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Antibacterial Effects of Herbal Extract Shampoo Combined with Synergistic Extracts

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Abstract: *This study aimed to develop an herbal shampoo formulation incorporating crude extracts from Cassia alata, Curcuma longa, Lawsonia inermis, Rhinacanthus nasutus, and Sapindus emarginatus and to evaluate their synergistic antibacterial effects. The extracts were tested against Pseudomonas aeruginosa TISTR 1467, Staphylococcus aureus TISTR 118, and Staphylococcus epidermidis TISTR 1845. The most antibacterial activity shampoo against Pseudomonas aeruginosa TISTR 1467 and Staphylococcus aureus TISTR 118 is the combination between the ethanol extracts of Lawsonia inermis and Curcuma longa, which show enhanced antibacterial activity from the original formula with a relative inhibitory zone of 4.64 and 4.76, respectively. For Staphylococcus epidermidis TISTR 1845, the most effective antibacterial activity occurred at the combination of the ethanol extracts of Rhinacanthus nasutus and Sapindus emarginatus with a relative inhibitory zone of 4.60. The Minimum Inhibitory Concentration (MIC) test identified the most effective formulation with a MIC of 10^{-8} mg/ml. Stability testing shows a high degree of color change in almost all herbal shampoos after being subjected to elevated temperatures. Additionally, consumer preference evaluations involving 30 participants revealed that a formulation containing Sapindus emarginatus with itself and Curcuma longa received the highest satisfaction scores regarding color, foam, and overall acceptance.*

Keywords: *Antibacterial activity, Extraction, Herbal shampoo, Medicinal plants.*

I. INTRODUCTION

Shampoo is a fundamental personal care product designed primarily to cleanse the hair and scalp by removing dirt, sebum, sweat, dead skin cells, environmental pollutants, and styling product residues [1]. The scalp accumulates oil and debris like the skin, leading to discomfort, unpleasant odor, and even scalp disorders such as dandruff or folliculitis if not adequately cleansed [2]. Current shampoos are formulated with additional functions: cleansing, moisturizing, conditioning, anti-dandruff, or hair-strengthening [3]. Shampoos maintain a clean and healthy scalp environment, are essential for hygiene, support optimal hair growth, and improve hair appearance, texture, and manageability [4].

Shampoos are diverse and suited for various hair types and concerns. Organic products and shampoos containing herbal extracts have become popular due to their multifunctional properties and eco-friendly approach [4]. They are also treated as moisturizers for the scalp and hair, strengthening hair and providing antioxidant effects from the incorporated herbal extracts, and they may have antibacterial activity [6].

Herbal shampoos incorporate plant-derived ingredients, such as extracts, oils, or powders, which are used for their beneficial effects on hair and scalp health. General herb ingredients include *Aloe vera* [7], *Hibiscus* [8], *Azadirachta indica* [9], *Eclipta prostrata* [10], *Acacia concinna* [11], *Punica granatum* [12], *Phyllanthus emblica*, and *Centella asiatica* [13]. These active herbal ingredients offer a variety of properties, including antimicrobial, anti-inflammatory, antioxidant, conditioning, and hair growth-promoting effects.

This study proposes the development of herbal shampoos incorporating extracts from Thai medicinal plants, including *Cassia alata* (L.) Roxb., *Lawsonia inermis* L., *Rhinacanthus nasutus* (L.) Kurz., *Sapindus emarginatus* Wall., and *Curcuma longa* L. Kurz. The formulation focuses on the synergistic combination

of these herbal extracts to evaluate their antibacterial activity against common skin pathogens, namely *Pseudomonas aeruginosa* TISTR 1467, *Staphylococcus aureus* TISTR 118, and *Staphylococcus epidermidis* TISTR 1845. In addition, the study assesses the stability of shampoo formulations and evaluates user preferences in terms of color, foam, and overall satisfaction through a consumer test involving 30 participants. This consumer-centric approach underscores end-users' integral role in developing herbal shampoo products that are both effective and well-accepted.

II. MATERIALS AND METHODS

A. Herbal Preparation

This study employed fresh Thai medicinal plants collected from various regions of Lopburi Province, Thailand, explicitly focusing on five distinct plant species. Each species underwent two forms of extraction: aqueous extraction using fresh material and ethanol extraction using dried material. The plant species analyzed in this research include *Cassia alata* (L.) Roxb. (commonly known as Ringworm Bush), with a focus on the leaves; *Lawsonia inermis* L. (Henna), utilizing all parts; *Rhinacanthus nasutus* (L.) Kurz. (White Crane Flower), employing the leaves; *Sapindus emarginatus* Wall. (Soap Nut Tree), which involves fruits and *Curcuma longa* L. (Turmeric), utilizing the rhizomes.

Medicinal plants were thoroughly washed to eliminate dust, soil, and impurities such as weevils and insects. Then, they were cut into smaller pieces and air-dried to prepare them for the next steps.

The fresh plants from the initial cleaning could be directly employed for aqueous extraction. Conversely, for the ethanol extraction process, the following protocol was followed: after the washing procedure outlined in the first phase, the finely chopped plants were dried in a hot air oven (Memmert, Universal oven UN30m, Germany) at a temperature of 60°C for 8 hours. Subsequently, the dried materials were ground into a fine powder capable of passing through a 30-mesh sieve (RETSCH® BASIC, Germany) or achieving a particle size smaller than 600 µm.

To ensure optimal extraction, the moisture content of the powdered materials was precisely measured. Before proceeding with the extraction process, the moisture content must not exceed 10%. This was achieved by weighing 2 g of each ground sample and measuring it with a moisture analyser (AND MX-50, Japan) set at 105°C. If any sample exceeded the 10% moisture threshold, it was subjected to additional drying at 60°C for another 8 hours, followed by retesting until the moisture content met the specified criteria.

B. Herbal Extract Preparation

1) ***Aqueous Extract Preparation:*** The fresh plants, weighing 500 g, were placed into a glass container. Distilled water, totaling 2,500 ml, was added to achieve a 1:5 herb-to-water ratio, ensuring the herbs were completely submerged. The mixture was then boiled at 100°C for 30 minutes to extract the active compounds from the plants. After boiling, the mixture was cooled and coarsely filtered through a muslin cloth to remove plant residues. The crude extract was further filtered twice using cotton.

Subsequently, the crude extract was transferred into an evaporating basin (Haldenwanger, Germany) and placed in a water bath (MEMMERT WNB22, Germany). The extract was evaporated at 95°C until all the solvent had evaporated entirely. The resulting concentrated crude extract was then stored in a glass container with a tight-fitting lid.

2) ***Ethanol Extract Preparation:*** The powdered plants were measured at 500 g. To ensure that the moisture content remained below 10%, the herbs were placed in a tightly sealed glass container. 2,500 ml of 95% ethanol was added to fully submerge the powdered plant material, maintaining a 1:5 herb-to-solvent ratio. The mixture was then macerated for 7 days, stirring the herbs in the solvent daily.

Following this period, the solvent was removed from the crude extract using a rotary evaporator (BUCHI, Japan) at 60°C and a pressure of 74.51 torr. This process effectively evaporated most of the solvent, although some residual solvent remained. The partially concentrated extract was then transferred to an evaporating basin, where the remaining solvent was further evaporated using a water bath set at 95°C until wholly evaporated. The final extract will be thick and viscous. It is crucial to store the extract in a tightly sealed glass bottle for the subsequent experimentation phase.

*C. **Synergistic Formulations***

Based on our previous study, this section explores synergistic interactions between crude extracts. Based on their antibacterial activity efficacy effects, the selected crude extracts are combined in a 1:1 ratio, as shown in Table I. This helps identify potential interactions that enhance the antibacterial activity of crude extracts.

Table I: Synergistic Formulation

Ethanol + Ethanol extract formulation	**Formula No.**
Cassia alata + *Lawsonia inermis*	1
Cassia alata + *Rhinacanthus nasutus*	2
Cassia alata + *Sapindus emarginatus*	3
Cassia alata + *Curcuma longa*	4
Lawsonia inermis + *Rhinacanthus nasutus*	5
Lawsonia inermis + *Sapindus emarginatus*	6
Lawsonia inermis + *Curcuma longa*	7
Rhinacanthus nasutus + *Sapindus emarginatus*	8
Rhinacanthus nasutus + *Curcuma longa*	9
Sapindus emarginatus + *Curcuma longa*	10
Aqueous + Ethanol extract formulation	
Sapindus emarginatus + *Cassia alata*	11
Sapindus emarginatus + *Lawsonia inermis*	12
Sapindus emarginatus + *Rhinacanthus nasutus*	13
Sapindus emarginatus + *Sapindus emarginatus*	14
Sapindus emarginatus + *Curcuma longa*	15

*D. **Product Development***

Begin by boiling 555 ml of water until it reaches a rolling boil. Once boiled, separate 1/5 of the water and carefully pour it into a plastic beaker. Next, add 300 g of sodium laureth sulfate to the beaker and gently stir with a plastic spatula to minimize foam formation. Slowly incorporate 40 g of cocamidopropyl betaine, continuing to stir gently to avoid creating additional foam.

In a separate container, dissolve 2 g of the pre-mixed herbal extract (as outlined in Table I) in 60 g of propylene glycol. Once dissolved, add this mixture to the primary solution and ensure it is mixed thoroughly.

Next, add 1 g of ethylenediaminetetraacetic acid (EDTA) to the primary mixture in another 1/5 hot water. Then, incorporate 10 g of propylene glycol and stir until thoroughly combined. Gradually dissolve 30 g of salt in another 1/5 of hot water and add it to the primary solution. Finally, pour in the remaining 2/5 of the water and mix well. Allow the solution to cool before transferring it into bottles.

*E. **Quality Assessment***

1) ***Cultural Media Preparation:*** Nutrient Broth (NB), Plate Count Agar (PCA), and Mueller Hinton Agar (MHA) are vital media used for the cultivation and testing of bacteria. To prepare NB, 13 g of NB powder is dissolved in 1,000 ml of distilled water and stirred until homogeneous. This mixture is

then transferred into a 250 ml Erlenmeyer flask, covered with cotton, and sterilized using an autoclave (HIRAYAMA, HICLAVE HVA-110, Japan) at 121°C and 15 psi for 15 minutes before cooling and storage.

For PCA preparation, 23.5 g of PCA powder is dissolved in 1,000 ml of distilled water by heating and stirring. The solution is then transferred into a 2,000 ml reagent bottle and autoclaved under the same conditions. After cooling to 45-50°C, 20-25 ml is poured into sterile 15x100 mm Petri dishes (Anumbra, Czech Republic) and allowed to solidify under UV light in a laminar airflow cabinet (FASTER BHA48, Italy) for storage.

Similarly, MHA is prepared by dissolving 38 g of MHA powder in 1,000 ml of distilled water, followed by heating, stirring, and autoclaving. Once cooled to 45-50°C, 20-25 ml is poured into sterile Petri dishes, solidified under UV light, and stored for further use.

2) ***Bacteria Preparation:*** Three pathogenic bacteria commonly linked to skin infections are *Pseudomonas aeruginosa* TISTR 1467, *Staphylococcus aureus* TISTR 118, and *Staphylococcus epidermidis* TISTR 1845. Each bacterial strain was cultured individually in a pre-prepared NB medium. The process commenced by transferring 1 ml of the bacterial stock culture into an Erlenmeyer flask containing 100 ml of NB. This mixture was then incubated at 37°C while shaking at 250 rpm for 24 hours. The resulting bacterial culture was subsequently stored for use in later experiments.

3) ***Antibacterial Activity of the Product:*** This experiment assesses the antibacterial activity of crude extracts by measuring the inhibition zones. Initially, bacterial cell suspensions are diluted using the Broth Dilution Method to achieve a concentration between 30 and 300 CFU/ml. This process involves performing a 10-fold serial dilution, starting with 1 ml of bacterial culture mixed with 9 ml of sterile distilled water and continuing until a final dilution of 10^{-10} is obtained. Following this, 100 µl from each dilution (ranging from 10^{-1} to 10^{-10}) is spread onto PCA plates using a sterile glass spreader, and the plates are incubated at 37°C for 18 hours before counting colonies with a hemocytometer (Menzel-Gläser Cubreobjetos, Germany).

The crude extracts are diluted to a concentration of 10 mg/ml for the aqueous extract. Then, volumes of 30 µl are applied to discs placed on MHA plates that have been inoculated with bacteria to evaluate their antibacterial activity. Similarly, ethanol extracts are dissolved in Dimethyl Sulfoxide (DMSO) to a final concentration of 1 mg/ml, with 30 µl applied to discs on MHA plates for antibacterial testing. The inhibition zones surrounding the discs are measured to identify the most effective extract concentration, which will be utilized for Minimum Inhibitory Concentration (MIC) testing.

4) ***Minimum Inhibitory Concentration (MIC):*** The MIC test identifies the lowest concentration of each crude extract that effectively exhibits antibacterial activity. Once the bacterial concentration falls within the 30-300 CFU/ml range, the bacterial suspension is combined with each crude extract at 10 mg/ml concentration. This mixture is serially diluted to 10^{-10} and then spread onto PCA. Finally, the number of viable bacterial cells is counted to determine the MIC, which indicates the minimum concentration of the crude extract necessary to exhibit antibacterial activity.

5) ***Stability of the Product:*** The physical properties of the shampoo were assessed after being stored at various temperatures: specifically, 4°C, 28°C, and 40°C. The storage temperature was changed monthly, starting at 28°C, followed by exposure to high temperature at 40°C, then low temperature at 4°C, and finally returning to room temperature at 28°C. This cycle was conducted over four months. The analysis of physical properties changes in the shampoo included measuring color variations with a spectrophotometer (HunterLab ColorFlex EZ, United States of America), pH variations using a pH meter (Consort, Belgium), and viscosity changes assessed with a viscometer (BROOKFIELD RVDVI+, United States of America). The CIELAB 76 color values obtained from the measurement were then used to calculate the degree of color change (ΔE_{76}) according to (1). L* is

a lightness, a* is a red-green axis, and b* is a yellow-blue axis at the initial and final stage of the colorimetric measurement.

$$\Delta E_{76}=\sqrt{\left(L_f^*-L_i^*\right)^2+\left(a_f^*-a_i^*\right)^2+\left(b_f^*-b_i^*\right)^2} \quad (1)$$

F. Preference Test

User satisfaction was evaluated by collecting statistical data, concentrating on three key variables: satisfaction with color, foam, overall preference, and satisfaction with a specific task. Responses from 30 participants were gathered using a 7-point hedonic scale. After the questionnaires were collected, statistical analysis was performed using ANOVA and Duncan's Multiple Range Test (DMRT) with SPSS version 16.

III. RESULTS

A. Synergistic Properties

Fig. 1 illustrates the synergistic effects of the crude extracts in antibacterial activity, specifically all crude ethanol extracts and aqueous extract of *Sapindus emarginatus* combined with all crude ethanol extracts. The test results indicate that the combined synergistic effects of these extracts enhanced their antibacterial effect.

The most effective synergistic interaction for antibacterial impact occurred at Formula No. 10. These extracts inhibited *P. aeruginosa* and *S. aureus* activity with the relative inhibitory zone of 1.85 (11.07 mm) and 1.68 (10.10 mm), respectively. The most effective synergistic combination for inhibiting *S. epidermidis* was Formulation No. 15, with a relative inhibitory zone of 2.07 (12.44 mm) (as shown in Fig. 2).

When the aqueous extract of *Sapindus emarginatus* was combined with the ethanol extracts, it was found that the aqueous extract did not significantly enhance the antibacterial effects of the ethanol extracts, except for Formula No. 15. This formulation exhibited the highest antibacterial activity, with relative inhibitory zones of 1.78, 1.52, and 2.07 (10.66, 9.10, and 12.44 mm) for *P. aeruginosa*, *S. aureus*, and *S. epidermidis*, respectively (as shown in Fig. 2).

The results from testing the MIC were aligned with the findings from the inhibitory zone tests of each extract, as shown in Fig. 3. The synergistic effect of Formula No. 7 demonstrated the highest efficacy activity in antibacterial activity in the strains *P. aeruginosa*, *S. aureus*, and *S. epidermidis*, with the lowest concentration capable of antibacterial activity being $1x10^{-4}$ mg/ml.

Regarding the synergistic effect of combining ethanol extracts with the aqueous extract of *Sapindus emarginatus*, the most effective combination for antibacterial activity is Formula No. 15. This combination had the lowest concentration capable of antibacterial activity of all three bacterial strains at $1x10^{-3}$, $1x10^{-4}$, and $1x10^{-4}$ mg/ml.

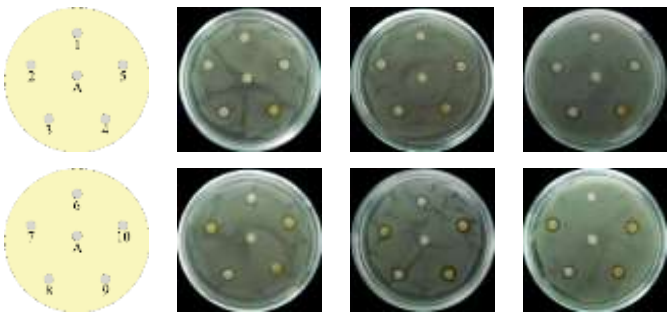

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Plate layout *P. aeruginosa* *S. aureus* *S. epidermidis*

Fig. 1 Synergistic effect of each extract (A) control, (1-15) each synergistic formula

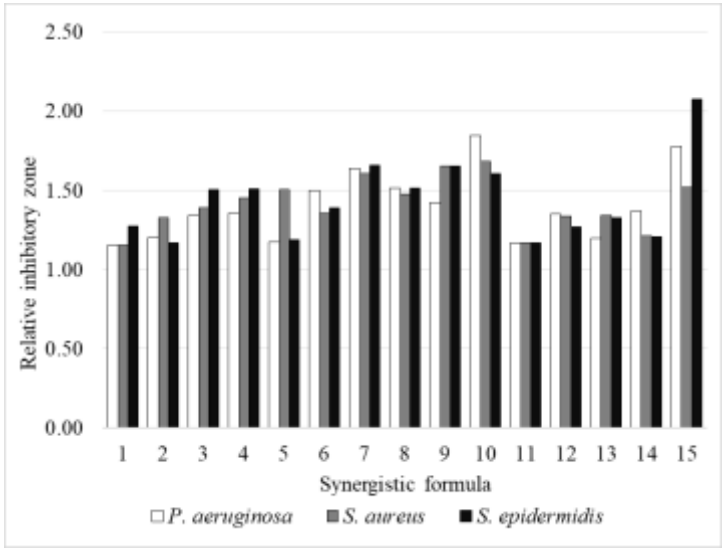

Fig. 2 Inhibitory zone of each synergistic formulation

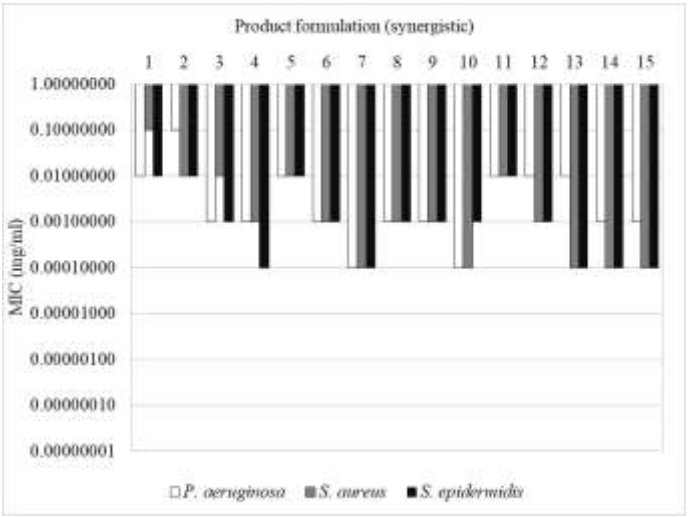

Fig. 3 MIC of each synergistic formulation

B. *Product Quality*

1) ***Product Appearance:*** Fig. 4 illustrates the shampoos formulated with various herbal extracts, ranging from Formula No. 1 to No. 15. These shampoos are classified into three categories based on their observed color: (1) dark color (Formula Nos. 2, 8, 9, and 13), (2) medium color intensity (Formula Nos. 1, 3, 4, 5, 6, 7, 11, and 12), and (3) light color (Formula Nos. 10, 14, and 15). The differences in coloration are due to the varying intensity of the herbal extracts used in each formulation.

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Fig. 4 Product appearance

2) ***Antibacterial Activity:*** Fig. 5 illustrates the antibacterial activity of all the shampoo formulations, indicating that each product could exhibit antibacterial activity. Fig. 6 illustrates the relative antibacterial activity effectiveness of each shampoo. It was noted that shampoos containing herbal extracts showed higher antibacterial activity compared to those without such extracts (Control). Formula No. 7 showed the highest relative inhibition against *P. aeruginosa* (4.64) and *S. aureus* (4.76). For *S. epidermidis*, Formula No. 8, composed of ethanol extracts of *Rhinacanthus nasutus* and *Sapindus emarginatus*, demonstrated the most significant effectiveness, with a relative inhibition zone of 4.60.

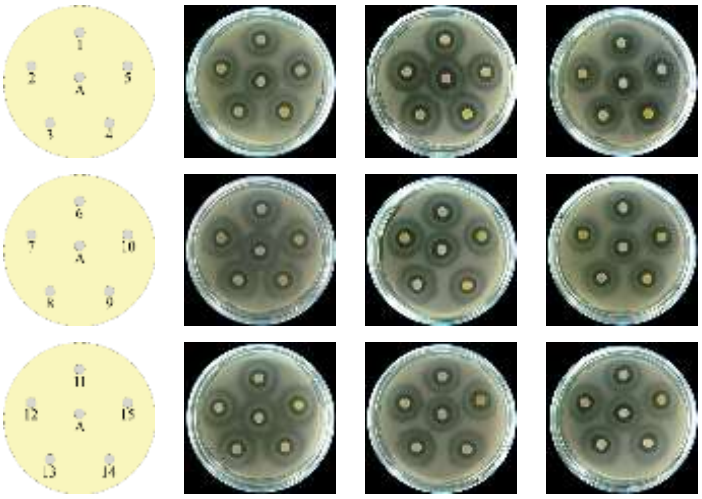

Fig. 5 Inhibitory zone of each product formula; (A) control, (1-15) each product formula

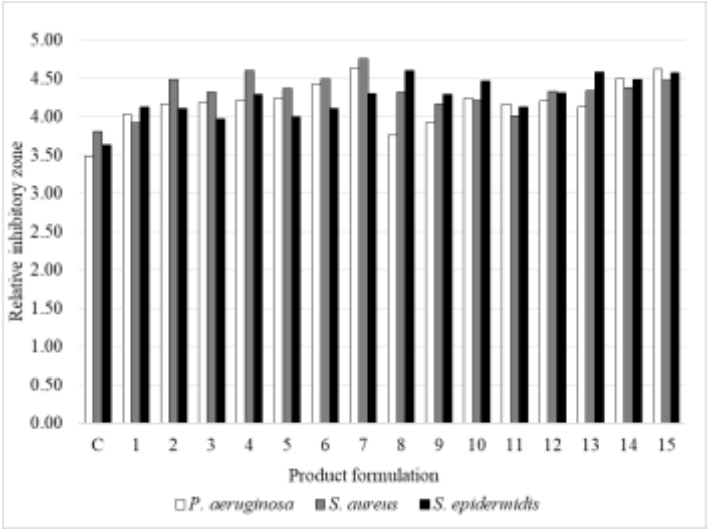

Fig. 6 Relative inhibitory zone of each product formula

3) ***Minimum Inhibitory Concentration (MIC):*** In the MIC test results in Fig. 7, Formula Nos. 7 and 15 exhibited the lowest extract concentrations necessary for antibacterial activity. Both formulas demonstrated antibacterial activity against all three bacterial strains at an impressively low concentration of 10^{-8} mg/ml. These findings align with the relative antibacterial effectiveness illustrated in Fig. 6.

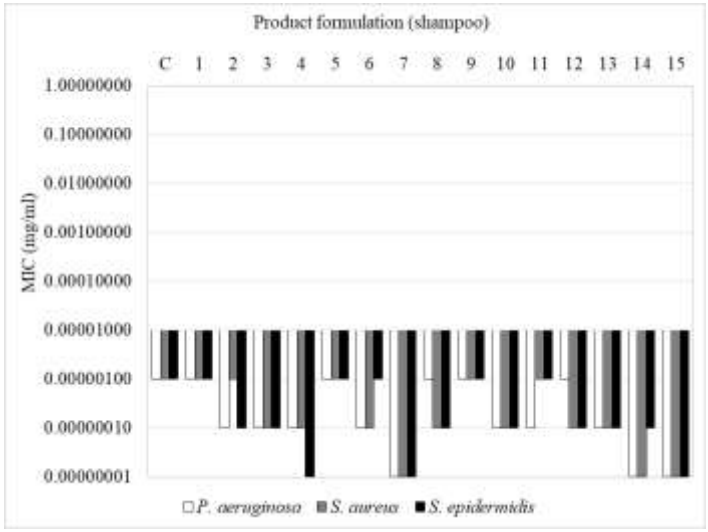

Fig. 7 MIC of each product formula

4) ***Stability of the Product:*** Fig. 8 shows the changes in pH and viscosity of all formulations. Adding herbal extracts mainly decreased pH, with the lowest value observed at 5.68 (Formula No. 14), which closes within the acceptable pH for application on human skin (pH 5.5). An exception was observed in Formula No. 5, where the pH increased slightly compared to the control (the maximum pH recorded was 6.66). Furthermore, the incorporation of herbal extracts generally led to a reduction in shampoo viscosity. After subjecting the shampoos to cyclic temperature variations, a decrease in viscosity was observed across all formulations, likely because of elevated temperature. Nevertheless, all formulations remained consistent, exhibiting no phase separation.

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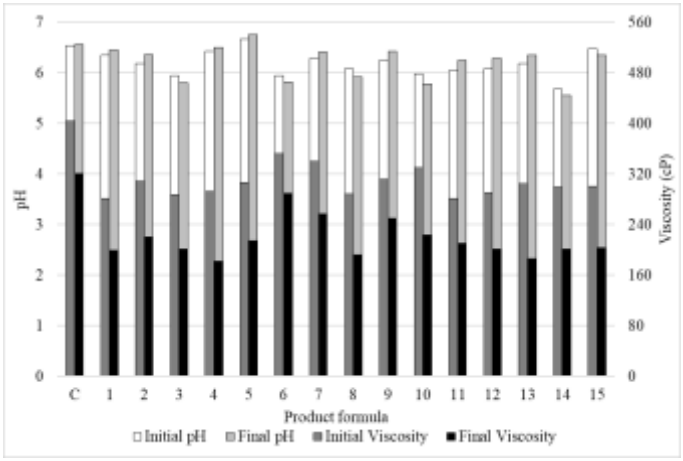

Fig. 8 pH and Viscosity of each product

Fig. 9 and Fig. 10 show the stability test results for all shampoo formulations, organized by their degree of color change (ΔE). After being stored under cyclic temperature conditions, all shampoo samples displayed some degree of color alteration—none remained utterly unchanged. Only four formulations demonstrated slight color changes: Control, Formula Nos. 2, 12, and 14. In contrast, the other formulations exhibited more significant color changes, with Formulation No. 3 showing the most conspicuous alteration.

Fig. 9 Color change of each product

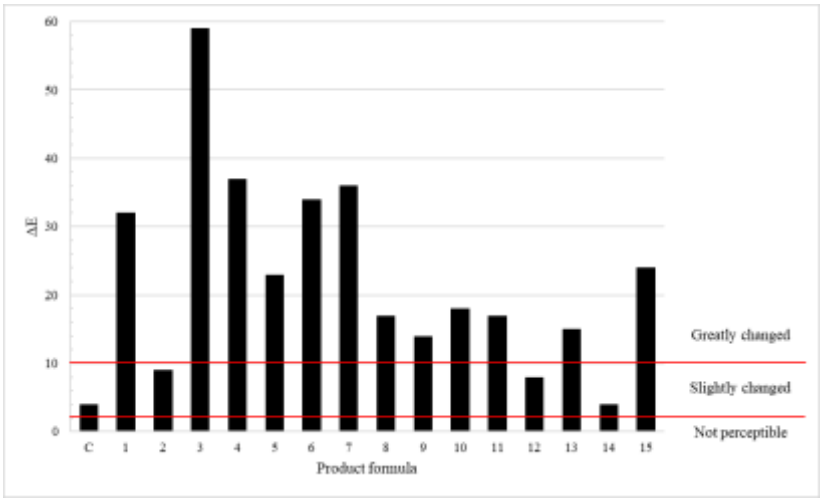

Fig. 10 ΔE of each product

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C. Preference Test

Table II outlines the ANOVA results concerning the color, foam, and overall preference for herbal shampoo products. The analysis revealed a statistically significant difference at a confidence level of $p \geq 0.05$. In conjunction with the findings from Duncan's Multiple Range Test (DMRT) displayed in Table III, the top three formulations that gathered the highest color, foam, and overall preference ratings were Formula Nos. 15, 10, and 14, in that order.

Table II: Anova Of The Preference Test

Color	Sum of Square	df	Mean Square	F	Sig.
Between Groups	420.898	14	30.064	25.320	0.000
Within Groups	516.500	435	1.187		
Total	937.398	449			
Foam					
Between Groups	489.031	14	34.931	29.208	0.000
Within Groups	520.233	435	1.196		
Total	1009.264	449			
Overall preference					
Between Groups	239.467	14	17.105	21.565	0.000
Within Groups	345.033	435	0.793		
Total	584.500	449			

Table III: Dmrt of the Preference Test

Product formula	Subset for alpha = .05											
	Color					Foam				Overall preference		
	1	2	3	4	5	1	2	3	4	1	2	3
8	4.033					3.833				4.333		
6	4.100					3.867				4.333		
7	4.100					3.867				4.467	4.467	
11	4.100					4.100				4.567	4.567	
12	4.100					4.167				4.567	4.567	
13	4.167	4.167				4.233				4.600	4.600	
9	4.267	4.267	4.267			4.300				4.667	4.667	
4	4.300	4.300	4.300			4.467	4.467				4.900	
5	4.333	4.333	4.333			4.467	4.467				4.933	
2		4.767	4.767	4.767			4.967	4.967			4.933	
1			4.867	4.867				5.167			4.967	
3				4.967				5.333			4.967	
14					6.400				6.600			6.300

10 15					0 6.767 6.767				0 6.667 7.000			0 6.300 6.667
Sig.	0.382	0.057	0.057	0.057	0.222	0.058	0.095	0.223	0.183	0.220	0.069	0.133

IV. CONCLUSIONS

- The synergistic effects of herbal extracts Formula Nos. 7, 10, and 15 demonstrated higher antibacterial activity than other extract combinations.
- Formula Nos. 7, 10, 14, and 15 exhibited superior antibacterial efficacies compared to other shampoo formulations when formulated into herbal extract-based shampoos.
- Based on the shampoo stability test, the original formula and Formula Nos. 2, 12, and 14 showed the best stability with minimal discoloration (ΔE) after being subjected to various storage temperatures, 4, 9, 8, and 4.
- Regarding overall preference among 30 participants, Formula Nos. 10, 14, and 15 were the top 3 most satisfied shampoos compared to the others.

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