Formulation of *Vernonia amygdalina* and *Calotropis procera* leaf extracts into a cream for the management of skin infections

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ABSTRACT

Background: The incidence of skin infections in West Africa and in fact globally continues to increase at an alarming rate.

Objective: This study was carried out to formulate the methanol extracts from *Vernonia amygdalina* and *Calotropis procera* into a topical cream for the treatment and management of skin infections.

Methods: Each extract was tested and thereafter combined in ratios 0:1, 1:3, 1:1, 3:1 and 1:0 *Vernonia: Calotropis* (V:C). The best extract combination based on results of zones of inhibition was then formulated into modified aqueous, cetomacrogol and vanishing cream bases. The creams were formulated at an extract concentration of 2.5, 5 and 10% w/w in the bases. The creams were further assessed for their physical and chemical properties. Antimicrobial activities of the creams were examined by the agar well diffusion method against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, and Trichophyton rubrum*. A combination of ketoconazole and neomycin was used as control.

Results: The creams exhibited zones of inhibition ranging from 8.00 ± 0.54 to 30.00 ± 0.00 mm. The extracts combined in ratio 3:1 V:C yielded the highest zones of inhibition ordinarily and in the cream across the test organisms. The cetomacrogol base was incompatible with the extract combinations, therefore unstable; the modified Aqueous cream base was stable with no antimicrobial activity while the vanishing cream base gave a stable cream with zones of inhibition against the test organisms.

Conclusion: These outcomes confirm that these extracts possess antimicrobial activities, and when incorporated into creams, their activities vary according to the cream bases employed with the vanishing cream base being the most effective.

Keywords: *Vernonia amygdalina, Calotropis procera*, antimicrobial, creams, skin infections

Formulation d'extraits de feuilles *Vernonia amygdalina* et *Calotropis procera* en crème pour la prise en charge d'infections cutanées

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RESUME

Contexte: l'incidence des infections de la peau en Afrique de l'Ouest et, en fait, sur le plan mondial, s'accroit à un rythme alarmant.

Objectif: Cette étude a été réalisée pour formuler les extraits de méthanol de *Vernonia amygdalina* et *Calotropis procera* en une crème topique pour le traitement et la prise en charge des infections cutanées.

Méthodes: Chaque extrait a été testé et ensuite combiné en rapports 0:1, 1:3, 1:1, 3:1 et 1:0 de *Vernonia: Calotropis* (V:C). La meilleure combinaison d'extraits basée sur les résultats des zones d'inhibition a ensuite été formulée dans des bases aqueuses modifiées de Cetomacrogol et de bases de crème de jour. Les crèmes ont été formulées par la suite à une concentration d'extrait de 2,5, 5 et 10% p/p dans les bases. Puis, les crèmes ont été évaluées pour leurs propriétés physiques et chimiques. Les activités antimicrobiennes des crèmes ont été examinées par la méthode de diffusion de gélose à l'agar contre *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* et *Trichophyton rubrum*. Une combinaison de ketoconazole et de néomycine a été utilisée comme témoin.

Résultats: Les crèmes ont montré des zones d'inhibition allant de 8,00 ± 0,54 à 30,00 ± 0,00 mm. Les extraits combinés en rapport 3:1 V:C ont donné les zones les plus élevées d'inhibition ordinairement et dans la crème à travers les organismes d'essai. La base de Cetomacrogol était incompatible avec les combinaisons d'extrait, donc instable; la base de crème aqueuse modifiée était stable sans activité antimicrobienne tandis que la base de crème de jour donnait une crème stable avec des zones d'inhibition contre les organismes d'essai.

Conclusion: ces résultats confirment que ces extraits possèdent des activités antimicrobiennes et, lorsqu'ils sont incorporés dans des crèmes, leurs activités varient en fonction des bases de crème employées avec la base de crème de jour comme étant les plus efficaces.

Mots-clés: *Vernonia amygdalina, Calotropis procera*, antimicrobien, crèmes, infections cutanées

INTRODUCTION

The skin is the largest organ of the body and constitutes the first line of defense against threats from the environment. It is made up of cells specialized for that purpose, comprising three layers; the epidermis, dermis and hypodermis.[1] The skin is therefore more exposed and susceptible to physical injuries and microbial attacks, as it serves to protect the body and prevent excessive water loss.[1] Skin diseases are one of the most common diseases affecting humans. It is responsible for illness in individuals of all ages, genders, cultures, with prevalence as high as 70%, especially in populations at risk.[2,3]Skin diseases can result in physical damages as well as death in very complicated cases.[4] Based on this, it is now ranking the fourth leading cause of nonfatal disease burden in the world today.[2] According to the International Classification of Diseases, skin diseases account for up to 1000 of the conditions on the list.[2] This goes to emphasize the global health burden attributable to skin diseases. However as disturbing as its epidemiology appears, skin diseases have still received less attention on both national and global health scenes.[2]There is a compelling need therefore, to deploy more research focus and health resources to see to the amelioration of the destructive impacts of skin diseases, especially in Africa. In Africa, particularly Nigeria, the standard of nutrition, health infrastructure and environmental sanitation is declining by the day contributing significantly to the intensity of the damages caused by skin diseases.[5,6] This demands that research communities in these greatly disturbed societies seek better and effective solutions to enhance the treatment and management of skin infections.

Despite the non-debatable efficacies of synthetic drugs in the management of various medical conditions, their side effects and challenges of affordability remain limitations that cannot be neglected, thereby causing a rapidly growing interest in natural remedies.[7] Plants and plant derived metabolites are becoming a better alternative in disease management due to their availability, reduced tendencies of adverse effects and a potency that is comparable to that of their synthetic counterparts.[8] There is an abundance of medicinal plants on the earth surface today and this has increased the interest in investigation into these plants and their extracts as candidates for new antimicrobial agents, as needed in the treatment of skin infections.[9] *Vernonia amygdalina* and *Calotropis procera* are widely available plants in West Africa and are been studied for their potentials in the management of skin infections.

Vernonia amygdalina is of the family Asteraceae.[10] It is commonly called bitter leaf (due to its bitter taste) and is consumed after extraction as a tonic based on ethnomedicinal claims for the treatment of various ailments or used as food condiments in local diets.[11] The leaf extract of *Vernonia amygdalina* is used in medicine as an antimalarial, antimicrobial, laxative, antihelminthic, and antithrombotic. In addition, both hypoglycemic and hypolipidaemic effects have been reported for *Vernonia* in diabetic hyperlipidemic and normoglycemic hyperlipidemic rats.[10] Phytochemical screening of the plant confirms the presence of saponins, glycosides and tannins as responsible for pugative effects, flavones for antioxidant activity and may be related to its actions against diabetes and artherosclerosis.[12]

Calotropis procera (Sodom apple) is a member of the plant family Asclepiadaceae, a shrub widely distributed in West Africa and other parts of the tropics.[13] Various parts of the plant contain cardenolide glycosides, calotropin, calotoxin, uscharin and uscharidin, and a base, choline. The plant also contains *o*-pyrocatechuic acid; cardenolides (calotropin, calotoxin, uscharin and uscharidin).[14] It has been used as a purgative, anti-helminthic, digestive, emetic, expectorant, sedative, blood purifier, and an antidote for snake poisoning and for the treatment of ulcers, tumors, leprosy, asthma, boils, dysentery, eczema, piles and diseases of liver, abdomen and spleen.[15]

The extracts of these plants in combination can be promising in the design of a pharmaceutical remedy for skin diseases due to their individual reputations. It is therefore the focus of this study to formulate the extracts into a cream for the management of skin infections. There are varieties of cream bases available for topical designs. The kind and nature of base used in formulating a topical dermatologic product greatly affects its effectiveness.[16] The choice of a cream base depends on the action desired, the nature of the active drug to be incorporated, its bioavailability and stability, and the requirement of shelf life of the finished formulation.[17]

MATERIALS AND METHODS

Leaves of *Vernonia amygdalina* and *Calotropis procera* (extract source) collected from Ilorin, in March, 2016 and authenticated at the herbarium of the Department of Plant Biology, University of Ilorin, Nigeria where voucher numbers UILH/001/1023 and UILH/002/962 were deposited respectively. Stock cultures of; *Staphylococcus aureus*, *Pseudomonas aeruginosa,*

Candida albicans, Aspergillus niger and Trichophyton rubrum (test organisms) obtained from the Department of Pharmaceutical Microbiology, University of Ilorin. Methanol (Guangdong Chemical Reagent, China) for extraction, liquid paraffin, white soft paraffin (BDH Chemicals Ltd., Poole England) for the cream bases, Sabouraud dextrose agar (SDA), nutrient agar (Biomalk, India) for agar diffusion tests.

Extraction of Plant

Extraction was done based on the method described by Adebayo and co-workers, 250 g of the powdered leaves of *Vernonia amygdalina* was macerated in 70% methanol and allowed to stand for four hours.[21] The mixture was decanted and more solvent added. This process was repeated three times and the final suspension was filtered through a large cloth sieve. The extract was then concentrated using a rotary evaporator and the resulting concentrate was freeze dried. The dried extract was then stored in a specimen bottle for later use. This procedure was repeated for *Calotropis procera*.

Antimicrobial Activity of Extract

Sensitivity tests were conducted on test organisms using varying concentrations (50 to 200 mg/mL) each of *Vernonia amygdalina* and *Calotropis procera.* The Agar Well Diffusion method as described by Russel and Furr with a little adjustment was used.[22] Based on the result of this sensitivity test, the extract concentration of 200 mg/mL of *Vernonia amygdalina* and *Calotropis procera* was selected for the antimicrobial activity test. For this test, 200 mg/mL of *Vernonia amygdalina*, 200 mg/mL of *Calotropis procera*, and combinations of these extracts in the ratio 3:1, 1:3 and 1:1 at a concentration of 200 mg/mL were tested against *Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus niger*, *Candida albicans* and *Trichophyton rubrum* using the agar well method. Griseofulvin was used as the positive control for the fungal tests; Amoxicillin/Clavulanate was used as positive control for the bacterial tests and 30% DMSO solution as the negative control.

Cream Formulation

Specific quantities of the extract were incorporated into the cream base by trituration in a mortar with a pestle.[24, 25] This process was repeated for each extract at predetermined concentrations in the cream base (Table 1). Each cream was scooped into an air tight cream jar and stored for further analysis and tests. Three concentrations of extract in the cream were used; 2.5, 5 and 10% w/w. For each of these concentrations, *Vernonia amygdalina* and *Calotropis procera* were combined at a ratio of 3:1 *Vernonia: Calotropis* (V:C) based on results of preliminary studies on the extract.[26] Also, creams containing 100% of each of the extracts were formulated.

Based on the preliminary results obtained on examination of the modified aqueous cream base (B.P) formulated creams, the extracts were formulated at the same concentrations of 2.5, 5 and 10% w/w in a vanishing cream base. The vanishing cream base, which was prepared by melting the oily ingredients (stearic acid 22.5%, triethanolamine 1.5%) to form the oily phase, heating the aqueous phase (glycerin 6%, potassium hydroxide 1%, water 69%) and mixing the phases on attainment of uniform temperature, is void of soft and liquid paraffin. The performances of these creams of different bases were thereafter evaluated and compared.

Table 1: Composition of creams formulated in aqueous cream and vanishing cream base

Creams	Constituents
Cream A	Vanishing Cream Base 20 g
10% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 7.5 mL
	Calotropis Extract 2.5 mL
Cream B	Vanishing Cream Base 20 g
5% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 3.75 mL
	Calotropis Extract 1.25 mL
Cream C	Vanishing Cream Base 20 g
2.5% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 1.8 mL
	Calotropis Extract 0.7 mL
Cream D	Vanishing Cream Base 10 g
10% w/w Extract with V:C ratio at 1:0	*Vernonia* Extract 5 mL
Cream E	Vanishing Cream Base 10 g
10% w/w Extract with V:C ratio at 0:1	*Calotropis* Extract 5 mL
Cream F	Vanishing Cream Base 10 g
5% w/w Extract with V:C ratio at 1:0	*Vernonia* Extract 2.5 mL
Cream G	Vanishing Cream Base 10 g
10% w/w Extract with V:C ratio at 0:1	*Calotropis* Extract 2.5 mL
Cream X	Aqueous Cream BP 20 g
10% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 7.5 mL
	Calotropis Extract 2.5 mL
Cream Y	Aqueous Cream BP 20 g
5% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 3.75 mL
	Calotropis Extract 1.25 mL
Cream Z	Aqueous Cream BP 20 g
2.5% w/w Extract with V:C ratio at 3:1	*Vernonia* Extract 1.8 mL
	Calotropis Extract 0.7 mL

Legend:
V:C – Ratio of Vernonia amygdalina extract to Calotropis procera extract

Evaluation of Creams

Physical Characterization

Organoleptic parameters such as appearance, colour, odour and texture were evaluated for each of the formulated creams. In addition, other physical evaluations such as ease of application of cream, ease of removal of cream, and feel of cream on the skin were conducted. The viscosities of the formulations were also determined by a NDJ-5S Digital Display Viscometer (Rinch, China).

pH Determination

The pH of each cream formulation was determined by a pH meter (Hanna, England). Each test was conducted in triplicates.

Stability Studies

All the developed formulations were subjected to stability testing for about three weeks. They were examined daily for physical deterioration and microbial contamination over this period. Room temperature of 28 °C was maintained throughout. Parameters of phase separation, colour, and texture were evaluated.

Microbiological Activity of Creams

The antimicrobial activity of each cream was determined by the Agar Well Diffusion method.[24] Nutrient Agar plates were streaked with standardized bacteria inoculums of *Staphylococcus aureus* and *Pseudomonas aeruginosa* while prepared SDA plates were streaked with fungal inoculums of *Candida albicans* and *Trichophyton rubrum*. Wells were thereafter bored into these holes with a 5 mm sterile cork borer to allow five holes on each plate. The bases of these wells were sealed with the appropriate agar preparation. The wells were thereafter filled with 0.5 g of each cream formulation (first for X, Y, Z and then for A, B, C, D, E, F and G) to be tested with the inclusion of the

control creams as necessary using a sterile scalpel. The plates for different organisms were allowed to stand for about 1 hour to allow diffusion. The bacteria plates were thereafter incubated 37 ℃ for 24 hours and the fungal plates at 27 ℃ for 72 hours. After the duration of incubation, diffusion patterns and zones of inhibition were measured with a transparent vernier caliper and results recorded for each cream sample. These tests were performed in triplicates.

Statistical Analysis

Results are presented as mean ± standard deviation. Statistical comparison was done by one-way analysis of variance (ANOVA). The difference between the means of zones of inhibitions of the different formulation and control was also evaluated. The statistical analysis was carried out with Graph pad prism version 7.0 software. Significant differences were set at $P < 0.05$.

Null Hypothesis (H_0) = There is no significant difference between the means of the antimicrobial activities of the creams A, B, C, D, E, F, G and the control cream used.

RESULTS

The organisms were sensitive to the extracts at 200 mg/mL; the extracts were therefore combined in five different combination ratios at 200 mg/mL. At 200 mg/mL of the extracts, the extract combination ratio of 3:1 *Vernonia*: *Calotropis* (V:C) gave the broadest zone of inhibition across all organisms, followed by the *Vernonia* only extract of V:C ratio 1:0.

The creams were greenish in colour, and the intensity increased with concentration of the extracts in the various bases. All the formulations were smooth to touch, easy to apply and washed off easily from the skin. The extracts were incompatible with the cetomacrogol cream base. The extracts separated out of the base after few minutes of formulation.

Table 2: Mean zone of inhibition of extract combinations against organisms

Extracts V: C	Zones of inhibition in mm				
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Aspergillus niger*	*Candida albicans*	*Trichophyton rubrum*
1: 0		16.67 ± 1.11	14.33 ± 0.74	30.00 ± 0.00	16.00 ± 0.53
3:1	18.00 ± 1.70	20.67 ± 1.06	18.33 ± 0.42	28.00 ± 1.14	22.43 ± 0.47
1: 1	12.00 ± 1.12	12.00 ± 0.82	15.00 ± 1.06	25.50 ± 0.25	20.33 ± 0.82
1:3	5.00 ± 1.24	15.67 ± 0.68	10.00 ± 1.00	14.00 ± 0.81	8.00 ± 0.26
0: 1	8.50 ± 0.01	24.00 ± 0.43	16.67 ± 0.72	16.00 ± 0.25	12.00 ± 0.00

Antimicrobial Activity of Creams

Antimicrobial tests of the creams at different concentrations gave zones of inhibition shown in Table 3 ranging from 8 to 30 mm with the highest activities being against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the lowest activities being against *Trichophyton rubrum*.

Table 3: Mean zone of inhibition of creams against test organisms

Creams	Inhibition zones against different organisms (mm)			
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Candida albicans***	***Trichophyton rubrum***
A	18.33 ± 1.20	24.00 ± 1.17	26.20 ± 0.82	28.67 ± 1.14
B	22.00 ± 0.00	15.70 ± 0.92	25.50 ± 0.65	24.30 ± 0.49
C	0.00	14.00 ± 1.00	0.00	0.00
D	30.00 ± 0.00	28.00 ± 1.18	16.00 ± 1.08	8.00 ± 0.54
E	28.67 ± 1.46	24.00 ± 0.00	14.67 ± 1.16	6.33 ± 0.82
F	16.00 ± 1.24	14.33 ± 0.65	18.00 ± 1.22	12.00 ± 0.68
G	13.33 ± 0.72	12.00 ± 0.00	14.00 ± 0.57	10.33 ± 0.26
X	-	-	-	-
Y	-	-	-	-
Z	-	-	-	-
V	0.00	0.00	0.00	0.00
2	30.00	24.00	25.00	23.00

Legend:
Cream V Negative Control with blank Vanishing Cream Base only
Cream 2 Positive Control with a Triple Action Cream

Statistical Analysis

The mean zone of inhibition obtained for the test organisms was compared across the formulated creams and controls, placing the sample size (n) at 9. Result of analysis of variance gave a P value < 0.05. This implies that there is a significant difference between the average antimicrobial activities of the creams in their various combinations and the control creams. The alternate hypothesis is therefore taken and the null rejected.

pH of Creams

The pH of the cream bases incorporated with extracts were in the range of 5.74 to 7.11 for vanishing cream and 5.6 to 6.25 for aqueous cream as compared to the control cream which gave a pH of 6.63.

Table 4: pH of Creams Formulated in Different Bases

Extract Concentrations in Creams	pH in Vanishing Cream Base	pH in Aqueous Cream Base
10% w/w (V:C 3:1)	7.11 ± 0.82	6.25 ± 0.47
5% w/w (V:C 3:1)	6.63 ± 0.55	6.23 ± 0.39
2.5% w/w (V:C 3:1)	5.82 ± 0.91	6.25 ± 0.86
0% w/w (V:C 3:1)	6.60 ± 0.29	5.60 ± 0.14
10% w/w (V:C 1:0)	6.13 ± 0.76	-
5% w/w (V:C 1:0)	5.74 ± 0.32	-
10% w/w (V:C 0:1)	5.90 ± 0.11	-
5% w/w (V:C 0:1)	6.42 ± 0.59	-
Control Cream (Triple Action Cream) pH 6.63		

Viscosities of Creams

Viscosities of the creams gave viscosity values ranging from 3.26 and 44.23 Pas except for Cream B which had a viscosity of 91.30 Pas at 60 rpm. Viscosity decreased with increasing speed of spindle rotation.

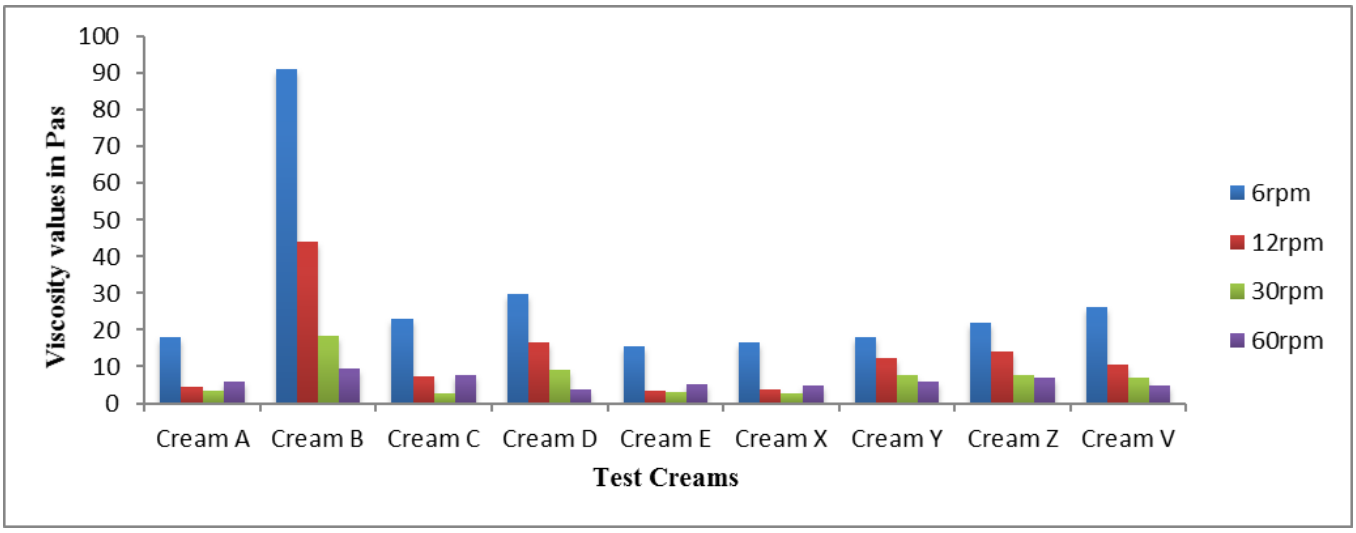

Figure 1: Viscosities of creams measured with spindle number 4

DISCUSSION

Preliminary studies were conducted on the extracts and their combined mixture to determine their activities at different concentrations. On this basis,200 mg/mL concentration was selected and a combination of the extracts in the ratio "3:1" *Vernonia* to *Calotropis* (V:C) widened the spectrum of activity of these extracts against both bacterial and fungal organisms. The extracts formulated in a Cetomacrogol cream base separated out of the base despite continuous trituration to ensure stability. This physical incompatibility can be attributed to the difference in the densities of the combined extracts as compared with the base resulting in creaming as postulated in Stokes's law. In addition, Cetomacrogol has an innate incompatibility tendency with plants containing phenolic compounds.[31] Sequel to this, a modified Aqueous cream formulation was formulated for the incorporation of the extracts.[27] The modification was the substitution of Sodium Lauryl Sulphate (SLS) as the emulsifying agent with polysorbate 80 as in the USP-NF on accounts of the irritation side effects induced by SLS.[27] This cream base yielded a stable cream that did not produce any zone of inhibition during the antimicrobial testing of the cream. The inhibited activity can be explained by the possible inhibition of drug release of some plant extracts by the presence of Liquid Paraffin in formulations.[32] The extracts were thereafter incorporated in a vanishing cream base in a bid to achieving an optimal stability and a better drug activity. The resulting creams were physically stable even after weeks of formulation in addition to the fact that they all diffused across the agar to produce zones of inhibition. An extract concentration of 200 mg/mL formulated at 10% w/w in the cream base at a V:C combination ratio of 3:1 gave the most optimal inhibition of microorganisms desired for therapeutic activity.

The antimicrobial activities of *Vernonia amygdalina* leaf extract in creams exhibited in Table 3 were in agreement with the research of Audu and co-workers; and Eraga and co-workers where the antibacterial activity of the extract was emphasized.[10,33] The combination of *Calotropis procera* was however used to achieve a broader antifungal activity thereby improving the antimicrobial effect of the cream formulation.[34] This implies that the extracts combined in appropriate proportions and concentrations in optimal cream bases such as a vanishing cream base widen the zones of inhibitions against a broader spectrum of organisms as seen with the bacterial and fungal species used in the test.

For viscosity measurements, the values gotten at spindle 4 are within the usual range of viscosity for semisolids as shown in Figure 1. This is in line with the results of viscosity obtained in a similar evaluation in the work of Dhase and co-workers; a viscosity of 27.025 Pas was gotten for the herbal cream formulated in a vanishing cream base.[35] He combined the ethanol extracts of *Curcuma caesia, Cyperus scariosus, Myristica fragrans, Linum usitatissimum, Triticum aestivum and Curcuma longa* with specific therapeutic properties into a vanishing cream base. The creams formulated had slightly acidic pH ranging between 5.6 and 6.6. It is desirable that the pH of a cream formulated for topical use be of slightly acidic pH, as potentiometric measurements carried out revealed that the skin's pH was slightly below 7.[36] Therefore, a slightly acidic pH as

obtained in the creams formulated will ensure better absorption through the skin, improved stability and efficiency of use.

In comparism with the control cream, the antimicrobial activities of the creams formulated from the combination of *Vernonia* and *Calotropis* extracts measured up approximately for cream A against *Pseudomonas aeruginosa* and *Candida albicans*. Creams A and B had higher activities than the control against *Trichophyton rubrum*, cream D had the same activity as the control cream against *Staphylococcus aureus* and a higher activity than the control against *Pseudomonas aeruginosa*. The choice of a triple action cream as control is due to the combination of Ketoconazole (10 mg in 15 g); an antifungal drug and Neomycin sulphate (5000 iu in 15 g); an antibacterial agent in the cream. These results demonstrate the potentials of the cream formulations from these extracts against test organisms commonly associated with various skin infections.

This research did not extend to isolate the specific principles responsible for the antimicrobial actions exhibited by the plant extracts. In subsequent works, attention can be channeled to isolating these fractions with specific solvents, characterizing them chemically and structurally and making them into stable topical dosage forms, in a bid to improving efficacy and reducing negative interactions. In addition, the color of the cream formulations can be made more appealing, and other physical characteristics improved.

CONCLUSION

All the creams formulated by the incorporation of different proportions of the methanol extracts of *Vernonia amygdalina* and *Calotropis procera* in a modified aqueous cream base did not yield any activity, the base is therefore not suitable for the combination of the extracts. The vanishing cream base exhibited antimicrobial activities comparable to and greater than the activities of the control cream in some cases as discussed. These extracts incorporated in cream bases therefore show great promises in the management of skin infections caused by *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, and Trichophyton rubrum*. Their activities however have been found to vary with the different kinds of cream bases used.

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