcus aureus*. While those in the rest of plates are *Staphylococcus epidermidis*.

Antimicrobial Activity in Mueller-Hinton Agar

As shown in Table III and Figures 2-5, the result from the Mueller-Hinton agar with different concentrations of diluted antiseptic active ingredients showed that chloroxylenol affected all six plates with all dilution rates, but the effect of 5% concentration was more substantial than 3% and 1% concentrations. The inhibition zones created by thymol were smaller than those created by chloroxylenol. Analysis of variance for the means of the inhibition zone among the tested substances revealed that significant differences ($p < 0.05$) exist among chloroxylenol and thymol. Moreover, the significant differences ($p < 0.05$) exist among the different concentrations used for the study with chloroxylenol 5% having more zones of inhibition than other concentrations against *S. aureus and S. epidermidis*.

Discussion

Microbial contamination of fingernails with resistant bacteria has become a global health problem. This is because human hand nails provide a favorable environment for different bacteria to grow[39]. Our study showed that the most prevalent bacteria in fingernails are *Staphylococcus aureus* and *Staphylococcus epidermidis*, which was agreed with results from studies of Wachukwu et al[40] and Nasrolahei et al[41] as *S. aureus* was accounted with the higher preva-

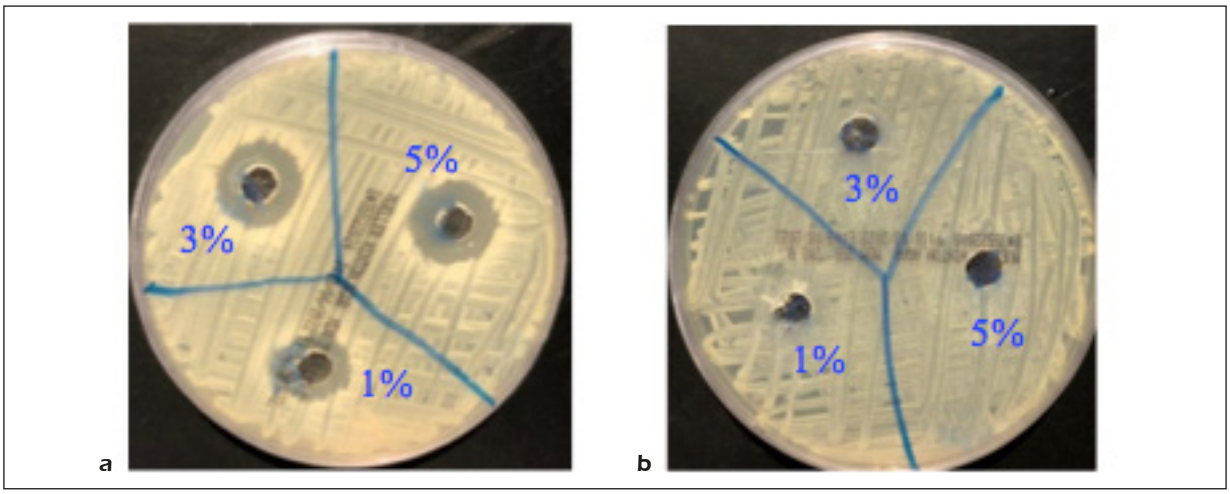

Figure 2. The antimicrobial activities on Mueller-Hinton agar (**a**); the effect of chloroxylenol, and (**b**); thymol in plate No. 4.

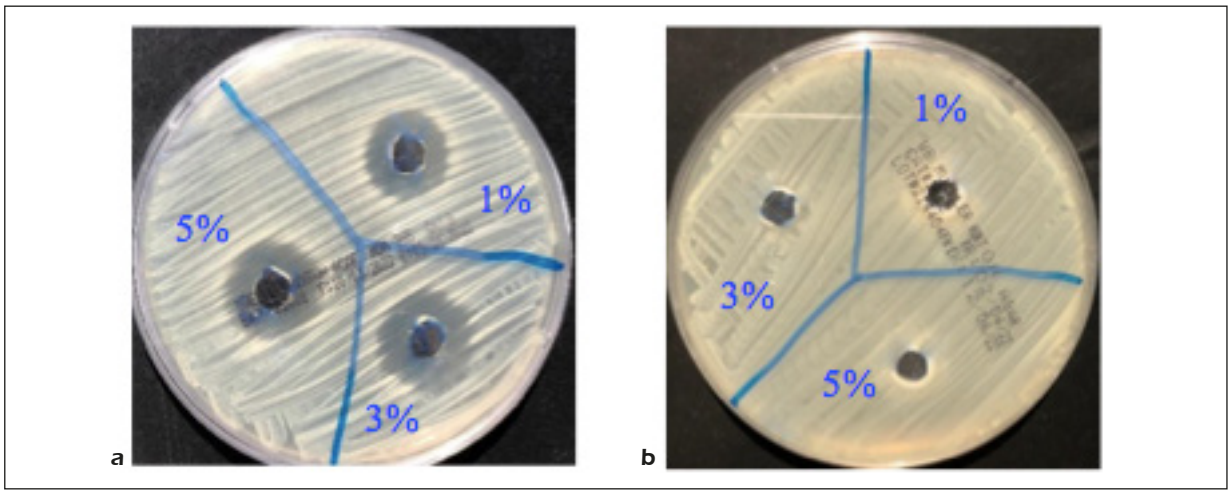

Figure 3. The antimicrobial activities on Mueller-Hinton agar (**a**); the effect of chloroxylenol, and (**b**); thymol in plate No. 2.

lence. In addition, Nahenthran et al[42] and, Tambekar and Tiwari[43] found that *Klebsiella spp, Enterobacter aerogenes,* and *Proteus spp* were prevalent in fingernails, and significant bacterial growth was presently found under fingernails over 1 mm in length.

The difference between bacteria is due to daily activities and the surrounding environment. The hygiene has a role in this[44]. Detecting *Staphylococcus aureus* may pose health risks to students because it causes enterotoxins that cause food poisoning[43]. Baron (1996)[44] stated that *Staphylococcus epidermidis* is one of the principal inhabitants of the skin. *Staphylococcus epidermidis*, in some regions, make up more than 90% of the resident aerial flora[8].

Nail hygiene is an essential aspect of daily life to prevent disease transmission because nails are home to many pathogenic and opportunistic bacteria, and the area under the nails is more difficult to clean. Therefore, hands must be cleaned well, especially the area under the nails. Good detergent cleansers must be chosen to eliminate bacteria[39].

In the present study, we tested chloroxylenol and thymol at different concentrations, as they are two

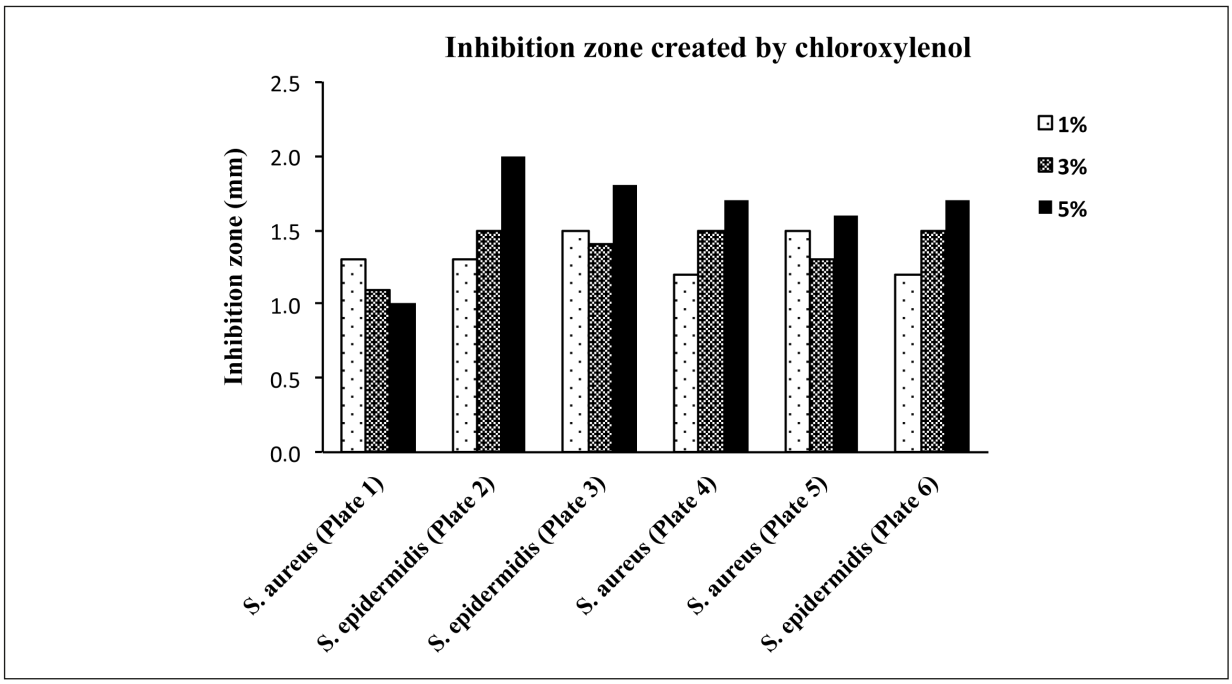

Figure 4. Inhibition zone created by different concentrations (1%. 3% and 5%) of chloroxylenol.

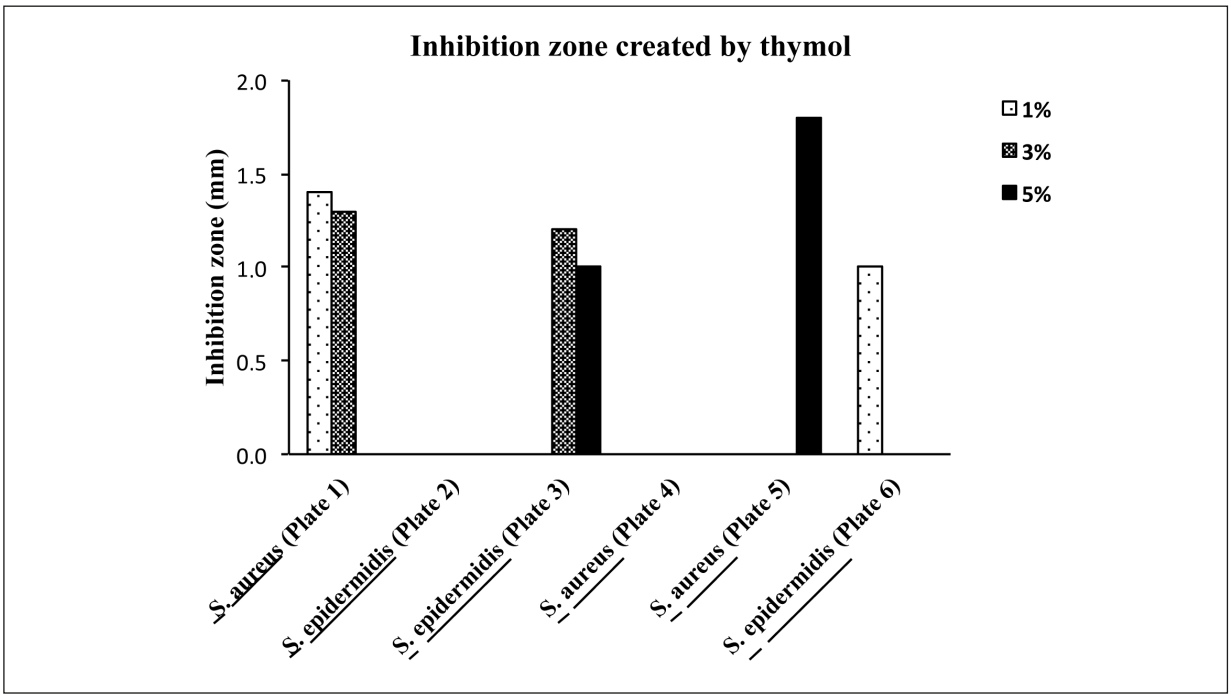

Figure 5. Inhibition zone created by different concentrations (1%. 3% and 5%) of thymol.

of the most common ingredients used in cleansers. The result was that both *Staphylococcus aureus* and *Staphylococcus epidermidis* are more sensitive to chloroxylenol. However, the inhibition zone around thymol was smaller than that of chloroxylenol.

Jouda et al[45] agreed that chloroxylenol was highly effective against Staphylococcus aureus with inhibition zones of 25-37 mm. In our study, the 5% concentration of chloroxylenol showed the best result with an average of 1.63 mm inhibition zone compared to the 3% chloroxylenol, which had an average of 1.38 mm. 1% chloroxylenol had the lowest inhibition zone with an average of 1.33 mm.

A study by Hayat and Munnawar[46] showed that *Staphylococcus aureus* is resistant to thymol-containing sanitizers while being sensitive to chloroxylenol-containing ones. These findings agreed with our result as thymol showed a weak effect on the *Staphylococcus aureus* compared to chloroxylenol which had a more substantial effect. On the other hand, Farzana et al[18] observed that *Staphylococcus aureus* is sensitive to thymol.

In the past, surgeries led to death from infection until phenol was discovered. Phenol can kill germs, but it causes severe burns on the skin. Disinfectants were developed based on phenol but with chemical modifications to reduce harmful effects and produce a safer product, such as chloroxylenol used as a skin disinfectant and for cleaning surgical instruments. It is also used in various types of creams and ointments. Papageorgiou and Chu[47] noted that it is non-irritating bactericidal against most Gram-positive bacteria but less effective against Gram-negative bacteria. Chloroxylon disrupts the cell wall thus allows the cell's contents to diffuse. When enough chloroxylenol enters the cell, the protein and DNA coagulate, leading to rapid cell death. Tap water contains traces of Mg^{2+}, Fe^{2+}, and $Ca2^{2+}$, which interact with chloroxylenol and reduce its effectiveness. Therefore, to kill all cells completely, the concentration must be high enough[48].

Thymol showed weak antibacterial activity in the present work. The inhibition zones created by thymol were smaller than those created by chloroxylenol. Thymol has a positive effect on human health. It is considered an antioxidant, anti-inflammatory, analgesic, and antispasmodic, antibacterial, antifungal, antiseptic, and antitumor. Thymol is a compound commonly found in many essential oils and inhibits Gram-positive and Gram-negative bacteria, including *Bacillus subtilis, E. coli, Klebsiella pneumonia,* and *S. aureus*[30]. It depolarizes the cytoplasmic membrane, disrupts cell membrane integrity, and decreases intracellular ATP levels[49,50]. In our study, the antibacterial efficacy of thymol on *S. aureus* is related to its concentration, as stated in a previous study[51].

Recommendations and Limitations

The detergents and sanitizers should have good ingredients such as chloroxylenol or thymol which can kill bacteria but not damage body tissues. In such a way, many immuno-compromised people can be protected from the transfer of pathogenic or opportunistic pathogens. In addition, it is highly recommended to explore strategies based on nanotechnology for the antibacterial ingredients used in detergents and hand washes to obscure their unwanted properties and enhance their antimicrobial activity.

This study was limited by its small sample size. The small sample size may affect the ability to detect the difference between the treatments. Another limitation of our study is the use of unmasked investigator for data collection. Finally, standardization of our methodology to culture all study groups eliminates the effect of this variable between groups and allows us to maintain our conclusions.

Conclusions

Fingernails greatly serve as a mean of pathogen transmission. Thus, effective hand hygiene can reduce infection and prevent diseases. The study revealed that the dominant species of bacteria present in the hands of undergraduate students of King Abdelaziz University, Saudi Arabia, are *S. aureus* and *S. epidermidis*. The use of hand sanitizers that contain chloroxylenol can help reduce both transient and pathogenic bacteria present under fingernails. *S. aureus* was the most isolated pathogenic bacteria. Chloroxylenol was the most effective antimicrobial agent. Therefore, the importance of conscientious hand cleaning with detergents containing chloroxylenol should be emphasized, and proper techniques should be underlined.

Authors' Contributions

All the authors have significantly contributed to the conceptualization and design of the study, the collection, analysis, and interpretation of data, the writing of the article or its critical revision for significant intellectual content, and the final approval of the version to be submitted.

Informed Consent

Written valid consent was obtained from all participants.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Data are contained within the article.

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Ethics Approval

Not applicable.

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