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Enhancing the antimicrobial activity of Herbal Moisturizer against S.aureus

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ABSTRACT

This study investigates the creation of bio-cosmetics, particularly herbal moisturizers with improved antimicrobial qualities, in response to growing consumer demand for safer, more sustainable, and eco-friendly cosmetic products. The goal of the research is to increase product efficacy by utilizing modern technologies and natural ingredients. Extracts from hibiscus (*Hibiscus rosa-sinensis*) and neem (*Azadirachta indica*) were used to create herbal moisturizers. Copper-based nanoparticles of hibiscus and neem extracts were created and then added to the moisturizer formulations to increase their antimicrobial activity.
Isolating and characterizing bacterial strains from human skin, including identifying Staphylococcus aureus as a target pathogen for antimicrobial testing, was a crucial component of this investigation. The identity of S. aureus was verified by biochemical tests, including methyl red, hydrogen sulfide, urease, nitrate, citrate, sugar fermentation, indole, and Voges-Proskauer tests.
The Kirby-Bauer disk diffusion method was used to assess the moisturizers' antimicrobial efficacy. The results showed that adding the herbal extracts as nanoparticles significantly increased their antimicrobial activity. In comparison to their respective plain herbal extract formulations, the moisturizers containing hibiscus and neem nanoparticles demonstrated a notably larger zone of inhibition against S. aureus. This suggests that these herbal ingredients' antimicrobial potency is successfully increased by nanoformulation. Contributing to the expanding field of green cosmetic innovation, this study demonstrates the potential of combining nanotechnology and traditional herbal knowledge to create novel and highly effective bio-cosmetic products with improved antimicrobial benefits.

KEYWORDS: Bio-cosmetics, Herbal moisturizers, Antimicrobial activity, Staphylococcus aureus, Hibiscus extract, Neem extract, Zone of inhibition, Nanotechnology, Gram staining

1. INTRODUCTION

In the recent years, the cosmetic sector has undergone a noticeable transformation in consumer preferences through growing awareness about product safety, environmental impact, and sustainability. Traditional cosmetics that are composed of synthetic compounds, are constantly being questioned for their long-term effects on health and the environment. Bio-cosmetics is an excellent alternative, blending biotechnology, nanotechnology, and practices that are gentle to the environment.
Bio-cosmetics are cosmetic products made from entirely natural ingredients that are derived from plants, animals, microbes, enzymes, insects, and organic crops that are free of pesticides and chemical fertilizers and used for topical skin, hair, face, and oral care [1] . These developments help achieve the objectives of the circular economy and pollution reduction by converting waste into useful inputs. It uses materials like vegetable waste, fruit peels, and spent oils in cosmetic formulations.
With an emphasis on how contemporary technologies are transforming the use of these well-known resources, this study intends to investigate the creation of bio- cosmetics from oils, organic residues, and waste materials from the food and textile industries. In order to support the expanding field of green cosmetic innovation, it also aims to assess the viability, sustainability, and safety of such practices.

2. MATERIAL AND METHOD

2.1 PREPARTION OF HERBAL MOISTURIZER

2.1.1 Preparation of neem extract- According to the protocol [2], 2 grams of neem leaves were accurately weighed and crushed using a mortar and pestle. The crushed material was then mixed with 20 mL of distilled water and heated in a boiling water bath for 30 minutes. After heating, the mixture was stored in a dark place for 3 days to allow for proper extraction.

2.1.2 Moisturizer- 10 mL of distilled water was taken in a beaker, to which 500 µL of glycerin and 10 mL of neem extract were added. A few drops of coconut oil were then incorporated, followed by the addition of 1 g of sodium alginate, with continuous mixing to ensure uniform consistency of the moisturizer.

Fig.1 Neem moisturizer

2.1.3 Preparation of hibiscus extract- 7.5g of hibiscus petals were crushed in a mortar and pestle .It was then mixed with 50 ml distilled water and heated in an induction for 30 minutes. Hibiscus extract was then obtained and filtered out for further use.

2.1.4 Moisturizer - 10 mL of distilled water was taken in a beaker, to which 500 µL of glycerin and 10 mL of hibiscus extract were added. A few drops of coconut oil were then incorporated, followed by the addition of 1 g of sodium alginate, with continuous mixing to ensure uniform consistency of the moisturizer.

Fig.2 Hibiscus moisturizer

2.2 FORMULATION OF NANOPARTICLES

2.2.1 NEEM NANOPARTICLE:

According to the protocol [3], 0.2394g of copper(II) sulfate (0.1M) salt was weighed using a weighing balance. It was then mixed with 15ml distilled water and was mixed thoroughly for 15 minutes on a heating plate. 1.25 ml of poly ethylene glycol was added to it and was heated was another 15 minutes. 10 ml of the prepared neem extract was added to it and further heated for 20 minutes.
pH of 7 was maintained by adding 0.1M sodium hydroxide drop by drop. It was then stored in the dark for 24 hours for the dark reaction to occur.
2 ml was taken in the eppendorf and was centrifuged for 10 minutes. The supernatant was discarded and 20µl of ammonia was added for preservation.

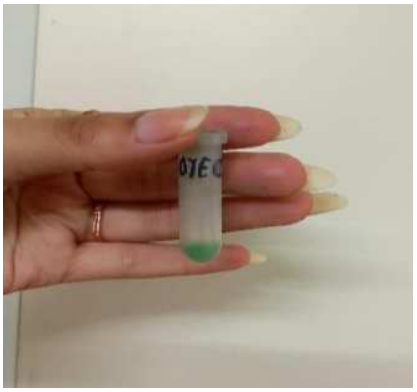


Fig.3 Neem nanoparticle

2.2.2 HIBISCUS NANOPARTICLE:

According to the protocol [4], 0.2394g of copper (II) sulfate (0.1M) salt was weighed using a weighing balance. It was then mixed with 15ml distilled water and was mixed thoroughly for 15 minutes on a heating plate. 1.25 ml of poly ethylene glycol was added to it and was heated was another 15 minutes. 10 ml of the prepared hibiscus extract was added to it and further heated for 20 minutes.
pH of 7 was maintained by adding 0.1M NaOH drop by drop. It was then stored in the dark for 24 hours for the dark reaction to occur.
2 ml was taken in the eppendorf and was centrifuged for 10 minutes. The supernatant was discarded and 20µl of ammonia was added for preservation.

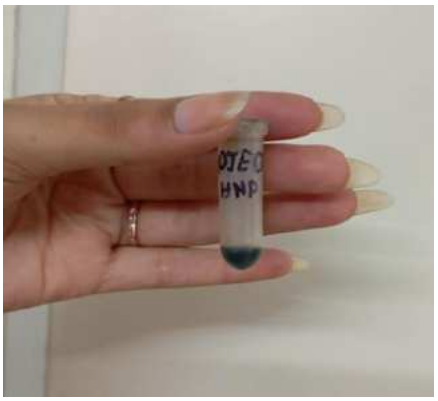


Fig.4 Hibiscus nanoparticle

2.3 ISOLATION OF BACTERIA

Certain isolation methods are needed for proteomic analysis of Gram-negative bacteria's cell envelope proteins. [5].According to culture-based research on skin microbiota, skin microbes may have an impact on wound healing [10], pathogen growth [9], immune responses [7, 8], skin characteristics [6], and even the attraction of disease vectors [11][12].

Bacteria was taken from the skin of a person having body ache with the help of a cotton swab and any further contamination was avoided. The bacteria was then made to grow on the nutrient agar medium. According to the protocol [13] ,

- Peptone- 5 g/L
- Sodium chloride- 5g/L
- Beef extract- 3 g/L
- Agar- 15 g/L

the above components were mixed in 0.020 L of distilled water and was kept for autoclave. After this, the molten medium was poured into the plates. Two plates were made to inoculate the bacteria.

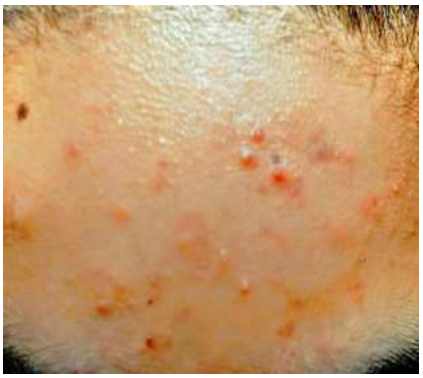


Fig.5 Infectious site

Bacteria was inoculated and kept in the incubator for the growth to occur. The plates were observed after 24 hours.
The grown colonies were carefully picked using an inoculating loop and were now made to grow on the second plate. The bacteria was now ready for further use and characterization of bacteria.

Fig.6 Bacterial colonies

2.4 STAINING OF BACTERIA

According to the protocol [14], 0.00001 L of distilled water was poured in the glass slide using a micro-pipette . The grown colonies of bacteria were carefully picked up from the plate using the inoculating loop and smeared to prepare a thin smear of bacterial culture. This was further air dried and heat-fix by flame.

- Flooded the slide with crystal violet for 1 minute, rinsed it with distilled water.
- Now, covered it with Gram's iodine for 1 minute.
- Used diluted ethanol as a decolorizer.
- Flooded the slide again with safranin counterstain for 1 minute and then rinsed it until clear.
- Air-dry it and then observed it under the microscope at 40X-45X.

Results are shown in table1.

TABLE 1:

BACTERIA	RESULT
B1	Gram-positive
B2	Gram-negative

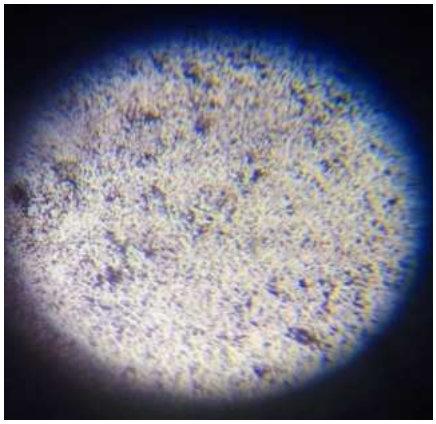
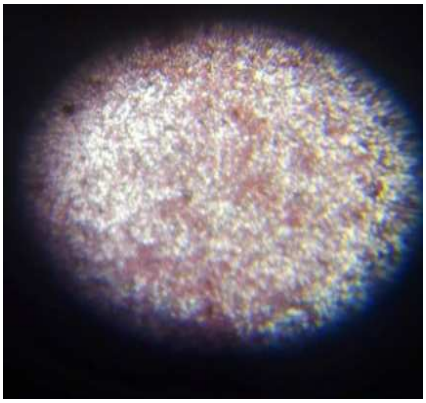


Fig.7(a) Gram-positive bacteria



(b) Gram-negative bacteria

2.5 CHARACTERIZATION OF BACTERIA

PROTOCOLS:

2.5.1 SUGAR TEST:

According to the protocol [15], eight different test tubes were taken, four for each type of bacteria(Bacteria 1 [B1], Bacteria 2 [B2]).

- Peptone- 5 g/L
- Beef extract- 3 g/L
- Sodium chloride - 5 g/L

all the above components were taken in the culture bottle with 0.040 L distilled water and were put on autoclave . After this, 5 mL was carefully poured in the eight test tubes.
Sugars such as dextrose, lactose, d-manitol and sucrose were carefully weighed and added to the test tubes . It was then put on autoclave . 200µl bacteria was inoculated in each and every tube and kept in incubator for 24 hours.
Few drops of phenol red was added in the test tubes.It was observed that the sugar test for both the bacteria was negative as the red color retained.

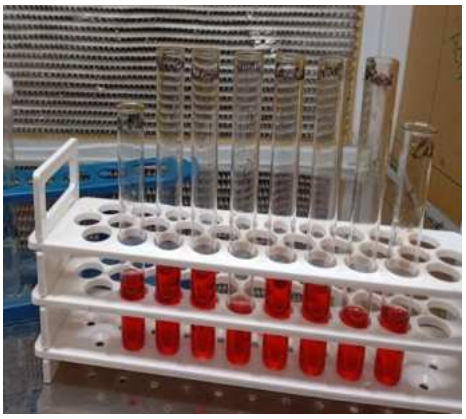


Fig.8 Sugar test

2.5.2 INDOLE TEST:

According to the protocol [16],

- Trypton- 10 g/L
- Sodium chloride- 5 g/L pH-

6.8

all the above components were added in the culture bottle with 0.010 L distilled water. It was then put on autoclave and 0.05 L was poured in two different test tubes. Both the bacteria(B1,B2) were inoculated in the test tubes(100µl). After this, it was kept in the incubator for 24 hours.

Few drops of Kovac's reagent was then added to both the test tubes. Both came out to be positive as a red ring was observed.

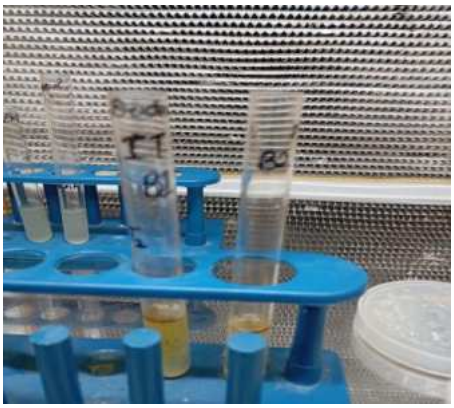


Fig.9 Indole test

2.5.3 HYDROGEN SULPHIDE TEST:

According to the protocol, [17]

- Peptone- 30 g/L
- Beef extract- 3 g/L
- Ferrous ammonium sulphate- 0.2 g/L
- Sodium thiosulphate- 0.025 g/L
- Agar- 3 g/L

pH- 7.3

all the above components are added in the culture bottle with 0.010 L distilled water and put on autoclave for sterilization. After this 0.05 L is poured in each test tube.Slants are prepared and 200µl of bacteria is inoculated in each of the test tube. The test tubes are then left in the incubator for 24 hours. Both came out to be negative because no black precipitate was observed.


(a) Bacteria 1 **(b) Bacteria 2**

Fig.10 Hydrogen sulphide test

2.5.4 UREASE TEST:

According to the protocol [18, 19]

- Peptone- 1 g/L
- Dextrose- 1 g/L
- Sodium chloride- 5 g/L
- Monopotassium phospate- 2 g/L
- Agar- 15 g/L

all the above components are put in a culture bottle with 0.010 L distilled water and put on an autoclave. After this 20 g/L urea is added and it is then poured in the two test tubes along with few drops of phenol red and slants are prepared.

200µl of each bacteria(B1, B2) is inoculated in each test tube and kept in the incubator for 24 hours. In bacteria 1, the result was negative because the red color retained and in bacteria 2 it came out to be positive as a deep red color was seen.


(a) Bacteria 1 **(b) Bacteria 2**

Fig.11 Urease test

2.5.5 NITRATE TEST:

According to the protocol [20],

- Peptone- 5 g/L

- Yeast extract- 3g/L
- Potassium nitrate- 1 g/L pH-
7

all the above components are added into a culture bottle with 0.010 L distilled water and put on an autoclave for sterilization. 0.05 l is poured into the two test tubes. 200µl bacteria (B1, B2) is inoculated in each test tube and left in the incubator for 24 hours.
Then sulphonic acid is diluted in 200µl distilled water and alpha naphthylamine is diluted in 200µl ethanol.

(a) Bacteria 1

(b) Bacteria 2

Fig.12 Nitrate test

2.5.6 CITRATE TEST:

According to the protocol [21,22],
- Sodium citrate- 2 g/L
- Sodium chloride- 5 g/L
- Ammonium dihydrogen phosphate- 1 g/L
- Dipotassium phosphate- 1 g/L
- Magnesium sulfate (heptahydrate)- 0.2 g/L
- Agar- 15 g/L

pH- 6.9

all the above components are added in the culture bottle with 0.010 L distilled water and put on an autoclave. Few drops of bromophenol blue and 0.05 L is poured in each test tubes. After that the slants are prepared. 200µl bacteria (B1, B2) is inoculated in each test tube and left in the incubator for 24 hours. For both the bacteria the results came out to be negative as the slants remains green in color.

(a) Bacteria 1

(b) Bacteria 2

Fig.13 Citrate test

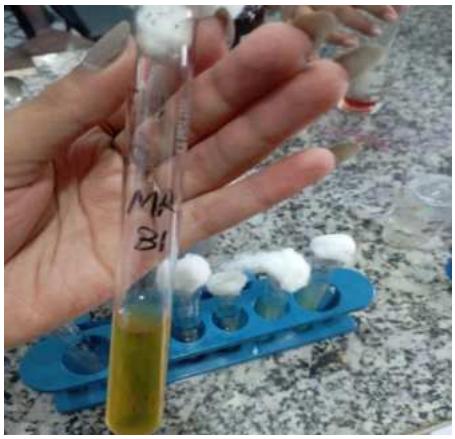
2.5.7 MR-VP TEST:

METHYL-RED TEST (MR TEST) :

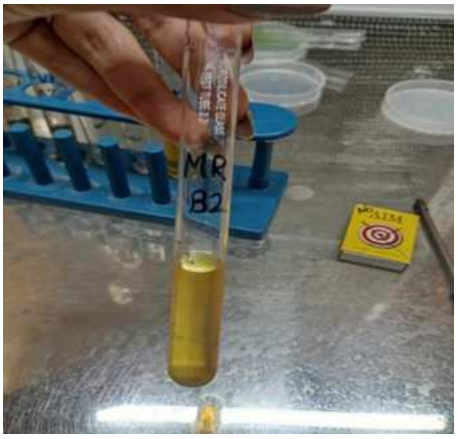
According to the protocol [23],

- Peptone- 7 g/L
- Dextrose- 5 g/L
- Dipotassium phosphate- 5 g/L pH ≈ 6.9 ± 0.2

all the above components are added to a culture bottle with 0.020 L distilled water and 0.010 L is separated for the MR-VP test. Results were negative as yellow color was retained.



(a) Bacteria 1



(b) Bacteria 2

Fig.14 Methyl-red test

METHYL RED- VOGES PROSKAUER TEST (MR-VP TEST):

The remaining 0.010 L is taken into another culture bottle , and the following components are added:

- Alpha naphthol- 5% in 0.01 L ethanol
- Potassium hydroxide- 40% in 0.01 L distilled water

Both the culture bottles are put on autoclave and 0.05 L is poured in the four test tubes. 3-5 drops of methyl red is added and 200µl bacteria (B1, B2) is inoculated in each
test tube and left in the incubator for 24 hours. The results were negative because there was no change in the yellow color.

(a) Bacteria 1

(b) Bacteria 2

Fig.15 Methyl red - Voges proskauer test

The results of the characterization of bacteria is shown in table 2.

TABLE 2:

TESTS	**B1**	**B2**
SUGAR TEST	Negative (red colour was retained)	Negative (red colour was retained)
INDOLE TEST	Positive (red coloured ring is seen)	Positive (red coloured ring is seen)
HYDROGEN SULPHIDE TEST	Negative (no black precipitate was seen)	Negative (no black precipitate was seen)
UREASE TEST	Negative (the red colour retained)	Positive (a deep red colour change was observed)
NITRATE TEST	Positive (a red coloured precipitate is observed)	Positive (a red coloured precipitate is observed)
CITRATE TEST	Negative (the slant remains green in colour)	Negative (the slant remains green in colour)
MR TEST	Negative (yellow colour was observed)	Negative (yellow colour was observed)

MR-VP TEST	Negative (yellow colour was observed)	Negative (yellow colour was observed)

2.6 ANTIMICROBIAL ACTIVITIES

OBJECTIVE:
To find out how well the moisturizer and its nanoparticle-enriched version inhibited microbial growth, their antimicrobial activity was tested against a few chosen bacterial strains.

TEST ORGANISM:
Staphylococcus aureus was used as model gram positive and gram negative bacteria.

METHOD:
The broth microdilution technique (Wiegand et al., 2008) and Kirby–Bauer disk diffusion method (Ericsson & Sherris, 1960) were used to evaluate the antimicrobial activity.[26]
According to the protocol [25], 0.020 L nutrient agar media(NAM) was prepared and put on an autoclave for sterilization. It is then poured into the petri dish (0.010 L in each petri dish) and left for a few minutes to solidify in the laminar.
After this both the bacteria (B1, B2) is swabbed with the help of a cotton swab in both the prepared NAM plates. [24]
The first paper discs is loaded with the moisturizer with the hibiscus extract and then the second one with the hibiscus extract moisturizer containing the prepared hibiscus nanoparticles . Third one is loaded with the neem extract moisturizer and the fourth one with the neem extract moisturizer containing the neem nanoparticles.
The discs are carefully placed on the NAM plate with the forceps . In the same way the other plate with the bacteria B2 is prepared.
Then it is placed in the incubator for 24 hours .

RESULT:
In comparison to the moisturizer with herbal extracts formulation, the moisturizer with nanoparticles showed a noticeably larger zone of inhibition. The plain version only displayed a smaller zone against S. aureus, whereas the NP-moisturizer displayed a larger zone.

The table 3 and 4 shows the formulated result.

TABLE 3:

TEST SAMPLE	**BACTERIA 1**	**BACTERIA 2**
Moisturizer with hibiscus extract	zone was very small	zone was very small
Moisturizer with hibiscus extract containing hibiscus nanoparticles	zone was much larger	zone was much larger

TABLE 4:

TEST SAMPLE	**BACTERIA 1**	**BACTERIA 2**
Moisturizer with neem extract	zone was very small	zone was very small

Moisturizer with neem extract containing neem nanoparticles	zone was much larger	zone was much larger

(a) Bacteria 1 **(b) Bacteria 2**

Fig.16 Antimicrobial activity of bacteria

3. RESULTS:

3.1 Characterization of Bacteria:

Gram staining and a battery of biochemical tests were used to characterize two bacterial isolates, B1 and B2, that were isolated from human skin.

3.2 Gram Staining: Bacteria 1 (B1) was determined to be Gram-positive and Bacteria 2 (B2) to be Gram-negative, as indicated in Table 1.

3.3 Biochemical Tests: Table 2 provides a summary of the biochemical test results.

3.3.1 Sugar Test: Both B1 and B2 produced negative results, maintaining their red hue, suggesting that the tested sugars—lactose, sucrose, D-mannitol, and dextrose—did not ferment.

3.3.2 Indole Test: The development of a red ring showed that both B1 and B2 were capable of producing indole from tryptophan, indicating positive results.

3.3.3 Hydrogen Sulfide Test: Both B1 and B2 produced no black precipitate, indicating that they do not produce hydrogen sulfide.

3.3.4 Urease Test: B2 displayed a positive result with a deep red color shift, indicating urease activity, whereas B1 displayed a negative result (red color retained).

3.3.5 Nitrate Test: Both B1 and B2 produced a red precipitate, indicating their positive results.

3.3.6 Citrate Test: Since the slant stayed green, both B1 and B2 tested negative, indicating that they are unable to use citrate as their only carbon source.

3.3.7 MR Test (Methyl Red Test): Both B1 and B2 produced stable acids through the fermentation of glucose, as evidenced by the yellow color that was seen.

3.3.8 MR-VP Test (Voges-Proskauer Test): B1 and B2 both tested positive, showing a yellow tint that indicated acetoin production.

In accordance with its Gram-positive status and positive urease test, as well as other biochemical profiles, B2 was determined to be Staphylococcus aureus based on these traits, especially the Gram stain and urease test. This is in line with the goal of testing against the model Gram-positive bacterium S. aureus.

3.4 Herbal moisturizers' antimicrobial properties
Using the disk diffusion method, the antimicrobial activity of the herbal moisturizers (with and without nanoparticles) was assessed against the isolated bacteria, including Staphylococcus aureus (B2).

The findings, which are shown in Tables 3 and 4, show that adding herbal extracts to the moisturizer as nanoparticles clearly increases its antimicrobial activity.

3.5 Moisturizer with Hibiscus Extract: Both Bacteria 1 and Bacteria 2 (S. aureus) were inhibited in a very small area by the hibiscus extract-containing plain moisturizer.

3.6 Moisturizer with Hibiscus Extract and Hibiscus Nanoparticles: On the other hand, the moisturizer enhanced with hibiscus nanoparticles demonstrated a significantly greater zone of inhibition against both bacterial strains, suggesting a substantially higher level of antimicrobial efficacy.

3.7 Neem Extract Moisturizer: In a similar vein, the neem extract-containing plain moisturizer showed a very small zone of inhibition against both S. aureus and Bacteria 1.

3.8 Moisturizer with Neem Extract and Neem Nanoparticles: The moisturizer with neem nanoparticles showed a wider zone of inhibition against both Bacteria 1 and Bacteria 2 (S. aureus), indicating the nanoformulation's improved antimicrobial activity.

Overall, the research consistently shows that the herbal moisturizers enhanced with nanoparticles (hibiscus and neem) have significantly higher antimicrobial activity than their counterparts made with plain herbal extracts, especially when it comes to Staphylococcus aureus. This implies that the antimicrobial compounds found in the

herbal extracts are more effectively delivered and have greater efficacy when using a nanotechnology approach.

4. CONCLUSION

This study effectively illustrated how using nanoparticle technology could improve the antimicrobial activity of herbal moisturizers. We made moisturizers with neem and hibiscus extracts, both in their conventional forms and in formulations enhanced with nanoparticles.

Staphylococcus aureus (B2) was validated as a pertinent Gram-positive test organism by our analysis of the isolated bacterial strains. Significantly, moisturizers containing neem and hibiscus nanoparticles demonstrated a significantly larger zone of inhibition against S. aureus than their conventional herbal extract counterparts, according to the antimicrobial efficacy assessment. This convincing finding implies that these herbal extracts' antimicrobial potency can be increased by encapsulating them in nanoparticles, most likely through increased stability, bioavailability, or penetration.

These findings underscore the promise of bio-cosmetics and nanotechnology in developing more effective and sustainable skincare solutions. The enhanced antimicrobial activity of these nanoparticle-enriched herbal moisturizers presents a viable pathway for creating products that offer better protection against bacterial skin infections, aligning with the growing consumer demand for natural, safe, and effective cosmetic alternatives.

5. ACKNOWLEDGEMENT

I want to sincerely thank Rapture Biotech, for giving me the priceless chance and resources to carry out this study. My sincere gratitude is extended to Dr. Manjit Kaur, whose outstanding leadership, steadfast assistance, and perceptive mentoring were crucial to this project at every turn. Her knowledge and support were essential to this study's successful conclusion. I also want to express my gratitude to the entire Rapture Biotech team for their support and collaboration during my time there.

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