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Original Research Article

Evaluation of Antimicrobial and Sporicidal Activities, and Stability Testing of Herbal Skin Wash Preparations from *Melaleuca cajuputi* subsp. *cumingiana* Essential Oils and *Kyllinga nemoralis* Aqueous Extract

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ABSTRACT

Herbal remedies with antimicrobial effects are derived from various plant parts. These treatments are commonly applied topically and can be found in cosmetic products, where they have been shown to exhibit antimicrobial properties. In this study, *Melaleuca cajuputi* subsp. *cumingiana* essential oils and *Kyllinga nemoralis* aqueous extract were used in preparation of the herbal skin wash. The skin wash was tested against bacterial vegetative cells, spores, and *Candida* species. The stability testing was also performed. The antimicrobial activity of the skin wash was evaluated using the disc diffusion method. Results showed that exposure of the tested bacteria to the herbal skin wash resulted in an inhibition zone with diameters ranging from 7.00 to 16.00 mm. The inhibition zone of the tested *Candida* species to the herbal skin wash was at the diameter ranging between 8.00 to 13.00 mm. The herbal skin wash, when utilized at its maximum concentration, demonstrated sporicidal efficacy against the spores of *B. subtilis* and *B. megaterium* after an exposure period of one hour. However, it did not exhibit similar activity against *B. cereus* and *B. pumilus*. Given that storage conditions are typically within the pH range of human skin, the herbal skin wash is considered stable and can be used for up to six months. In conclusion, *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash shows a promising potential antimicrobial activity against wide range of microorganisms and thus has potential to be developed into a commercial herbal antimicrobial skin wash.

Keywords: Herbal skin wash; Antimicrobial; Sporicidal; *M. cajuputi* subsp. *cumingiana*; *K. nemoralis*

Introduction

Maintaining skin health through proper skincare practices is essential, as the skin is the largest and most complex organ in the human body, playing vital roles in overall health. The deterioration of skin structure can compromise its functions, especially its barrier function, increasing the risk of foreign substances, including bacteria, penetrating the skin and causing infections.[1] Nowadays, the market is saturated with numerous commercial skincare products, many of which contain chemical and synthetic components, such as parabens and triclosan, known for their toxic effects. Parabens, alkyl esters derived from 4-hydroxybenzoic acid, have been extensively used as preservatives in pharmaceuticals, food products, and cosmetics due to their effective antimicrobial properties. Data has shown indirect but significant associations between parabens and conditions such as breast cancer and endometriosis.

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While parabens are not the primary cause of cancer, they remain a concern.[2] Extensive research into the toxicity and negative effects of chemical compounds in skincare products has led to significant advancements in developing plant-based alternatives.[3,4]

Plant-based skincare products are primarily formulated with natural plant materials as their key ingredients, rather than synthetic or chemical substances. Although plant-based products have a long history of use in skincare, recent advancements in research and technology have provided deeper insights into their properties and benefits. The antibacterial, antioxidant, and anti-inflammatory properties of phytochemicals in plants are vital for promoting and maintaining skin health. As a result, chemical ingredients commonly used in skincare products can be effectively replaced with plant-derived alternatives.[5] While plant-based substances are often extracted from plant leaves, they are not limited to this source. Depending on their specific phytochemical properties, these substances may also be obtained from fruits, flowers, rhizomes, and herbs.[6]

The inherent qualities of plant-based skincare products make them gentler on the skin, reducing the likelihood of irritation. Additionally, these products are suitable for sensitive skin types, as many plants possess hypoallergenic properties.[7] Individuals with acne or eczema are often advised to use plant-based skincare products, such as chamomile, which is commonly incorporated into moisturizers. Known for its anti-inflammatory properties, chamomile effectively addresses these skin conditions while minimizing the risk of adverse effects.[8] As a result, plant-based skincare has become a popular choice among consumers due to its effectiveness, reduced risk of harm, and superior safety compared to chemical and synthetic products.

Plant-derived antimicrobial agents may have limited efficacy compared to conventional antibiotics. However, the effectiveness of antibiotics is often hindered by challenges such as drug resistance, side effects, and a lack of specificity.[9] Some plant-based pharmaceuticals exhibit inhibitory properties even at minimal dosages, particularly against Gram-positive bacteria, while being less likely to be toxic to animal cells.[10] The rise of antibiotic resistance in bacteria and fungi presents a significant public health challenge, highlighting the need for alternative solutions such as plant-derived antimicrobials.[11]

To enhance antimicrobial effectiveness against multidrug-resistant bacteria and mitigate the development of resistance, researchers have explored the synergistic use of essential oils with antibiotics.[12,13,14] Certain studies suggest that antimicrobial agents and essential oils target multiple sites within the pathogen, while others attribute their effects to increased chemical complexity.[15,16] Additionally, essential oils may improve skin penetration and facilitate the diffusion of antibiotics through bactericidal cell membranes. They may also inhibit the efflux pump in Gram-negative bacteria, further enhancing antibiotic efficacy.[17,18] The combined use of conventional medications and essential oils has become common among patients. However, this practice may inadvertently enhance or reduce the effectiveness of their treatments.[19] *M. cajuputi* essential oils are commonly used in alternative medicine for treating wounds, colds, and inflammation. Several studies have reported significant antimicrobial effects of *M. cajuputi* extracts against bacteria, viruses, protozoa, and fungi.[20,21] The plant also exhibits notable insecticidal and antioxidant properties.[22,23,24] Investigations of *K. nemoralis* root extracts show antimicrobial potency against broad-spectrum bacterial strains, including MRSA, and herpes simplex virus type 1 (HSV-1).[25,26] These inhibitory effects on pathogens are likely due to the plant's phytochemical composition, which includes tannins, phytosterols, sitosterols, saponins, anthraquinones, glycosides, and oleanolic compounds.[27] The findings suggest that *K. nemoralis* is a promising source of antioxidants and could serve as a basis for drug development. Given the global challenge of antimicrobial resistance to commercial medications, medicinal plants offer a valuable avenue for new drug development. This study aimed to evaluate the antimicrobial and sporicidal activities, as well as the stability of herbal skin wash preparations formulated from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract.

Materials and Methods

Plant collection

M. cajuputi subsp. *cumingiana* leaves and *K. nemoralis* roots were collected from a wild source in Kuala Nerus, Terengganu. Plant identification and authentication was performed by Herbarium Universiti Sultan Zainal Abidin. Voucher ID UniSZA/A/000000045 for *M. cajuputi* subsp. *cumingiana* and Voucher ID UniSZA/A/000000044 for *K. nemoralis* were preserved for the voucher specimen in the herbarium of Universiti Sultan Zainal Abidin.

Essential oils and aqueous extraction

Essential oils were prepared using steam distillation process from 3 kg dried leaves of *M. cajuputi* subsp. *cumingiana.*[28] The oils were obtained after a 5 hours of steam distillation process and were carefully extracted from the distillation receiver using a pipette. The purified essential oil was transferred to an amber vial, weighed, and stored at 4°C until it was ready for bioassay testing. The fresh roots of *K. nemoralis* were dried in a shed and then ground using an electric grinder. The powder was stored in an airtight container until it was ready for extraction. 50g of ground dried plant material were mixed with 150 mL of sterilized distilled water, heated below the boiling point, and stirred for 2 to 3 hours. The extract was filtered using Whatman No. 1 filter paper. The aqueous extract was frozen and then subjected to a strong vacuum for further processing. The aqueous extract (200 mL) was completely frozen at -85°C for 4 to 5 hours, resulting in a solid product without causing denaturation of enzymes or any chemical alterations.[25]

Preparation of herbal skin wash

K. nemoralis aqueous extract (0.2%) was mixed to prepare the herbal skin wash at a concentration of 100 mg/mL. A specific amount of thickener or emulsifier (guar gum), solubilizing agent (Tween 20), and preservative (germall plus) was added to the mixture. All ingredients were melted and thoroughly mixed. After heating was stopped, *M. cajuputi* subsp. *cumingiana* essential oils (0.1%) were added to the solution. The herbal skin wash was then packaged in bottles, ready for use.[29]

Test organism

The bacterial species used as test organisms were *Staphylococcus aureus* (ATCC 11632), clinical isolate methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecalis*, *Streptococcus epidermidis* (ATCC 12228), *Streptococcus agalactiae*, *Streptococcus pyogenes* (ATCC 12344), *Propionibacterium acne*, *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Proteus mirabilis*, clinical isolate *Salmonella* Typhi, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Bacillus cereus* (ATCC33019), *Bacillus subtilis* (ATCC6633), *Bacillus pumilus* (ATCC14884), and *B. megaterium* (ATCC14581). The *Candida* species used as test organisms were *Candida albicans* (ATCC 10231), *Candida glabrata* (ATCC 2001), and *Candida tropicalis* (ATCC 13803). All the stock cultures were obtained from the Microbiology Laboratory, Faculty of Medicine, Universiti Sultan Zainal Abidin, except for *Bacillus* species, which were obtained from the Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia.

Antibacterial Assay

The disc diffusion method was used to measure the extent of bacterial inhibition by the herbal skin wash preparations containing *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract. Mueller-Hinton agar (MHA) plates were inoculated with a bacterial species standardized to 0.5 McFarland. Blank, sterile discs were impregnated with 20 µL of each herbal skin wash at concentrations of 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL, and then placed on the agar surface. Two controls were used. Chloramphenicol (30 µg/mL) as the positive control and sterile distilled water as the negative control. The MHA plates were incubated for 24 hours at 37°C in an aerobic atmosphere. The experiment was repeated three times. Following incubation, the size of the inhibition zones around the tested discs, the positive control, and the negative control was measured, and the results were documented.[25]

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC assay was performed in a 96-well microplate, where the herbal skin wash underwent two-fold serial dilutions, with final concentrations ranging from 0.781 mg/mL to 100 mg/mL. The bacterial stock solution was added to each well, and the plate was incubated for 24 hours at 37°C. After incubation, 50 µL of MTT solution was added, followed by an additional 2 hours incubation. The experiments were conducted in triplicate to ensure accuracy. The presence of a yellow color in the solution indicates the absence of visible growth, whereas a purple color indicates observable growth of the tested bacteria.[30] After the MIC was established, the sub-culturing procedure was immediately performed to determine the MBC by transferring the suspension from each MIC well onto an MHA plate. The MBC was determined by mixing 50 µL of the suspensions from wells showing no growth during the MIC incubation with 150 µL of fresh broth. The mixtures were reincubated at 37°C for 48 hours. The suspension was then plated onto NA and incubated at 37°C for 24 hours. No growth on the NA plate indicated the MBC endpoint.[31]

Anticandida assay

The anticandidal assay of the herbal skin wash against the tested *Candida* species was evaluated using an agar well diffusion method. Sterilized sabouraud dextrose agar (SDA) plates were inoculated with the *Candida* species at a concentration corresponding to 0.5 McFarland standards. A volume of 20 µL from each herbal skin wash, at concentrations of 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL, was applied to sterile, blank discs measuring 6 mm in diameter, which were then placed on the surface of the agar. Fluconazole (5 mg/mL) was used as a positive control, and sterile distilled water as a negative control. The plates were incubated at 28°C for 24 hours. The

effectiveness of the herbal skin wash against the tested *Candida* species was evaluated by measuring the inhibition zone diameters in millimeters. The assay was repeated three times for confirmation.[32]

Minimum Inhibitory Concentration (MIC) and Minimum Candidacidal Concentration (MCC)
The broth dilution method was used to determine the MIC of the herbal skin wash against the tested *Candida* species, with MIC values assessed after 48 hours at 35°C. The MIC endpoint was visually identified as the lowest concentration showing 100% inhibition. For the MCC, 10 µL from wells with no visible growth were transferred to SDA and incubated at 35°C for 48 hours. The MCC represents the concentration that prevents candida growth.[32]

Sporicidal assay
The spore suspension was thawed and diluted 1:100 in a 1% PBS solution (pH 7) to create an initial suspension of *Bacillus* species spores (10^6-10^7 spores/mL). The herbal skin wash was added to achieve final concentrations of 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL. Chlorhexidine (10%) was used as the positive control, and DMSO (10%) as the negative control. Each concentration was incubated for 1 hour in a water bath at 37°C to assess sporicidal activity. After incubation, the solution was centrifuged at 12,000 × g for 5 minutes at 4°C, then rinsed twice with 1% PBS to remove bacterial residue. The pellets were suspended in 100 µL of 1% PBS, serially diluted, and spread onto nutrient agar plates. After incubation at 30°C for 24 hours, colony-forming units (CFUs) were enumerated. The sporicidal activity was calculated by comparing the log10 CFU/mL values of the test solution to the control group, with reductions in CFUs indicating sporicidal effectiveness.[33]

Stability study
A stability study was conducted to evaluate the qualities and characteristics of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract in the herbal skin wash, examining how these properties alter over time under different environmental conditions. The objective was to determine the shelf life of the product and ensure its efficacy, safety, and quality throughout its intended usage. The National Pharmaceutical Regulatory Agency Malaysia (NPRA) guidelines were followed to conduct this stability testing. Three batches of the herbal skin wash were stored in three different environments with varying temperatures over a period of six months. The products were placed in a cool area, an air-conditioned area, and a room temperature area. The stability of the herbal skin wash was evaluated by observing its appearance, colour, odour, pH, and texture. The herbal skin wash was visually inspected at regular intervals after preparation under different conditions. Any changes in colour, clarity, consistency, or signs of separation (layering of ingredients) or sedimentation were observed. The herbal skin wash should maintain its original colour, with no significant fading or darkening, which could indicate degradation of the herbal ingredients or oxidation. For the odour examination, the herbal skin wash was smelled initially to record its fragrance. The smell was reassessed at regular intervals, and any development of an unpleasant, rancid, or off-putting smell was noted. Such changes may indicate spoilage, microbial contamination, or breakdown of volatile herbal compounds. The product should retain a consistent herbal fragrance. A change in odour may signal the degradation of the natural ingredients. For pH inspection, the pH of the herbal skin wash was measured using a pH meter or pH strips, and the pH test was conducted at various intervals (immediately after preparation and after 6 months). The texture of the skin wash was evaluated by feeling the product for viscosity and consistency. The spreadability and smoothness when applied to the skin were assessed by taking a small amount and spreading it on the hand or on a flat surface. The product's consistency was observed to check whether it had thickened or become too runny over time. The texture should remain consistent without becoming excessively thick or watery. It should be easy to apply and should not separate or change in texture. If the texture changes significantly, it could indicate the product is unstable.[34]

Statistical analysis
The experiments in this study were conducted in triplicate to ensure accuracy and reliability. Data were analyzed using Microsoft Excel 2019 and presented as the mean ± standard deviation (SD). A statistical significance of 95% was observed between treatments, with a p-value less than 0.05, determined through Tukey's test.

Results and Discussion

Antibacterial activity
The results of the study indicated herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract exhibits notable antibacterial activity against the majority of tested bacteria, except *E. faecalis*, *P. mirabilis*, *B. cereus*, and *B. pumilus*. This herbal skin wash at a concentration of 100 mg/mL showed inhibitory effects on various bacteria including *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes*, *S. agalactiae*, *P. acnes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S.* Typhi, *S. dysenteriae*, *B. subtilis*, and *B. megaterium*. The inhibition zones observed ranged from 7 to 16 mm, respectively (Table 1).
The herbal skin wash demonstrated significant antibacterial activity, especially against Gram-positive bacteria. This difference in effectiveness is likely due to the structure of the Gram-negative bacterial cell wall, which provides greater resistance to the antimicrobial agents. Gram-negative bacteria possess a highly efficient permeability barrier in the form of a thin lipopolysaccharide outer membrane, which can impede the infiltration of the plant extract. The findings from this study are consistent with most previous research. Prior reports have indicated that Gram-negative bacteria generally show heightened resistance to plant-derived antimicrobials and may exhibit reduced susceptibility compared to Gram-positive bacteria.[35,36] In contrast, Gram-positive bacteria have a more accessible peptidoglycan layer that forms a mesh-like structure, allowing easier permeation by plant extracts compared to the outer membrane of Gram-negative bacteria, which provides greater resistance to such compounds.[37] The primary reason for the reported antibacterial activity of this herbal skin wash is the presence of bioactive compounds in *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract. Antimicrobial activities are associated with chemical substances that are either derived from biological sources or synthesized chemically. The results found in this study are both supported and contradicted by data reported in the literature. The results of this study align with previous research showing that *M. cajuputi* essential oils have inhibitory effects against *S. aureus*, *S. pyogenes*, MRSA, *K. pneumoniae*, and *E. coli*. Additionally, 19 compounds were identified in the essential oils extracted from *M. cajuputi* leaves, contributing to its antimicrobial properties. The predominant components present in the essential oils were found to be p-Cymene, a type of monoterpene, followed by linalool and caryophyllene. The primary antibacterial compound in *M. cajuputi* essential oils is p-Cymene.[28] Additionally, *M. cajuputi* essential oils have been proven to be effective for dermatological use, particularly in treating skin diseases such as acne.[17]
The antibacterial properties of *M. cajuputi* essential oils can be attributed to the presence of bioactive compounds like 1,8-cineole and limonene, which are effective against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *P. acnes*, *E. coli*, MRSA, *S. pyogenes*, as well as fungal pathogens like *C. albicans* and *T. rubrum*.[38,39]
The *K. nemoralis* plant extracts were found to contain medicinal phytochemicals such as total phenols, flavonoids, saponins, and tannins. These compounds are known for their physiological and therapeutic properties, including antimicrobial activity. Tannins, for instance, inhibit protein synthesis, while flavonoids and saponins show potent effects against a variety of microorganisms.[30,40] Tannins, which are polyphenolic compounds, have the ability to bind to proline-rich proteins, inhibiting protein synthesis. This mechanism contributes to their antimicrobial properties, as they can interfere with bacterial growth and function.

Table 1: Zone of inhibition (mm) of herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract against test bacteria

Test bacteria	Zone of Inhibition (mm)									
	Herbal Skin Wash Concentration (mg/mL)									
	100	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**Positive control (Chloramphenicol 30 µg/mL)**	**Negative control (Distilled water)**
S. aureus	13.00 ± 0.11	9.00 ± 0.15	7.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	30.00 ± 0.50	6.00 ± 0.00
MRSA	12.00 ± 1.12	8.00 ± 0.55	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	25.00 ± 0.25	6.00 ± 0.00
E. faecalis	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	25.00 ± 0.55	6.00 ± 0.00
S. epidermidis	15.00 ± 2.10	11.00 ± 1.00	9.00 ± 0.080	8.00 ± 0.70	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	37.00 ± 0.85	6.00 ± 0.00
S. agalactiae	15.00 ± 0.00	13.00 ± 0.00	10.00 ± 0.00	8.00 ± 1.00	7.00 ± 1.55	7.00 ± 0.55	6.00 ± 0.00	6.00 ± 0.00	27.00 ± 0.06	6.00 ± 0.00
S. pyogenes	16.00 ± 0.60	15.00 ± 0.10	14.00 ± 0.30	12.00 ± 0.90	11.00 ± 0.80	10.00 ± 1.60	9.00 ± 0.00	6.00 ± 0.00	31.00 ± 0.03	6.00 ± 0.00
P. acnes	9.50 ± 0.40	9.00 ± 0.15	7.00 ± 2.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	27.00 ± 0.90	6.00 ± 0.00
E. coli	7.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	24.00 ± 0.25	6.00 ± 0.00
K. pneumoniae	8.00 ± 0.50	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	37.00 ± 0.00	6.00 ± 0.00
P. mirabilis	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	27.00 ± 0.65	6.00 ± 0.00
S. Typhi	7.00 ± 0.16	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	33.00 ± 0.00	6.00 ± 0.00
P. aeruginosa	12.00 ± 0.04	10.00 ± 0.05	7.00 ± 1.55	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	23.00 ± 0.00	6.00 ± 0.00
S. dysenteriae	7.00 ± 1.80	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	27.00 ± 0.00	6.00 ± 0.00
B. cereus	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	27.00 ± 0.00	6.00 ± 0.00
B. subtilis	8.00 ± 1.56	7.00 ± 1.90	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	23.00 ± 0.00	6.00 ± 0.00
B. pumilus	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	26.00 ± 0.00	6.00 ± 0.00
B. megaterium	8.00 ± 0.09	7.00 ± 0.02	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	25.00 ± 0.00	6.00 ± 0.00

*Data are means of three replicates (n = 3) ± standard error

These compounds, along with other phytochemicals in *K. nemoralis*, help explain the plant's medicinal potential.[41,42] Additionally, these compounds have also demonstrated efficacy in inhibiting bacterial growth and displaying antibacterial properties.[43,44] Flavonoids are hydroxylated polyphenolic compounds produced by plants in response to microbial infections. Extensive research has highlighted their potent antimicrobial activity against a wide range of microorganisms *in vitro*.[45] Saponins, which are glycosides, have been shown to inhibit the growth of both Gram-positive and Gram-negative bacteria.[46] The interaction between two or more drugs or active compounds can result in synergism, which refers to an increase in efficacy greater than that of either compound alone. The combined use of plant extracts has the potential to enhance pharmacological efficacy through synergism, simultaneous targeting of multiple pathways, reduced dosage requirements for individual extracts, and fewer occurrences of side effects.[47] Therefore, the synergistic combination of bioactive compounds from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract in this herbal skin wash formulation could significantly enhance its inhibitory effects against the tested bacteria.

MIC and MBC

Given the significant antibacterial effect observed in the disk diffusion method against the tested bacterial strains, the MIC was determined for the herbal skin wash preparations containing *M. cajuputi* subsp. *cumingiana* essential oil and *K. nemoralis* aqueous extract. MIC values for this herbal skin wash ranged from 0 to 100 mg/mL against the tested bacteria (Table 2). MBC testing was performed to determine the lowest concentration of the herbal skin wash required to kill the tested bacteria. MBC values for this herbal skin wash ranged from 0 to 100 mg/mL against the tested bacteria. The tested bacteria most susceptible to this herbal skin wash in general was *S. pyogenes*, while *E. faecalis*, *P. mirabilis*, *B. cereus*, and *B. pumilus* were the most resistant among the tested bacteria. In this study, the MBC/MIC ratio was used to assess antibacterial activity. Bacteriostatic effects were defined as those with MBC/MIC ratio greater than 4, while bactericidal effects were

considered to occur when this ratio was less than 4.[48] These results indicate the potency of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash as antibacterial agents through a bactericidal mechanism. The high antibacterial potency of these extracts may be attributed to the bioactive compounds they contain.

Table 2: MIC and MBC values of herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract against tested bacteria

Tested bacteria	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
S. aureus	25	25	1
MRSA	50	50	1
E. faecalis	0	0	0
S. epidermidis	12.5	12.5	1
S. agalactiae	3.12	3.12	1
S. pyogenes	1.563	1.563	1
P. acnes	25	25	1
E. coli	100	100	1
K. pneumoniae	100	100	1
P. mirabilis	0	0	0
S. Typhi	100	100	1
P. aeruginosa	25	25	1
S. dysenteriae	100	100	1
B. cereus	0	0	1
B. subtilis	50	50	1
B. pumilus	0	0	0
B. megaterium	50	50	1

*MBC/MIC = Bacteriostatic effects were defined as those with an MBC/MIC ratio greater than 4, while bactericidal effects were considered to occur when this ratio was less than 4

Anticandidal assay

Anticandidal activity was also examined to evaluate the biological effects of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash on *C. albicans*, *C. glabrata*, and *C. tropicalis*. Among the tested *Candida* species, antifungal activity was observed with the application of the herbal skin wash against *C. albicans* and *C. glabrata* only. The inhibition zones observed ranged from 8 to 13 mm, respectively. No inhibitory activity was observed against *C. tropicalis* at any concentration of the herbal skin wash. The effect of the herbal skin wash was compared to the standard antifungal agent, fluconazole (5 mg/mL), and the negative control, sterile distilled water (Table 3). Similar results have been reported for other species within the same genus of the Myrtaceae family. The MFC/MIC ratio of *M. cajuputi* Powell essential oils against FLC-resistant *C. albicans* clinical isolates ranges from 1 to 2.[49] The anticandidal activity of the *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash may be attributed to the complex mixture of biologically active compounds they contain. These compounds vary significantly depending on the plant's chemotype and the location where it was harvested.

Previous research identified a total of nineteen phytochemical substances in the essential oils of *M. cajuputi*, including p-cymene, linalool, terpinolene, and terpinene-4-ol. P-cymene has been found to impact the cell membrane of fungi, inhibiting biofilm production and affecting fungal growth and morphology.[50] Linalool and terpenoids have been found to hinder the growth of *C. albicans* by impacting the integrity of its membranes.[51] Terpinene-4-ol, also present in *M. cajuputi* essential oils, suppresses fungal species by stimulating mitochondrial activity within the cell.[52]

Table 3: Zone of inhibition (mm) for disc diffusion method of herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract against test *Candida* species

Tested *Candida* species	Zone of Inhibition (mm)									
	Herbal Skin Wash Concentration (mg/mL)									
	100	50	25	12.5	6.25	3.125	1.563	0.781	Control positive (fluconazole 5 mg/mL)	Control negative (distilled water)
C. albicans	13.00 ± 0.11	9.00 ± 0.15	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	30.00 ± 0.50	6.00 ± 0.00
C. glabrata	12.00 ± 1.12	8.00 ± 0.55	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	25.00 ± 0.25	6.00 ± 0.00
C. tropicalis	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	25.00 ± 0.55	6.00 ± 0.00

*Data are means of three replicates (n = 3) ± standard error

A previous study found that the essential oils of *M. cajuputi* and their components can modify the permeability and fluidity of the *C. albicans* membrane. This effect is achieved by altering the membrane's characteristics and compromising its associated functions.[53] Phytochemical screening tests revealed the presence of saponins, steroids, and terpenoids as secondary metabolites in the root extract of *K. nemoralis*.[25] Secondary metabolites, such as saponins, which are present in each plant extract, have been reported to exhibit antimicrobial activity. Saponins interfere with the biosynthesis of ergosterol in fungal cell membranes, damage mitochondria, and regulate energy and lipid metabolism.[54] Steroids are toxic to fungi by affecting fungal growth, morphology, virulence, and drug resistance.[55] Terpenoid phenols demonstrate potent antifungal activity against a wide range of pathogens, including *C. albicans*. Terpenoids are essential molecules for plant life, acting as structural components or as important tools that help plants adapt to their environment. They play a crucial role in various plant life processes.[56] Thus, the inhibition of *Candida* species. growth is a significant finding, as the mixture of phytochemical substances from *M. cajuputi* subsp. *cumingiana* essential oils and *K.*

nemoralis aqueous extract in the herbal skin wash formulation inhibited the growth of the tested *Candida* species synergistically.

MIC and MCC

The MIC and MCC values for *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract in the herbal skin wash ranged from 0 to 50 mg/mL against the tested *Candida* species (Table 4). MCC is defined as the minimum concentration of a substance that causes the death of 99.9% of the *Candida* species inoculum.[57] The MCC/MIC ratio was calculated to assess antifungal activity, where fungistatic activity is defined when the MCC/MIC ratio is ≥4, and fungicidal activity is defined when the MCC/MIC ratio is <4.[58] The MIC and MFC ranged from 0 to 50 mg/mL, respectively. Based on the MCC/MIC ratio calculation, the ratios ranged from 0 to 1, indicating that the antifungal activity of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash was fungicidal.

Table 4: MIC and MCC values of herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract against tested bacteria

Tested *Candida* species	MIC (mg/mL)	MCC (mg/mL)	MCC/MIC
C. albicans	50	50	1
C. glabrata	50	50	1
C. tropicalis	0	0	0

*MCC/MIC = fungistatic activity is defined when the MCC/MIC ratio is ≥4, and fungicidal activity is defined when the MCC/MIC ratio is <4

Sporicidal assay

The sporicidal activity of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash was tested at different concentrations (1.563, 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL) for 1 hour. The reduction in the viability of *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* spores at different concentrations after 1 hour of incubation is shown in Table 5. At higher concentrations, the herbal skin wash showed weak sporicidal activity, with a reduction in the number of *B. subtilis* and *B. megaterium* spores at the highest concentration (100 mg/mL). However, the herbal skin wash at any concentration did not cause a reduction in the spore count for *B. cereus* and *B. pumilus* spores.

The phytochemical components found in medicinal plants are linked to their sporicidal properties. The previous study found that the combination of herb extract and organic acid effectively killed *B. subtilis* spores completely at the minimum concentration used in their research. It was suggested, the combination of herb extract and organic acid managed to penetrate spore coat of *B. subtilis* and destroys its protein.[59] In addition, *Euphorbia tirucalli* extract exhibit inhibition activity towards the growth of vegetative cells and sporicidal activity against spores of *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* spores.[32] On the contrary, in this study *M. cajuputi* essential oils and *K. nemoralis* aqueous extract herbal skin wash has antibacterial activity against vegetative cells and inhibit the germination of *B. subtilis* and *B. megaterium* at concentrations, 100 mg/mL and 50 mg/mL respectively, but did not has antibacterial activity against vegetative cells and inhibit the germination of *B. cereus* and *B. pumilus* at any concentrations of herbal skin wash. In fact, the variations in the tested bacteria used and the concentrations employed make the straightforward comparison are difficult.

Table 5: Sporicidal activity of herbal skin wash preparations from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract against *Bacillus* species

Test *Bacillus* species	CFU/mL of spores							Positive control (Chlorhexidine 10%)	Negative control (Distilled water)
	Herbal Skin Wash Concentration (mg/mL)								
	100	50	25	12.5	6.25	3.125	1.563		
B. cereus	n.a	n.a	n.a	n.a	n.a	n.a	n.a	17.00 ± 0.00	n.a
B. subtilis	0.80 ± 0.02	n.a	n.a	n.a	n.a	n.a	n.a	17.00 ± 0.00	n.a
B. pumilus	n.a	n.a	n.a	n.a	n.a	n.a	n.a	17.50 ± 0.00	n.a
B. megaterium	0.50 ± 0.05	n.a	n.a	n.a	n.a	n.a	n.a	18.00 ± 0.00	n.a

*Data are means of three replicates (n = 3) ± standard error; n.a: not active (no inhibition).

The resistance of *Bacillus* species is influenced by multiple factors. The resistance of spores to chemicals is primarily attributed to the low permeability of their inner membrane.[60] The various layers of spores and their unique compositions contribute significantly to the resistance mechanisms of *Bacillus* species spores. In particular, the peptidoglycan layer of the spore acts as a substantial barrier against small hydrophilic molecules found in antimicrobial agents.[61] p-Cymene, the primary antimicrobial component in *M. cajuputi* subsp. *cumingiana* essential oils, has been shown in numerous studies to exhibit antibacterial, antiviral, and antifungal properties.[28] Additionally, *K. nemoralis* aqueous extract contains high levels of total phenols, flavonoids, flavonols, and tannins, which contribute to its antimicrobial properties.[62] Therefore, these compounds could also be responsible for the sporicidal activities observed in the current study.

Stability study

A stability study was conducted to evaluate the physical characteristics of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous herbal skin wash over time under three distinct environmental conditions. Three sets of the herbal skin wash were stored in environments with varying temperatures for a duration of six months: a refrigerated area at 4°C, an air-conditioned area at 22°C, and a room at a normal temperature of 37°C. Assessments were conducted on day 1 and after six months to evaluate the texture, color, odor, and pH of the samples (Table 6). There were no variations in the color of the herbal skin wash across the three batches, as it retained its original pale-yellow hue and liquid texture. The herbal skin wash effectively deodorized and freshened the room with its woody and camphoraceous aroma, maintaining these properties even after storage at three different temperatures for up to six months. The pH of the *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash was evaluated at the three storage temperatures. Initially, the pH of the skin wash was 4.80. After six months, a slight reduction in pH was observed under all storage conditions. The pH values were measured as 4.46, 4.48, and 4.50 for the samples stored in a cool area (4°C), an air-conditioned area (22°C), and a room-temperature area

(37°C), respectively. Despite the reduction, the pH range of the herbal skin wash remained within the optimal range for human skin, which typically falls between 4 and 6.[63] Maintaining the pH of the skin within its normal range is essential for preserving and protecting the skin's barrier function. A healthy and intact skin barrier optimizes the skin's performance and resilience.[64] Alterations in skin pH can lead to various skin issues and contribute to the development of conditions such as irritation. Previous studies have shown that an increase in skin pH disrupts the protective barrier, leading to higher levels of serine proteases and reduced levels of enzymes responsible for ceramide production.[65] Conversely, a decrease in skin pH, resulting in increased acidity, can trigger typical skin flora, such as *S. aureus*, to exhibit more aggressive behavior.[66] Since the pH of the *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash remains within the optimal range of human skin during storage, it is considered stable and suitable for use for up to six months.

Table 6: The characteristics of *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract herbal skin wash at different durations and storage condition

Temperature / storage	Parameter	Day 1	Six months
Cool area	Colour	pale-yellow	pale-yellow
	Odour	woody and camphoraceous aroma	woody and camphoraceous aroma
	pH	4.46	4.46
	Texture	liquid	liquid
Air conditioning area	Colour	pale-yellow	pale-yellow
	Odour	woody and camphoraceous aroma	woody and camphoraceous aroma
	pH	4.48	4.48
	Texture	liquid	liquid
Room temperature area	Colour	pale-yellow	pale-yellow
	Odour	woody and camphoraceous aroma	woody and camphoraceous aroma
	pH	4.50	4.50
	Texture	liquid	liquid

Conclusion

The formulated herbal skin wash derived from *M. cajuputi* subsp. *cumingiana* essential oils and *K. nemoralis* aqueous extract exhibited significant antibacterial, anticandidal, and sporicidal activity against a broad range of bacterial strains and *Candida* species. As the formulation demonstrated comparable effectiveness to commercial standards and met all other quality parameters, it can be concluded that the developed herbal skin wash is well-standardized and holds potential as a viable alternative to the chemical-based skin washes currently available on the market.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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