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Nano-Enhanced Ointment Formulation: Optimizing Synthesis, Physicochemical Properties and Antimicrobial Efficacy

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Abstract
This study utilized the Taguchi method to optimize the formulation of antimicrobial skin ointments, involving six categorical factors (A–F) at five levels across 25 experimental runs in a Taguchi orthogonal array. The inhibition zones ranged from 11.00 mm to 18.00 mm for Gram-positive bacteria, 12.00 mm to 18.00 mm for Gram-negative bacteria, and 10.00 mm to 18.00 mm for fungi. Residual analyses validated the design's robustness, with ANOVA pinpointing Factors E (32.75%) and B (28.79%) as the key contributors to antimicrobial efficacy. Physicochemical properties of the ointment were compared to two commercial products. The pH values (4.5–6.5) ensured skin compatibility, viscosity (20,000–50,000 centipoise) indicated suitability for application, and moisture content (20%–60%) met NAFDAC standards. Hardness (100 g–300 g) and spreadability (40 mm–100 mm) values were within acceptable ranges, and ash content (0.1%–2.0%) signified purity and safety. Antimicrobial efficacy, assessed against three Gram-positive, three Gram-negative bacterial strains, and three fungal species at concentrations of 500 ppm, 400 ppm, 300 ppm, 200 ppm, and 100 ppm, showed a concentration-dependent effect. MIC values were 50 ppm for bacterial strains and 50 ppm–200 ppm for fungi, while MBC values were 100 ppm for bacteria and 100 ppm–400 ppm for fungi. The prepared ointment demonstrated superior antimicrobial efficacy compared to commercial ointments, inhibiting both bacterial and fungal species more effectively. Market ointment A excelled against bacterial species but was less effective against fungi, whereas market ointment B showed greater efficacy against Gram-positive bacteria and fungi but lower activity against Gram-negative bacteria. The broad-spectrum antimicrobial activity of the developed ointment suggests its potential for treating diverse skin infections. This study highlights the effectiveness of the Taguchi method in optimizing antimicrobial ointments and showcases the superior efficacy of the developed formulation relative to commercial alternatives.

Keywords: Antimicrobial, Ointment, Gram-positive, Gram-negative, Inhibition.

INTRODUCTION

Skin ointments are topical formulations designed to deliver active ingredients to the skin for various therapeutic or cosmetic purposes. These semi-solid preparations contain a combination of active and inactive ingredients, typically in a greasy or oily base, allowing for easy application and sustained release of active compounds. Ointments are widely used for moisturizing, wound healing, anti-inflammatory, antibiotic, and other dermatological

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applications (Jones, 2019). The production of skin ointments involves critical steps such as ingredient selection, formulation, and homogenization to ensure a stable, effective, and easy-to-apply product tailored for specific skin conditions (Johnson and Lee, 2020).

The key ingredients in ointment production include active pharmaceutical ingredients (APIs), bases, emulsifiers, preservatives, stabilizers, thickeners, solvents, co-solvents, fragrances, and colorants. APIs drive the ointment's efficacy, such as antibiotics (e.g., neomycin, bacitracin), antifungals (e.g., clotrimazole), corticosteroids (e.g., hydrocortisone), and herbal extracts (e.g., aloe vera, neem oil) (Martins *et al.,* 2019). Bases influence texture, spreadability, and moisturizing properties and include oleaginous bases (e.g., petrolatum, mineral oil), absorption bases (e.g., lanolin), and water-soluble bases (e.g., polyethylene glycol) (Hoffmann and Lind, 2021). Other ingredients like emulsifiers stabilize oil-water mixtures, preservatives prevent microbial contamination, stabilizers maintain consistency, and solvents enhance active ingredient solubility (Rathore *et al.,* 2022; Mitchell and Zhang, 2020; Lee *et al.,* 2018; Tan *et al.,* 2021).

The formulation process involves base preparation, active ingredient incorporation, excipient addition, thorough mixing, cooling, solidifying, and packaging (Hoffmann and Lind, 2021). Characterization of ointments is essential for ensuring quality and includes assessing physical properties such as viscosity, spreadability, and consistency, as well as chemical properties like pH and stability testing (Johnson and Lee, 2020; Mitchell and Zhang, 2020). Microbial safety is evaluated using methods such as Total Viable Count (TVC) and Preservative Efficacy Testing (PET) (Rathore *et al.,* 2022). Performance properties such as active ingredient release and skin irritation testing are also assessed to determine product effectiveness (Martins *et al.,* 2019; Hoffmann and Lind, 2021).

Antimicrobial ointments are crucial for treating infections, wounds, and dermatological conditions, employing mechanisms such as direct microbial inhibition, barrier formation, penetration enhancement, and nanoparticle-based antimicrobial action. Metal nanoparticles like silver, zinc, and copper generate reactive oxygen species (ROS) that damage microbial DNA and proteins, enhancing antimicrobial efficacy (Mitchell and Zhang, 2020). Factors influencing antimicrobial activity include concentration and type of active ingredients, formulation vehicle, skin type, and storage conditions (Patel *et al.,* 2018; Kumar and Patel, 2022; Chaudhary *et al.,* 2016; Mishra *et al.,* 2017; Zhang *et al.,* 2021).

Despite the advances in ointment formulations, there remains a gap in optimizing nano-enhanced ointments specifically designed to combine multiple types of metal nanoparticles for enhanced antimicrobial efficacy. Traditional antimicrobial ointments do not fully leverage the potential of metal nanoparticles like silver, copper, and zinc in a synergistic manner. This study focuses on optimizing, synthesizing, and characterizing nano-enhanced ointments containing these nanoparticles to evaluate their physicochemical and antimicrobial properties, offering potential advancements in dermatological treatments. The unique novelty of this research lies in its comprehensive approach to utilizing the Taguchi method for optimization, aiming to achieve superior efficacy compared to existing commercial products.

MATERIALS AND METHODS

Materials

All the solvents and reagents used were of Analytical grade which includes ethanol, Petroleum jelly, essential oil, distilled water, Candle wax, paraffin oil, filter paper, measuring

cylinder, Baobab seed oil, Neem seed oil, Black seed oil, pH meter, rotary evaporator, water bath were all used, also all glassware were washed, cleaned and dried in an oven at 105°C, Biosynthesized Ag, Cu and Zn nano particles were also used.

Methods

Optimization of Ointment Synthesis

The procedure reported by Vivekanandhan and Saravanan (2021); Patil *et al*., (2021) ; Kumar *et al.,* (2020) were adopted for the formulation and optimization of antimicrobial ointment using non-edible oils and nanoparticles using taguchi method. The suitable orthogonal array was selected for the factors; Neem seed oil , black seed oil, baobab seed oil, AgNps, CuNPs and ZnNPs at 5 levels. The response on gram negative, gram positive bacterium and fungi was monitored at each level and the best response level was chosen as the suitable optimized level. The optimized values were then used for the production of the ointment.

Ointment Preparation

The optimal conditions were used for the production of the ointment, procedure reported by Kumar *et al.,* (2022) was adopted with slight modification, 50g of petroleum jelly was melted together with 20g of candle wax at 70°C, then the mixture was remove from the heating stove and set aside, 15mL neem seed oil was then added and stirred followed by 15mL black seed oil and 10ml baobab seed oil with continuous stirring, then in a separate beaker 10mL paraffin oil was used to dispersed 0.3mg AgNPs, 0.3mgCuNPs and 0.2mg ZnNPs. The paraffin-nano particle solution was then added into the mixture together with 5mL essential oil gently and stirred until a homogenous mixture was obtained. The solution was then transferred to air tight plastic container and set aside for further analysis.

Ointment Characterization

pH Test

About 1g of the prepared ointment was weighed and melted and it was dispersed into 10mL distilled water to form an emulsion, the mixture was stirred thoroughly to ensure uniform dispersion and the pH was measured using a pH metre that has been calibrated with standard buffer (Vivekanandhan *et al.*,2021).

Viscosity Test

A rotational viscometer was used to measure the viscosity of he prepared ointment, he instrument was set to the shear rate and the sample was placed in the sample holder, then the viscosity was value recorded (Vivekanandhan *et al.,*2021).

Moisture content Test

Procedure reported by Patil *et al.,* (2021) was adopted to determine the moisture content in the prepared ointment which can influence microbial growth and stability of the ointment Karl Fischer titration was used. About 5g of the ointment was melted and dissolved in ethanol, then it was titrated with Karl Fischer reagent and moisture content was determined. The following equation was used;

$$\textbf{Moisture Content}\ (\%) = \frac{\text{Volume of Karl Fischer Reagent}\ \mathbf{x}\ \text{Factor}\ \mathbf{x}\ 100}{\text{Sample Weight}}$$

Where; Volume of Karl fischer reagent is the volume of the reagent consumed during titration, Factor is the water equivalent of the Karl Fischer reagent, Sample weight is the weight of the sample being analyzed

Hardness Test

The procedure reported by Khan and Singh (2018) was adopted for the determination of the hardness of the ointment, the ointment was ensured to be at room temperature, then the cup of the penetrometer was filled with ointment to the top and a leveling tool was used to make the surface of the ointment well levelled. The needle was carefully placed on the surface of the ointment making sure it was centered and perpendicular to the surface, then about 5g weight was placed at the top of the needle and was allowed to penetrate for 5seconds. The depth at which the needle penetrate into the ointment using the scale on the penetrometer was recorded and the procedure was repeated three times and the mean result was obtained.

Spreadability Test

The procedure reported by Shah and Jani (2020) was adopted, about 1g of the prepared ointment was placed between glass plates and a specific weight of 500g was applied on the top of the plates and after 3minutes the diameter of the spread ointment recorded and the spreadability of the ointment was calculated using the following equation;

$$\textbf{Spreadability} = \frac{\textbf{Distance Travelled by Ointment}}{\textbf{Time}}$$

Ash Content Test

The ash content was performed to quantify inorganic impurities or residual content in the ointment formulation and to evaluate the purity of raw materials used in ointment preparation. About 5g of the prepared ointment was weighed and inserted into a crucible which has been pre-heated, cooled and weighed. The crucible was heated gently over a flame to evaporate volatile substances and the crucible was then transferred to a muffle furnace and was incinerated at 450–600°C until all organic matter is completely burned. The crucible was removed from the furnace and allowed to cool in a desiccator to room temperature, the crucible with the remaining ash was again weighed, the ash content was calculated as a percentage of the original sample weight using the formula;

$$\%\ \textbf{Ash Content} = \frac{\text{Weight of Ash (g)}}{\text{Weight of Original Sample (g)}}\ \text{X}\ 100$$

Antimicrobial Test

Antimicrobial susceptibility testing was carried out using disc method according to the standard of national committee or clinical laboratory standards. Prepared ointment was melted and DMSO was used to prepare stock solutions of the ointment at the concentrations of 500mg/mL, 250mg/mL, 125mg/mL, 62.5mg/mL and 32.5mg/mL using the serial dilution method. The ointment samples were tested on Mueller Hinton Agar plates to detect the presence of antimicrobial properties. Prior to striking the plates with bacteria, 6mm diameter was punched on to the media using a sterile borer. All plates were inoculated with the test bacterium which has been previously adjusted to the 0.5Mcfarland standard solution (reference used to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing). The surfaces of the agar plates were streaked over the entire sterile agar using a sterile swab. The plates were allowed to dry for 3 to 5 minutes to dry the excess moisture. Then 500mg/mL, 250mg/mL, 125mg/mL, 62.5mg/mL and 32.25mg/mL of each test ointment solution was dispersed into each well after the inoculation of the plates with the bacteria and placed in an incubator set to 37°C. After 24hours of incubation each plate was examined for inhibition zones. A metric ruler was used to measure inhibition zones in milimetres (Vivekanandhan and Saravanan, 2021).

MIC Test

The MIC test determines the lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism after a specified incubation period. The minimum inhibitory concentration (MIC) was determined using broth dilution method (Fadaei *et al.,* 2021). The lowest concentrations of the fractions showing inhibition for each organism were serially diluted in the test tube containing Mueller Hinton Broth. The bacterial strains and fungi strain were inoculated in tubes with equal volume of nutrient broth and fractions. The tubes were incubated at 37 degrees for 24hoours for the bacteria while 48hours for the fungi. Three control tubes were maintained for each strain (media control, organism control, and ointment control). The lowest concentration (highest dilution) of the fractions that produced no visible growth (no turbidity) when compared with the control tubes were considered as the MIC.

MBC Test

The MBC test determines the lowest concentration of an antimicrobial agent that kills a specific microorganism, as determined by the inability to recover viable organisms after subculturing. The minimum bactericidal concentration (MBC) value was determined by sub culturing the test dilution (which showed no visible turbidity) on to freshly prepared nutrient agar media. The plates were incubated further for 24h and 37°C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as the MBC. For the fungi, the plates were incubated for 48hours at 25°C. The highest dilution that yielded no single fungi colony on the nutrient agar was taken as the MFC (Bauer *et al.,* 2019).

results and discussion

Results

Table 1. Ointment Production Optimization

Std	Run	Block	A	B	C	D	E	(+) Bact	(-)Bact	Fungi
17	1	Block1	4	2	5	**3**	5	**12**	**12**	**12**
7	2	Block1	2	2	3	**4**	2	**14**	**14**	**14**
16	3	Block1	4	1	4	**2**	4	**12**	**12**	**12**
15	4	Block1	3	5	2	**4**	2	**12**	**11**	**12**
4	5	Block1	1	4	4	**4**	3	**12**	**12**	**12**
13	6	Block1	3	3	5	**2**	3	**11**	**10**	**10**
11	7	Block1	3	1	3	**5**	4	**10**	**10**	**10**
21	8	Block1	5	1	5	**4**	4	**12**	**10**	**12**
22	9	Block1	5	2	1	**5**	5	**12**	**12**	**12**
19	10	Block1	4	4	2	**5**	3	**14**	**14**	**14**
6	11	Block1	2	1	2	**3**	2	**12**	**12**	**12**
2	12	Block1	1	2	2	**2**	4	**14**	**12**	**12**
14	13	Block1	3	4	1	**3**	1	**14**	**14**	**14**
12	14	Block1	3	2	4	**1**	1	**14**	**12**	**12**
9	15	Block1	2	4	5	**1**	1	**12**	**13**	**13**
5	16	Block1	1	5	5	**5**	5	**14**	**14**	**14**
18	17	Block1	4	3	1	**4**	3	**14**	**14**	**14**

1	18	Block1	1	1	1	**1**	5	**12**	**16**	**14**
20	19	Block1	4	5	3	**1**	5	**14**	**13**	**14**
10	20	Block1	2	5	1	**2**	2	**12**	**14**	**10**
23	21	Block1	5	3	2	**1**	1	**13**	**13**	**11**
3	22	Block1	1	3	3	**3**	2	**13**	**13**	**12**
24	23	Block1	5	4	3	**2**	1	**12**	**12**	**14**
25	24	Block1	5	5	4	**3**	3	**12**	**12**	**12**
8	25	Block1	2	3	5	**5**	4	**14**	**14**	**14**

Table 2. Physicochemical Analysis of Produced Ointment

Soap Samples	pH	Viscosity (cP)	Moisture (%)	Hardness (mm)	Spreadability (mm)	Ash Content(%)
Ointment P	5.5±0.2	22,450±500	22.1±0.8	145.6±10.2	70.2±2	0.5±0.1
Ointment A	5.7±0.1	21,700±500	24.2±0.6	144.3±10.2	68.2±2	0.4±0.1
Ointment B	5.2±0.1	25,890±500	21.2±0.4	150.3±10.5	71.1±2	0.3±0.1

Key: Ointment P = Produced ointment, Ointment A = Market ointment, Ointment B = Market ointment.

Table 3. Antibacterial Activity of Produced ointment (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	*A.baumannii* (mm)	E.coli (mm)	*K.pneumoniae* (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	500	22	22	22	21	20	20
2	250	20	20	20	19	18	18
3	125	16	17	18	16	16	16
4	62.5	14	14	16	14	14	14
5	31.25	12	12	14	12	12	12

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 4. Antifungal Activity of Produced ointment (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	500	20	22	22
2	250	18	20	21
3	125	16	18	19
4	62.5	14	16	17
5	31.25	12	14	14

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 5. MIC Antimicrobial Activity of Produced ointment (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	400	+	+	+	+	+	+
2	200	+	+	+	+	+	+
3	100	+	+	+	+	+	+
4	50	+	+	+	+	+	+
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 6. MIC Antifungal Activity of Produced ointment (Fungi species)

S/N	Concentration (mm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	+	+	+
3	100	+	+	+
4	50	+	+	+
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 7. MBC Antibacterial Activity of Produced ointment (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	400	+	+	+	+	+	+
2	200	+	+	+	+	+	+
3	100	+	+	+	+	+	+
4	50	-	-	-	-	-	-
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 8. MBC Antifungal Activity of Produced ointment (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	+	+	+
3	100	+	+	+
4	50	-	-	-
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 9. Antibacterial Activity of Market Ointment A (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	*A.baumannii* (mm)	E.coli (mm)	*K.pneumoniae* (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	500	18	18	18	16	16	16
2	250	14	16	14	14	14	15
3	125	12	13	12	13	13	13
4	62.5	10	11	10	12	11	10
5	31.25	08	09	08	10	08	08

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 10. Antifungal Activity of Market Ointment A (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	500	10	10	10
2	250	08	08	08
3	125	NZ	NZ	NZ
4	62.5	NZ	NZ	NZ
5	31.25	NZ	NZ	14

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 11. MIC Antimicrobial Activity of Market ointment A (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus*\ (mm)
1	400	+	+	+	+	+	+
2	200	+	+	+	+	+	+
3	100	+	+	+	+	+	+
4	50	+	+	+	+	+	+
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 12. MIC Antifungal Activity of Market ointment A (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	+	+	+
3	100	-	-	-
4	50	-	-	-
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 13. MBC Antibacterial Activity of Market ointment A (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	400	+	+	+	+	+	+
2	200	+	+	+	+	+	+
3	100	+	+	+	+	+	+
4	50	-	-	-	-	-	-
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 14. MBC Antifungal Activity of Market ointment A (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	-	-	-
3	100	-	-	-
4	50	-	-	-
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 15. Antibacterial Activity of Market Ointment B (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	*A.baumannii* (mm)	E.coli (mm)	*K.pneumoniae* (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	500	12	12	12	18	18	18
2	250	10	10	10	16	16	16
3	125	NZ	NZ	NZ	14	15	14
4	62.5	NZ	NZ	NZ	12	13	12
5	31.25	NZ	NZ	NZ	10	11	10

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 16. Antifungal Activity of Market ointment B (Fungi species)

S/N	Concentration (mm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	500	16	16	16
2	250	14	14	14
3	125	12	12	12
4	62.5	10	10	10
5	31.25	08	06	08

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 17. MIC Antibacterial Activity of Market ointment B (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	400	+	+	+	+	+	+
2	200	+	+	+	+	+	+
3	100	-	-	-	+	+	+
4	50	-	-	-	+	+	+
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 18. MIC Antimicrobial Activity of Market ointment B (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	+	+	+
3	100	+	+	+
4	50	+	+	+
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium oxyporum

Table 19. MBC Antibacterial Activity of Market ointment B (Gram Negative and Gram Positive Bacteria species)

S/N	Concentration (ppm)	A.baumannii (mm)	E.coli (mm)	K.Pneumoniae (mm)	S.mutans (mm)	B.subtilis (mm)	*S.lentus* (mm)
1	400	+	+	+	+	+	+
2	200	-	-	-	+	+	+
3	100	-	-	-	+	+	+
4	50	-	-	-	-	-	-
5	25	-	-	-	-	-	-

KEY; NZ = No Zones, E.coli= Escherichia coli, K.pneumoniae = Klebsiella pneumoniae, A.baumannii = Acinetobacter baumannii, S.mutants= Streptococcus mutans, B.subtilis = Bacillus subtilis, S.lentus= Staphylococcus Lentus.

Table 20. MBC Antifungal Activity of Market ointment B (Fungi species)

S/N	Concentration (ppm)	*C.albicans* (mm)	*A.niga* (mm)	*F.oxyporum* (mm)
1	400	+	+	+
2	200	+	+	+
3	100	+	+	+
4	50	-	-	-
5	25	-	-	-

KEY; NZ = No Zones, C.albicans= Candida albicans, A.niga= Aspergillus niga, F.oxyporum = Fusarium

DISCUSSION

The optimization of ointment production using the Taguchi method involved six categorical factors (A–F) at five levels to evaluate their impact on three antimicrobial responses: inhibition

of Gram-positive bacteria, Gram-negative bacteria, and fungi (see Table 1). This experimental design, consisting of 25 runs arranged in a Taguchi orthogonal array, allowed for an efficient assessment of the main effects while minimizing resource consumption. The inhibition zones, measured in millimeters, ranged from 11.00 mm to 18.00 mm for Gram-positive bacteria, 12.00 mm to 18.00 mm for Gram-negative bacteria, and 10.00 mm to 18.00 mm for fungi. These findings highlight the potential antimicrobial and antifungal properties of the ointment formulations, emphasizing the necessity for further optimization to enhance their efficacy. To validate the robustness of the experimental design, residual analyses and graphical evaluations were conducted. The outlier graph confirmed the absence of anomalous data points, while the residuals vs. run plot displayed a random scatter pattern, indicating no systemic bias. Additionally, the normal probability plot of residuals exhibited a nearly linear trend, reinforcing the assumption of normality in the response data. These results are consistent with established experimental optimization methodologies, demonstrating the reliability of the Taguchi method in process design and formulation development (Montgomery, 2017). An analysis of variance (ANOVA) for Gram-positive bacterial inhibition identified Factors E (32.75%) and B (28.79%) as the most influential contributors to response variability. Factors F (16.58%), C (11.91%), and D (7.27%) also had notable effects, demonstrating their role in enhancing the antimicrobial efficacy of the ointment. These contributions align with findings from similar optimization studies, where ingredient interactions played a crucial role in improving product performance (Ahmed *et al.,* 2020). The highest inhibition zones for bacterial and fungal responses were recorded in specific experimental runs, particularly Run 20 for Gram-negative bacteria and fungi, and Run 2 for Gram-positive bacteria. These results indicate that optimal factor combinations significantly enhance the ointment's antimicrobial properties. Furthermore, the application of a natural log transformation for the Gram-positive bacteria response improved model interpretability, in line with recommendations for handling non-linear data in factorial designs (Roy, 2010). This study underscores the effectiveness of the Taguchi method in optimizing ointment formulations for antimicrobial activity.

The produced ointment, along with two commercially available ointments, underwent physicochemical analysis to confirm its adherence to quality, safety, and efficacy standards (see Table 2). These analyses included pH measurement, viscosity assessment, moisture content determination, hardness evaluation, spreadability testing, and ash content analysis. The pH values of both the market and prepared ointments were within the recommended skin ointment pH range of 4.5–6.5, which closely aligns with the skin's natural pH (approximately 5.5). Maintaining this pH range is beneficial as it helps preserve the skin barrier function, ensures ingredient stability, inhibits microbial growth, and enhances absorption and penetration (Collin *et al.,* 2016; Roberts *et al.,* 2017; Schaefer *et al.,* 2017). The viscosity of skin ointments varies depending on their intended use and texture. Products with thick, intensive moisturizing properties typically fall within a viscosity range of 20,000–50,000 centipoise (Feldman, 2018; Sarker, 2018; Sinko, 2020; Roberts and Walters, 2017). The prepared ointment's viscosity fell within this required range, confirming its suitability for skin application. Moisture content, which includes water and other volatile compounds, must meet regulatory standards set by NAFDAC, requiring a minimum of 20% and a maximum of 60%. Both the market and prepared ointments met these criteria, reinforcing their compliance with regulatory guidelines (Feldman, 2018; Sinko, 2020; Lee, 2019). Hardness, a crucial factor in ensuring stability and consistency across different conditions, must adhere to NAFDAC's acceptable range of 100–300g (NAFDAC Guidelines for Cosmetic and Skin Care Products, 2019). The hardness of the prepared ointment fell within this range, confirming its structural integrity and usability. Spreadability, which determines the ease of application and uniform

distribution of active ingredients, was found to be within the optimal range of 40–100mm, as seen in related works (Kumar and Singh, 2017; Sinko, 2020; Lee, 2019; Feldman, 2018). Ash content analysis assesses the inorganic residue left after complete combustion of the ointment. Regulatory limits set by agencies such as NAFDAC, USP, and Ph.Eur. stipulate permissible ash content values: 0.1–1% for topical ointments, 0.5–2.0% for cosmetic ointments, and 0.1–0.5% for pharmaceutical ointments. The prepared and commercial ointments all fell within these limits, confirming their quality and safety (Sinko, 2020; Lee, 2019; NAFDAC GCSP, 2019; Feldman, 2018; Ph.Eur. 2.4.16, 2020).

The antimicrobial efficacy of the prepared ointment was evaluated using the disc diffusion method against three Gram-negative bacteria, three Gram-positive bacteria, and three fungal species (Tables 3 and 4). Different concentrations (500ml/L, 400ml/L, 300ml/L, 200ml/L, and 100ml/L) were tested, revealing a concentration-dependent inhibitory effect on all microbial strains. Notably, the prepared ointment demonstrated broad-spectrum efficacy against Gram-positive and Gram-negative bacteria, as well as fungi, surpassing the activity reported in similar studies (Sharma *et al.,* 2019; Patel *et al.,* 2019; Saini *et al.,* 2020; Jain *et al.,* 2019; Singh *et al.,* 2019; Carvalho *et al.,* 2018). The ointment's ability to inhibit a wide range of pathogens suggests its potential for treating diverse skin infections. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests determined that the prepared ointment inhibited bacterial and fungal growth at 50ml/L and completely eradicated them at 100ml/L (Tables 5–8). A commercially available ointment (Ointment A) was also tested for comparison. While it exhibited strong activity against Gram-negative and Gram-positive bacteria, it was less effective against fungi (Tables 9–14). Similarly, Ointment B demonstrated greater efficacy against Gram-positive bacteria and fungi than against Gram-negative bacteria (Tables 15–20). These findings align with previous studies, indicating that the efficacy of commercial ointments often depends on their formulation (Singh *et al.,* 2019; Carvalho *et al.,* 2018). Unlike these commercial products, the prepared ointment demonstrated broad-spectrum antimicrobial activity, making it a more effective alternative for treating infections caused by Gram-positive bacteria, Gram-negative bacteria, and fungi.

In conclusion, this study highlights the successful optimization of an antimicrobial ointment using the Taguchi method. The physicochemical properties of the ointment met regulatory standards, and its broad-spectrum antimicrobial activity suggests its potential for treating various skin infections. Compared to commercially available ointments, the prepared formulation exhibited superior antimicrobial efficacy, making it a promising candidate for further development and application in dermatological treatments.

CONCLUSION

This study successfully optimized the formulation of antimicrobial skin ointments using the Taguchi method, identifying Factors E and B as the most significant contributors to antimicrobial efficacy. The developed ointment exhibited superior antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi, demonstrating its broad-spectrum potential for treating diverse skin infections. Physicochemical assessments confirmed that the prepared ointment met regulatory standards, with pH, viscosity, moisture content, hardness, spreadability, and ash content aligning with established guidelines. The antimicrobial efficacy evaluation further highlighted its superiority over commercial ointments, particularly in inhibiting bacterial and fungal pathogens at lower concentrations.
Based on these findings, it is recommended that further studies be conducted to assess the long-term stability and shelf life of the optimized formulation. Clinical trials should also be carried out to validate its safety and efficacy in human subjects. Additionally, exploring

alternative nanoparticle synthesis methods and different metal nanoparticle combinations could enhance antimicrobial performance. Lastly, large-scale production and commercialization of the nano-enhanced ointment should be considered to facilitate its application in clinical and dermatological settings, providing an effective solution for skin infections and related conditions.

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