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EVALUATION OF TOPICAL ANTIMICROBIAL OINTMENT FORMULATIONS OF ESSENTIAL OIL OF *LIPPIA MULTIFLORA* MOLDENKE

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Abstract

Background: Essential oil of *Lippia multiflora* (lippia oil) has been reported to possess antibacterial and antifungal activities. This study was aimed at developing an effective topical formulation of the oil for the treatment of skin infections.
Materials and Methods: Lippia oil was extracted from leaves of *L. multiflora* by hydrodistillation. Different concentrations of the oil were incorporated into six different ointment bases. Tween 80 was incorporated at different concentrations into ointments containing 10 %w/w lippia oil; a concentration at which minimum antimicrobial activity was observed in the ointment bases. The viscosity, spreadability and stability of the formulations were determined. Antimicrobial activities of lippia oil in the formulations were determined against selected bacteria and fungi using the agar diffusion assay method. 10 %w/w salicylic acid formulations were used as reference products.
Results: Incorporation of lippia oil into the ointment bases led to reduction in their viscosity with increase in spreadability. None of the formulations showed antimicrobial activity at lippia oil content ≤ 5 %w/w. Inclusion of Tween 80 in the formulations significantly increased the antimicrobial activities of the oil ($P<0.05$). The antimicrobial activities of 10 %w/w lippia oil formulated in absorption bases containing 6 %w/w Tween 80 was significantly higher ($P<0.05$) than those formulated in hydrophobic bases. Lippia oil ointment formulations showed greater antimicrobial activities than salicylic acid ointments. Two of the lippia oil ointment formulations bled when subjected to centrifugal force.
Conclusion: Ointment formulations of lippia oil (10 %w/w) in absorption base (Hydrous Wool fat Ointment BP or Simple Ointment BP) containing 6 %w/w Tween 80 were found stable and very effective in inhibiting growth of selected pathogens implicated in skin infections.

Key words: *Lippia multiflora*, Essential oil, Ointment formulation, Antimicrobial activity

Introduction

Lippia multiflora Moldenke (Family Verbaneceae) is a stout woody, perennial essential oil yielding shrub mainly distributed throughout tropical Africa as well as South and Central America (Irvine, 1961; Pascual et al., 2001). Phytochemical studies by several researchers have shown the presence of essential oil (lippia oil) especially in the aerial part of the plant (Kunle et al., 2003; Owolabi et al., 2009; Ogunwande et al., 2012). While there is a great degree of variability in the chemical composition of the oil, major composition reported among others are; 1, 8-cineole, linalool, β-pinene, α-terpineol, carvacol, thymol, limonene, γ-terpineneol, ρ-cymene and geraniol (Valentin et al., 1995; Oladimeji et al., 2001; Kasali et al., 2004; Agnaniet et al., 2004).

Therapeutic uses of *L. multiflora* plant have been suggested to be largely due to the essential oil contained in the plant (Pascual et al., 2001). Oladimeji et al. (2000) reported the pediculocidal and scabicidal properties of the essential oil of *L. multiflora*. In another study, Oladimeji et al. (2005) demonstrated that the emulsion formulations of lippia oil was more effective and safer than the orthodox benzyl benzoate emulsion used in treating scabies. The antimicrobial activities of carvocol and thymol, which are major components of lippia oil, have also been reported (Kunle et al., 2003; Botelho et al., 2007). Due to the oil's rich chemical composition, Kunle and Egharevba (2012) had suggested that the oil could be a good pharmaceutical raw material for disinfectants, antiseptic, antifungal, and antibacterial products. Thus, as proven in vitro (Bassole et al., 2003; Oladimeji et al., 2004; Mevy et al., 2006), lippia oil may represent a promising and affordable topical agent for the treatment of skin infections.

A step towards enhancing the relevance of lippia oil in the treatment of skin infections is its formulation into appropriate dosage forms, one of which is the ointment form. By definition, medicated ointments for external application to the skin or mucous membrane contain medicament(s) dissolved, dispersed or emulsified in a suitable vehicle called ointment base (Allen, Jr. et al., 2005; Oyedele, 2007). Ointment bases may be hydrocarbons (oleaginous), absorption bases, water removable and water soluble type (Carter, 1987; Betageri and Prabhu, 2002). Apart from the therapeutic effect of the medicaments contained within the ointment bases, they are usually very moisturizing and good for dry skin with very low risk of sensitization and irritation (Betageri and Prabhu, 2002; Allen, Jr. et al., 2005). However, the selection of appropriate ointment bases to be used in the formulation of medicated ointments requires a careful assessment of the desired quality of the formulation (Allen, Jr. et al., 2005). Generally, epidermic ointments are required to produce their action on the surface of the skin, acting as protectives antiseptics and parasiticides (Carter, 1987). Its effectiveness depends on the extent of release of medication from the ointment base (Najmuddin et al., 2010).

In the present study, attempts were made to develop an effective lippia oil ointment for the treatment of skin infections using some select ointment bases among those indicated in the pharmacopoeia (Pharmaceutical Codex, 1979) as the vehicle.

Materials and Methods

Plant Material and Extraction

The leaves of *L. multiflora* Moldenke were collected from wild plants growing in Ipetumodu, near Ile-Ife, South Western part of Nigeria in August, 2013 and authenticated by Prof. H. C. Illoh (Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria) by comparison with the voucher specimens (IFE 586, IFE 1850 and IFE 6238) deposited at the Herbarium of Obafemi Awolowo University, Ile-Ife,

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Nigeria. The essential oil of the lippia leaves (lippia oil) was obtained by hydrodistillation using British Pharmacopoeia method (BP, 1988). The extracted lippia oil was stored fully filled in brown bottles at 6^{O}C.

Preparation of Ointment Bases

Six ointment bases, comprising of three absorption bases (Hydrous Wool Fat Ointment BP [HWO], Wool Alcohol Ointment BP [WAO], Simple Ointment BP [SO]); two hydrocarbon bases (Paraffin Ointment BP [PO], White Soft Paraffin BP [WSP]); and one emulsifying basis (Cetomacrogol Emulsifying Ointment BP [CEO]) were studied (Table 1). Each of the ointment bases was prepared using the official methods (Pharmaceutical Codex, 1979). The ointment bases were packed in glass jars with closely fitted screw caps and stored at 25^{O}C pending further analysis.

Table 1: Composition of ointment bases (%w/w)

Constituents	Ointment base/composition (%w/w)					
	Hydrous Wool Fat Ointment BP [HWO]	Simple Ointment BP [SO]	Wool Alcohol Ointment BP [WAO]	Paraffin Ointment BP [PO]	White Soft Paraffin BP [WSP]	Cetomacrogol Emulsifying Ointment BP [CEO]
Hydrous wool fat BP	50	-	-	-	-	-
Yellow soft paraffin	50	-	10	-	-	-
Wool alcohols	-	-	6	-	-	-
White soft paraffin	-	85	-	90	100	50
Hard paraffin	-	5	24	3	-	-
White beeswax	-	-	-	2	-	-
Liquid paraffin	-	-	60	-	-	20
Cetostearyl alcohol	-	5	-	5	-	-
Wool fat	-	5	-	-	-	-
Cetromacrogol emulsifying wax BP	-	-	-	-	-	30

Preparation of Ointments Containing Lippia Oil (Medicated Ointments)

Lippia oil at concentrations of 1.0 %w/w, 5.0 %w/w, 10.0 %w/w and 20.0 %w/w were incorporated into the ointment bases using levigation method with tile and stainless steel spatula (Pharmaceutical Codex, 1979). Tween 80 at concentrations of 1.0 %w/w, 2.0 %w/w, 4.0 %w/w and 6.0 %w/w were further incorporated into another sets of ointments containing 10.0 %w/w lippia oil. The medicated ointments were packed in glass jars with closely fitted screw caps, labeled and stored at 25^{O}C pending further analysis.

Preparation of 10.0 %W/W Salicylic Acid Ointments

The ointment bases indicated in Table 1 were used in the preparation of the salicylic acid ointments. Finely grinded and sifted (mesh size 180 µ) salicylic acid was incorporated into the ointment bases at a concentration of 10.0 %w/w using levigation method as earlier described. Salicylic acid (10.0 %w/w) was also incorporated into another set of ointment bases containing 2.0 % and 6.0 % w/w Tween 80. The salicylic acid ointments were also packed in glass jars and stored as indicated for lippia oil ointments pending further analysis.

Analysis of the Bland and Medicated Ointments

The melting point range of each of the ointment bases (bland ointments) was determined using Stuart Melting Point Apparatus SMP 10 (Bibby Scientific Limited, Stone, Staffordshire, UK) and the BP 1988 method.

The Bulk densities (weight per millimeter) of the ointment bases were determined by weighing 10 g of each of the ointment bases into a 25 ml measuring cylinder whose internal diameter (d cm) had been previously determined with a venier caliper. The measuring cylinder was placed in a water bath and the ointment basis melted to a state of complete fluidity, and was allowed to congeal at 25^{o}C. The height (h cm) of the congealed ointment basis in the measuring cylinder was measured with venier caliper. The Bulk density (gcm^{-3}) of the ointment basis was calculated using equation 1 below. Each result was an average of three determinations.

$$\text{Bulk density} = \frac{10\ g \times 4}{\pi d^2 h} \qquad \text{Equation} \quad 1$$

Determination of Homogeneity

All the ointment formulations were tested for homogeneity by visual inspection after incorporation of the lippia oil.

Determination of Spontaneous Bleeding in Medicated Ointments

The method used by Oyedele (2007) was adopted. Medicated ointments containing 10 %w/w lippia oil in combination with 6 %w/w Tween 80 were observed visually for possible separation of the oil from the ointment 24 h after preparation and storage for 28 days at 25^{o}C.

The resistance of medicated ointments to bleeding as a result of external force (a measure of stability) was determined using the modified method of Oyedele (2007). Triplicate 2g-samples of each of the medicated ointments containing 6 %w/w Tween 80 and 10 %w/w lippia oil were subjected to centrifugal force of 2500 rpm for 30 min using Centrifuge Model 90-1 (Search-Tech Instruments). The samples

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were thereafter examined carefully for sign of instability and bleeding. The nature of the bleeding fluid was determined by its miscibility with methylene blue solution or Sudan red solution indicating an aqueous or oily fluid, respectively (Oyedele, 2007).

Determination of Viscosity of the Ointments

The viscosity of each ointment formulation was determined using Rion Viscometer (Viscotester VT – 04) with spindle number 2. A 50 g sample of the ointment was used for each determination. The spindle of the viscometer was inserted into the ointment placed in an 80 ml beaker with internal diameter 4.72 cm and 6.93 cm height. The first reading was taken at 5 seconds (recorded as time zero), while subsequent readings were taken at intervals of 10 seconds for a total of 60 seconds duration. The determinations were carried out at 25^{o}C. The results were average of at least three determinations. The viscosity of medicated ointments containing 6 %w/w Tween 80 and 10 %w/w Lippia oil were determined at 7 days interval for a period of 28 days.

Spreadability Measurement

The Spreadability of the ointment was determined 48 h after preparation using two parallel glass plates method (Sera and Ramana, 2006). 1 g of the ointment was placed between the two 20 cm x 20 cm horizontal plates, with the upper plate weighing 125 g. The diameter of spread recorded as the spreadability (Φ) was measured at 1 min in millimeter in the vertical, diagonal and horizontal axes of the upper plate. The average values of Φ were graded from very stiff (Φ $\leq$ 25 mm) through semi-stiff (Φ $>$ 25mm but $\leq$ 50 mm); semi-fluid (Φ $>$ 50 mm but $<$ 70 mm) to fluid (Φ $\geq$ 70 mm) ointment based on Arvouter-Grand et al. (1995) classification. The temperature of the experiment was maintained at 25^{o} ± 1^{o}C.

The spreading indices of the ointments were determined by De Paula et al. (1998) method. 1g sample of each ointment was placed at the centre of a glass plate of 20 cm x 20 cm with a thickness of 3 mm. Glass plates of known weight were subsequently placed over the sample at 1 min intervals. The spreading areas reached by the sample were measured in millimeter in the vertical, diagonal and horizontal axes of the plates. The results were expressed in terms of the spreading area as a function of the applied weight according to the equation.

$$S_a = \frac{\pi d^2}{4} \qquad \text{Equation 2}$$

S*a* is the spreading area (mm^2) resulting from applied weight *a* (g), and d is the mean diameter (mm) reached by the sample. The spreading area (S*a*) was plotted against the plate weight to obtain the spreading index (S*i*) from the slope of the linear portion of the plot. The temperature of the experiment was maintained at 25^{o} ± 1^{o}C.

Antimicrobial Activity and Agar Diffusion Assay

The microbial strains used were from stocks of culture collections maintained in our laboratory. Bacteria were maintained on nutrient agar slants, and fungi on Sabouraud Dextrose agar slants at 4^{o}C and subcultured monthly.

The experiments were performed in two phases. The first phase was to select the most effective minimum antimicrobial concentration of lippia oil to be used for formulation. For this phase, four organisms were used: Methicillin Resistant *S. aureus* [MRSA] (ATCC 43300), *Escherichia coli* (ATCC 25922), *Clostridium sporogenes* (NCIB 532) and *Candida pseudotropicalis* (NCYC 6). The concentrations of lippia oil used were: 0, 1.0, 5.0, 10.0 and 20 %w/w.

Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml sterile distilled water in a test tube and mixed thoroughly, using an electric shaker, for uniform distribution to give a turbidity of 0.5McFarland standard equivalent to an inoculum size of 1 x 10^{5}cfu/ml. A sterile cotton swab was then used to spread the resulting suspension uniformly on the surface of oven-dried Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates (Sterillin) for bacteria and fungi, respectively. These were incubated for an hour at 37^{o}C for bacteria for the purpose of acclimatization of growth.

Holes of 9 mm diameter each were made in the agar plates using sterile metal cup-borer and two drops of the oil were placed in each hole which already had agar as cover for the base. This was kept at room temperature for 1 hour for diffusion into the agar medium. The plates were then accordingly incubated at 37^{o}C for 24 h for the bacterial strains and at 25^{o}C for 72 h for the fungal strains. The antimicrobial activities were evaluated by noting the zones of inhibition against the test organisms. The determinations were done in duplicates.

The second phase of evaluation of antimicrobial activities of the ointment formulations involved the use of 10.0 %w/w lippia oil ointments to which 1.0, 2.0, 4.0 and 6.0 %w/w Tween 80 were incorporated. Salicylic acid (10.0%w/w) formulated in the same bases as the lippia oil containing 2.0 and 6.0 %w/w Tween 80 were used as control. *P. aeruginosa* ATCC 10145, *S. aureus* NCTC 6571, *C. albicans* ATCC 24433 and *C. pseudotropicalis* NCYC 6 were used for this phase of the experiment. The agar cup plates were prepared as above and a sterile syringe was used to introduce 150mg of each preparation into each cup. The release of the lippia oil and the salicylic acid from ointment bases and the antimicrobial activities were evaluated by noting the zones of inhibition against the test organisms. The determinations were also done in duplicates.

Statistical Analysis

Experimental results were expressed as mean value ± standard deviation. The results were subjected to either Student's t test or analysis of variance with P–values $<$ 0.05 considered statistically significant. Where required, correlation coefficient and coefficient of determination were calculated.

Results

Physical Properties of Bland and Medicated Ointments

The physical properties of the selected ointment bases (bland ointments) are indicated in Table 2. The hydrocarbon bases [PO, WSP] had the highest viscosity values. The viscosity of PO (hydrocarbon base) was about four times that of WAO, an absorption base. While the PO

had the lowest bulk density, there was no significant difference ($P > 0.05$) between the bulk densities of all the bases. The relative standard deviation calculated for the bases was 0.023.

The Spreadability values (ϕ) of the ointment bases were less than 50 mm, with HWO having the highest value (Table 2). The correlation coefficient between the viscosity and spreadability (Φ) of the ointment bases was very low ($r = -0.5594$; $r^2 = 0.3130$). This was also reflected in low correlation between the viscosity and spreading index of the ointments ($r = -0.4251$; $r^2 = 0.1807$).

The incorporation of lippia oil into the ointment bases resulted in concentration dependent decrease in the viscosities of the ointments (Figure 1). The effect of shearing time on the viscosity of ointment bases containing 10 %w/w also indicated a time dependent decrease in the viscosities of the ointments (Figure 2). Further reduction in the viscosity of the ointments was observed when Tween 80 was incorporated into the ointment bases containing 10 %w/w lippia oil (Figure 3).

Results in Table 3 showed that incorporation of Tween 80 into ointments containing 10 %w/w lippia oil further increased the spreadability of the ointments when compared with the values in Table 2. For example, a spreadability value greater than 50 mm was obtained for HWO base containing 6 %w/w Tween 80 and 10 %w/w lippia oil (Table 3) as against 31.8 mm obtained for the bland HWO base Table 2). Increase in the spreading index (S*i*) of the lippia oil ointments was observed on incorporation of Tween 80 to the products (Table 3). The spreading indices were greater than 1.200 for all ointments containing 10 %w/w lippia oil in combination with 6 % w/w Tween 80.

The stability of lippia oil ointment formulations to which 6 %w/w Tween 80 was incorporated is indicated in Table 4. There was increase in the viscosity of the ointment formulation on storage up to day 14 and 21 respectively, for the hydrocarbon ointment bases [PO, WSP] and absorption [HWO, SO, WAO]/ emulsifying ointment bases [CEO] after which the viscosity remained constant. The medicated ointments did not show any sign of bleeding during the 28 days storage. However on centrifugation, WAO and CEO ointment bases containing combinations of 6 %w/w Tween 80 and 10 %w/w lippia oil showed sign of bleeding, with an oily layer separated on top of the ointments.

Table 2: Physical properties of ointment bases

Ointment base code	Melting range (^oC)	Bulk density (gcm^{-3})#	Viscosity (Poise)	Spread diameter (Φ) (mm)	Classification of spreadability*	Spreading Index (S*i*) (mm^2g^{-1})
HWO	36.8 – 51.2	0.850 ± 0.004	1075 ± 18	31.8 ± 0.5	Semi-stiff	1.306 (0.998)
SO	38.6 – 55.3	0.807 ± 0.016	1446 ± 26	26.4 ± 2.5	Semi-stiff	1.071 (0.997)
WAO	37.1 – 54.8	0.812 ± 0.005	629 ± 24	27.5 ± 0.8	Semi-stiff	1.022 (0.995)
PO	39.3 – 57.1	0.795 ± 0.005	2208 ± 28	24.3 ± 2.7	Very stiff	0.900 (0.989)
WSP	38.1 – 56.5	0.808 ± 0.004	1956 ± 26	25.3 ± 2.5	Semi-stiff	1.083 (0.982)
CEO	39.0 – 54.5	0.818 ± 0.002	1152 ± 21	24.8 ± 1.8	Very stiff	0.912 (0.982)

Figures in parentheses are coefficients of determination (r^2)
* Classification based on Arvoutet-Grand et al. (1995)
# Relative Standard Deviation (RSD) = 0.023

Table 3: Effect of inclusion of Lippia oil (10 %w/w) and Tween 80 (6 %w/w) on the Spread-ability of the ointments

Ointment basis code	Spread diameter (Φ) (mm)		Spreading Index (S*i*) (mm^2g^{-1})	
	10 %w/w Lippia oil	10 %w/w Lippia oil plus 6 %w/w Tween 80	10 %w/w Lippia oil*	10 %w/w Lippia oil plus 6 %w/w Tween 80*
HWO	41.3 ± 1.0	51.9 ± 1.7	1.607 (0.986)	1.719 (0.952)
SO	35.0 ± 1.5	47.3 ± 2.0	1.389 (0.998)	1.607 (0.964)
WAO	29.1 ± 1.0	37.2 ± 0.6	1.100 (0.870)	1.515 (0.958)
PO	31.5 ± 1.1	34.8 ± 1.3	1.290 (0.992)	1.374 (0.952)
WSP	29.9 ± 2.6	32.4 ± 0.8	1.131 (0.994)	1.207 (0.937)
CEO	25.7 ± 1.5	33.4 ± 0.5	1.015 (0.990)	1.305 (0.969)

Figures in parentheses are coefficients of determination (r^2)

Table 4: Stability of ointment bases containing 10 % w/w Lippia oil and 6 %w/w Tween 80

Ointment basis code	Storage period (Day)/ Viscosity value (Poise)					Bleeding tendency on storage for 28 days	Bleeding tendency on centrifugation at 2500 rpm for 30 min
	1	7	14	21	28		
HWO	561 ± 14	617 ± 16	657 ± 18	660 ± 12	660 ± 15	Stable	Stable
SO	735 ± 18	883 ± 21	905 ± 20	908 ± 15	905 ± 13	Stable	Stable
WAO	210 ± 13	231 ± 13	243 ±16	243 ± 11	245 ± 16	Stable	Not stable
PO	867 ± 22	997 ± 20	1002 ± 17	1003 ± 22	998 ± 15	Stable	Stable
WSP	960 ±14	993 ± 17	1049 ± 23	1047 ± 20	1042 ±18	Stable	Stable
CEO	518 ± 15	578 ± 21	642 ± 19	695 ± 15	693 ± 17	Stable	Not stable

Antimicrobial Activities

The antimicrobial activities of various concentration of lippia oil in the ointment bases against the selected pathogens are indicated in Table 5. Lippia oil in 50 % methanol-water served as the positive control, while the bland ointment bases were used as negative control. The lippia oil demonstrated concentration dependent antimicrobial activity in the methanol-water medium. In contrast to the methanol-water

medium, lippia oil showed no activity against any of the selected pathogens at concentration ≤ 5 % w/w in all the ointment bases. The general trend of the susceptibility of pathogens to lippia oil was *C. pseudotropicalis* > MRSA > *Clostridium* spp. > *E. coli*. The lippia oil showed greater activity against the pathogens in the absorption bases [HWO, SO, WAO] and emulsifying base [CEO] than the hydrocarbon bases [PO, WSP].

Table 5: Antimicrobial activity of Lippia oil in various ointment bases against selected pathogens

Base/Lippia oil (%) Methanol-Water	Zone of inhibition (mm)			
	MRSA (ATCC 43300)	*E. coli* (ATCC 25922)	*Clostridium sporogenes* (NCIB 532)	*C. pseudotropicalis* (NCYC 6)
0	0	0	0	0
1	2.1	0.8	1.5	2.9
5	6.1	2.0	4.0	8.1
10	7.9	3.1	5.9	15.6
20	14	6	9.5	31.0
HWO (0, 1, 5)	0	0	0	0
10	2.4	1.5	2.3	4.7
20	3.9	2.3	3.5	6.9
SO (0, 1, 5)	0	0	0	0
10	2.3	1.3	2.2	4.0
20	3.7	2.0	3.4	6.5
WAO (0, 1, 5)	0	0	0	0
10	3.0	1.6	2.7	5.2
20	4.6	2.4	3.9	7.8
PO (0, 1, 5)	0	0	0	0
10	1.4	1.0	1.3	3.4
20	2.4	1.6	2.0	5.2
WSP (0, 1, 5)	0	0	0	0
10	1.6	0.9	1.1	3.6
20	2.3	1.6	2.0	5.1
CEO (0, 1, 5)	0	0	0	0
10	4.3	2.4	3.1	5.8
20	6.6	3.0	4.6	9.6

MRSA = Methicillin Resistant *S. aureus*; Values exclude size of pore hole (9 mm)

The inclusion of Tween 80 (2 – 6 %w/w) in the ointment bases led to significant increase ($P < 0.05$) in the activities of 10 %w/w lippia oil against the selected skin pathogens (Table 6). Irrespective of the type of skin pathogens, the absorption bases [HWO, SO, WAO] containing Tween 80 released the lippia oil better than the hydrocarbon bases as indicated in the value of the zones of inhibition by the oil. The highest activity of lippia oil in the ointment bases in combination with 6 %w/w Tween 80 was observed against *C. pseudotropicalis* (Table 6). A correlation coefficient between the viscosities of the ointments and zones of inhibition against *C. pseudotropicalis* was -0.6245 ($r^2 = 0.3900$), while that between the spreading indices of the ointments and the same microorganism was 0.7689 ($r^2 = 0.5912$).

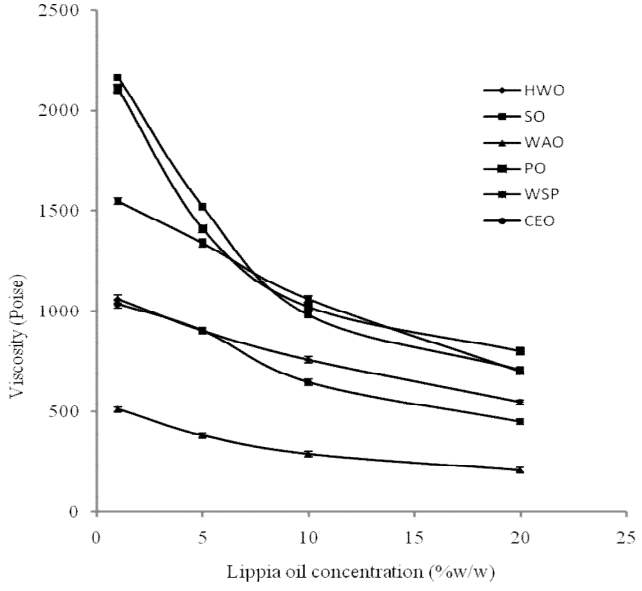

Figure 1: Effect of lippia oil concentration on viscosity of the ointment bases

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Table 6: Comparative antimicrobial activities of 10 %w/w Lippia oil and 10 %w/w salicylic acid in various ointment bases in combination with Tween 80 against pathogens that cause skin infections

Ointment basis/medication	Tween 80 concentration (%w/w)/Zones of inhibition (mm)																			
	P. aeruginosa (ATCC 10145)					*S. aureus* (NCTC 6571)					*C. pseudotropicalis* (NCYC 6)					*C. albicans* (ATCC 24433)				
(A) Lippia oil (10 %w/w)	**0**	**1**	**2**	**4**	**6**	**0**	**1**	**2**	**4**	**6**	**0**	**1**	**2**	**4**	**6**	**0**	**1**	**2**	**4**	**6**
HWO	0	8	8	11	12	6	26	26	30	38	11	30	30	38	40	0	14	14	25	28
SO	0	6	8	11	12	10	25	28	32	38	14	32	36	40	45	9	18	18	20	25
WAO	6	8	11	11	12	8	18	20	20	24	14	24	31	38	40	8	14	18	20	24
PO	0	6	8	8	11	0	13	15	20	24	0	18	20	28	32	0	8	11	13	18
WSP	0	0	0	6	8	0	0	8	10	12	0	0	8	12	16	0	0	0	8	10
CEO	0	8	10	8	10	11	18	24	31	35	0	22	27	34	38	0	15	18	20	24
(B) Salicylic acid (10 %w/w)																				
HWO	0	-	8	-	10	0	-	14	-	18	0	-	18	-	20	0	-	12	-	15
SO	0	-	6	-	12	10	-	19	-	23	13	-	20	-	30	10	-	14	-	18
WAO	0	-	8	-	12	0	-	11	-	12	0	-	10	-	14	0	-	8	-	12
PO	0	-	8	-	10	0	-	14	-	18	0	-	18	-	20	0	-	12	-	15
WSP	0	-	0	-	10	0	-	0	-	12	0	-	0	-	11	0	-	0	-	10
CEO	0	-	0	-	8	10	-	11	-	21	0	-	15	-	26	0	-	10	-	17

- Not determined; Values exclude size of pore hole (9 mm)

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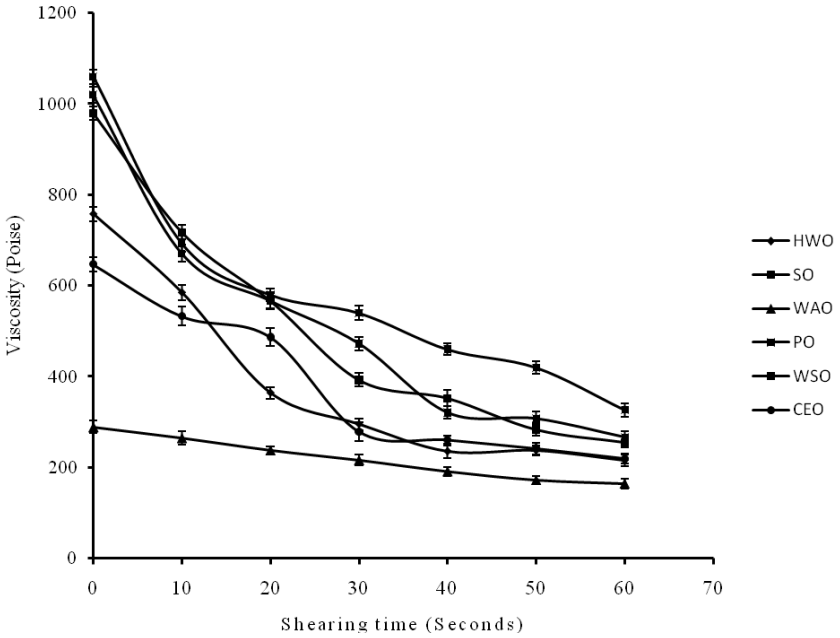

Figure 2: Effect of shearing time on viscosity of ointment formulations containing 10 %w/w lippia oil

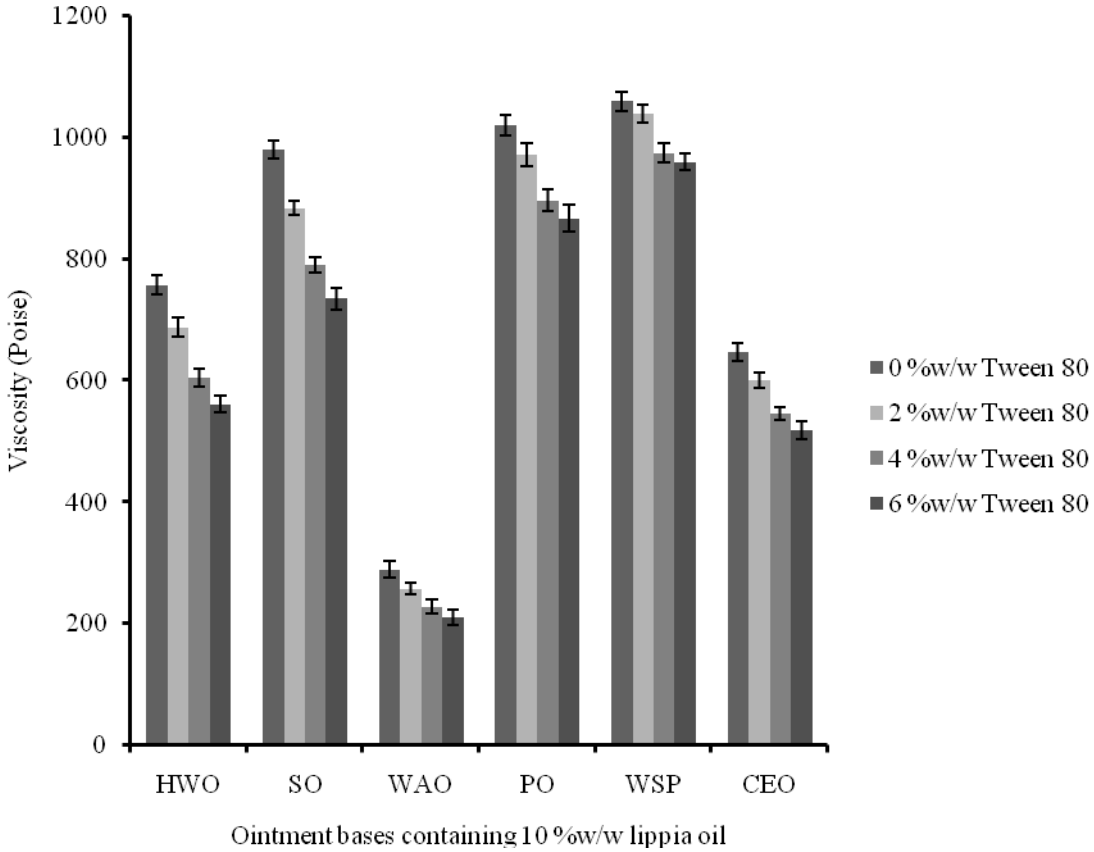

Figure 3: Effect of different concentrations of Tween 80 on viscosity of ointments containing 10 %w/w lippia oil

The antimicrobial activities of 10 %w/w salicylic acid contained in the ointment bases to which 2 or 6 %w/w Tween 80 was incorporated are indicated in Table 6. The results showed that ointment formulations containing 10 %w/w lippia oil in combination with Tween 80 had significantly higher activity ($P < 0.05$) against *S. aureus*, *C pseudotropicalis* and *C. albicans* than those formulations containing 10 %w/w salicylic acid. However, the activities of the two agents in the different bases against *P. aeruginosa* were not significantly different ($P > 0.05$).

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Discussion

Medicated ointments utilize bases that act as vehicles in delivering the drug, impart emollient and lubricant properties to the preparation. The base of a tropical medication is as important as the medication itself. As indicated in Table 2, there was variation in the physical properties of the ointment bases which depended on their constituents (Table 1). The melting range of the ointment bases was an aggregate of the melting points of their constituents (Batagori and Prabhu, 2002). Blending of increasing quantities of white soft paraffin with other oleaginous materials produced ointment bases of various consistencies and melting range [SO, PO, CEO] as indicated in Tables 1 and 2. The high melting range and viscosity of PO was due to the presence of hard paraffin, beeswax and cetostearyl alcohol in the ointment base, which increased the consistency of the base. As normal human body temperature is about 37.5^{o}C, it implies that ointment base with higher melting point than the body temperature may not melt or spread early to release the drug content.

The bulk density was an indication of the relative packing and the porosity of the congealed ointment base. The higher the value, the less bulky the ointment base, and the smaller the volume of container required for the package. While there was variation in the bulk densities of the ointments bases due to variation in their constituents (Table 2), the low relative standard deviation (RSD) calculated for the bulk densities of the ointment bases indicated no significant difference ($P < 0.05$) in their packaging requirement.

The viscosity of the ointment bases indicated in Table 2 showed that hydrocarbon bases (PO, WSP) are more viscous than the absorption bases [HWO, SO, WAO]. The high liquid paraffin constituents (60 %w/w) of WAO could be responsible for the low viscosity, while the high viscosity of PO could be due to stiffening agents (cetostearyl alcohol, beeswax and hard paraffin) incorporated into the base. The addition of lippia oil to the ointment bases led to decrease in their viscosities (Figure 1). This may be due to disruption of the congealing property of the constituents of the ointment bases. Similar finding has been reported when lippia oil was added to oleaginous phase of emulsion (Oladimeji, 2003). The extent of reduction in the viscosity of the ointments was found to depend on the concentration of the lippia oil and the constituents of the ointment bases.

The rheological behavior of ointment bases containing 10 %w/w lippia oil showed a pseudo-plastic system on continuous shearing (Figure 2). Such a rheological behaviour enables the ointment to spread easily on application. Further reduction in the viscosity of the ointments was observed on incorporation of Tween 80 to 10 %w/w lippia oil ointment formulations (Figure 3). The Tween 80 reduced the tackiness of the ointment and made them more pliable as evident from the decrease in their viscosity values and increase in their spreadability. Thus, the inclusion of both lippia oil and Tween 80 provide synergistic effect in the reduction of viscosity and increase in spreadability of the ointments.

The spreadability (Φ) of the bland ointment bases was very poor (Table 2). The values Φ were graded from very stiff [PO, CEO] to semi-stiff [HWO, SO, WAO, WSP] ointments. Spreadability was the term used to denote the extent of area to which the ointment readily spreads on application to the skin or affected part (Kavitha et al., 2013). It is an important factor in therapy and has been shown as index of ease of application (Jelvehgari et al., 2007). Larger spread diameter indicates relative ease of spreading of the ointment on the skin (Table 2). The incorporation of lippia oil into the ointment bases improved the spreadability of the ointments (Table 3) which may be as a result of decrease in the viscosity of the ointments.

Another parameter that has been used in determination of spreadability of an ointment was the spreading index (S*i*) (De Paula et al., 1998). Here, the variation of the spread area (mm^2) as a function of the applied weight to 1 g of the ointment was analysed, with the slope used as the response factor (Spreading index, S*i*) shown to be directly related to the spreadability (Barakat, 2010). There was a high correlation (r^2 = 0.9716) between the spreadability (Φ) and the spreading index (S*i*) of the medicated ointments containing 10 %w/w lippia oil (Table 3) to justify the use of spreading index (S*i*) in the classification of spreadability of the ointments. Thus, using a linear regression equation ($Si = 0.03967\Phi - 0.01750$; $r^2 = 0.9716$), values of S*i* were graded as: very stiff ($Si \leq 0.97$ mm^2g^{-1}); semi-stiff ($Si > 0.97$ mm^2g^{-1} but ≤ 1.97 mm^2g^{-1}); semi-fluid ($Si > 1.97$ mm^2g^{-1} but < 2.76 mm^2g^{-1}) or fluid ($Si \geq 2.76$ mm^2g^{-1}) ointment. These values correspond with the spreadability classification of ointments by Arvouter-Grand et al. (1995).

The main instability in ointments are bleeding and change in consistency due to aging or changes in temperature (Oyedele, 2007).This study revealed that the viscosity of the medicated ointments increased on storage (Table 4) within the first 14 days of preparation. Such increase in viscosity was expected as ointments have considerable degree of structure that requires some days to develop after preparation. The increase in the viscosity of the ointments may also be due to rebuilding of the ointment structures that were disrupted on addition of lippia oil. The degree of viscosity recovery differs in the ointments, with the hydrocarbon based ointments [PO, WSP] reaching a steady viscosity state on day 14 of the preparation (Table 4). Temperature stress was avoided in the determination of the stability of the ointments containing lippia oil since previous studies indicated that volatility of the lippia oil increased by 6.7 folds at temperature above 37.7^{o}C (Oladimeji, 2003). The use of centrifugal force as an alternative to temperature stress led to bleeding in WAO and CEO which are the only ointments with liquid paraffin constituents (Tables 1 and 4). In contrast to the findings of Oyedele (2007), bleeding did not occur in HWO at the level of stress condition employed.

The diameters of zones of inhibition obtained for different concentrations of lippia oil in 50 % methanol-water medium (Table 5) confirmed our earlier reports on the impressive antimicrobial activities of the oil against a wide range of microorganisms (Oladimeji et al., 2001 and 2004). The zones of inhibition against Gram positive bacteria (MRSA) and fungi were larger than those of Gram negative bacteria (*E. coli*). This was in conformity with earlier findings on the antimicrobial activities of lippia oil (Oladimeji et al., 2004). The formulation of the lippia oil into an ointment was to make relevant the antimicrobial effects of the oil in the treatment of skin infections. The ointment bases chosen as vehicle for the lippia oil were standard (Pharmacopoeia) ointment bases, which represent established formulations known to be stable (Oyedele, 2007). The usefulness of the bases in the formulation of lippia oil ointments was demonstrated by inhibition of microbial growth in agar plates. Comparatively, the antimicrobial activities of lippia oil in all the ointment bases were significantly lesser ($P < 0.05$) than those obtained in the 50 % methanol-water medium (Table 5). Lippia oil concentration ≤ 5 %w/w in the ointment bases did not show inhibitory activities against any of the selected pathogens. This confirmed some earlier reports that the type of ointment base could affect the antimicrobial activity of the incorporated drug (Olowosulu et al., 2005; Orafidiya et al., 2001; Alalor et al., 2012). For the lippia oil to be effective, it must diffuse from the ointment base into the agar medium. However, lippia oil being lipophilic has been shown to be completely miscible with the ointment bases, making its release to be very low. Based on the inhibition zones, the release of lippia oil from the ointment bases could be ranked as: CEO>WAO>SO=HWO>PO=WSP. The trend in releasing lippia oil from the bases followed the polarity of the bases; CEO is an emulsifying base whereas WAO, SO, and HWO are absorption bases. Their constituents, especially the wool fats (containing cholesterol) and wool alcohol rendered the bases hydrophilic and enhanced the release of the lippia oil. The high hydrophobic nature of PO and WSP will lead to high partitioning of lippia oil within the bases and reduced its diffusion to the agar medium. Factors such as high viscosity and low spreadability in

addition to high melting points of the two hydrophobic ointments [PO and WSP] (Table 2) could also affect the release of lippia oil from the bases.

The possibility of enhancing the release of lippia oil from the bases was evaluated by incorporating Tween 80 at different concentrations to ointments containing 10 %w/w lippia oil (Table 6). The microorganisms selected for use in the study have been reported to be involved in the infections of the skin and mucous membrane (Gilbert and Allison, 2004). Tween 80 has surface activities and therefore, the significant increase ($P < 0.05$) in the inhibitory activities of the 10 %w/w lippia oil on incorporation of Tween 80 to the formulations could be due to increase in the hydrophilicity of the bases, in addition to reduction in their viscosity (Figure 4) and increase in the their spreadability (Table 3). These factors must have assisted in the diffusion of the lippia oil through the bases into the agar medium.

The antimicrobial activities of salicylic acid used as positive control was significantly ($P < 0.05$) lower than those of lippia oil in all the ointment bases (Table 6). Salicylic acid is a keratolytic substance with bacteriostatic and fungicidal properties (Pharmaceutical Codex, 1979). It was formulated as Salicylic Acid Ointment in Pharmaceutical Codex (1979) using wool alcohols ointment as the base. A comparative antimicrobial activities of 10 %w/w salicylic acid with that of 10 %w/w lippia oil in wool alcohols ointment (WAO) showed that lippia oil was significantly ($P < 0.05$) more effective than salicylic acid against all the selected skin pathogens (Table 6).

Conclusion

The results of this study showed that incorporation of lippia oil into ointment bases led to reduction in viscosity and increase in spreadability of the bases. The lippia oil showed differential antimicrobial activities in the ointment bases which was ascribed to the observable differences in the hydrophilicity, viscosity and spreadability of the ointments. Generally, the antimicrobial activities of lippia oil in the absorption bases were significantly greater than those in the hydrophobic bases. The inclusion of Tween 80 in the formulations enhanced the antimicrobial activities of lippia oil. Considering the stability and release properties of the ointment bases, 10 % w/w lippia oil in Hydrous Wool Fat Ointment BP or Simple Ointment BP with incorporation of 6 %w/w Tween 80 would be an effective topical formulation for the treatment of skin infections caused by pathogens such as *C. pseudotropicalis*, *S. aureus*, *C. albicans* and *P. aeruginosa*. These formulations were more effective than 10 %w/w salicylic acid ointment using the same bases.

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