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Evaluation of Acute and Sub-Chronic Toxicities and the Effect of Ointment Bases on the Antimicrobial Potency of the Ethanolic Extracts of *Alchornea cordifolia* Leaf and *Terminalia superba* Stem Bark

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ABSTRACT

The problem of resistance of dermatological infections to some antimicrobial agents available in the market has constrained man to search for newer compounds of natural origin with potent antimicrobials. *Alchornea cordifolia* leaf and *Terminalia superba* stem bark are used in African folkloric medicine and have been documented to have antimicrobial properties. It is important to ascertain the potency of their extracts against microorganisms, their toxicities and the type of ointment base most suitable for the formulation of these plant extracts into topical antimicrobials. This study was designed to evaluate the effect of ointment bases on the antimicrobial potency of the ethanolic extracts of *A. cordifolia* leaf and *T. superba* stem bark. The *in - vitro* antimicrobial activity of the extracts and their minimum inhibitory concentration (MIC) were determined against some microorganisms using the agar diffusion method. The physical properties of the creams formulated with these extracts were evaluated using standard procedures. The extracts exhibited good antibacterial activity. The MIC (mg/mL) of *A. cordifolia* extract against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were 12.8, 12.8, 25.6 and 12.8, respectively. The MIC (mg/ mL) of *T. superba* extract against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* were 25.6, 25.6, > 25.6 and 25.6, respectively. The topical formulation prepared using the soft water washable base released antibacterial activity of the extracts better than the formulations prepared using other bases. The soft water washable base is the most suitable ointment base for topical antimicrobials formulation of the extracts.

Keywords: *Alchornea cordifolia*, *Terminalia superba*, Antimicrobial potency, Ointment bases.

Introduction

In developing countries, the major causes of diseases are the poor quality of accessible drinking
Water, contaminated food, poor standard of personal hygiene and lack of appropriate sanitation.[1] Antimicrobials are the mainstay of extract treatment strategy of infectious diseases worldwide.[2] Regardless of this fact, the problems of antimicrobial resistance and toxicity have triggered interest in research for newer antimicrobial compounds of natural origin and likely to be less toxic to man.[3] Apart from the problem of resistance, environmental degradation, cost and pollution associated with irrational use of orthodox medicines have necessitated renewed interest in the use of medicinal plants as sources of effective and safer alternatives in the management of human infections.[4] Plants occupy a very important place in modern medicine as they are used as either raw materials for drugs or as a template for discovery and synthesis of drugs.[5] According to the World Health Organization[2], medicinal plants can provide the best alternative source for obtaining a variety of drugs, since they possess a variety of bioactive principles known as phytochemicals[6] which make them potential sources of antimicrobial agents.[7,8] These phytochemicals include alkaloids, flavonoids, terpenoids, glycosides, tannins and saponins.[9] Topical antibiotics are the mainstay of the treatment of skin infections caused by bacterial, fungal and viral organisms and these agents are available as creams, ointments, powders and sprays.[10,11]

Alchornea cordifolia Muell. Arg. belongs to Euphorbiaceae family and is a traditional medicinal plant widely distributed in West Africa including Nigeria. Its common English name ischristmas bush.[12] The plant is used for ethnomedicinal purposes against wounds, ulcers, and sores.

Terminalia superba is of Combretaceae family. It is commonly called yellow pine and is a large tree which is native to tropical western Africa.[13] The plants, *A. cordifolia*and *T. superba*are used traditionally against infections and various health conditions. Investigations have been carried out on the Phytochemical constituents and antimicrobial activities of *A. cordifolia* leaf extracts.[14-17] Bits of information exist regarding its acute toxicities. Nevertheless, there had not been records of investigations regarding the phytochemical screening and acute toxicity evaluations of *T. superba* stem bark extract. There are no records of acute toxicities and antimicrobial activities of the combination of *A. cordifolia* leaves and *T. superba* stem bark extract.

Since studies have shown that the type of ointment base and formulation process affect topical drug bioavailability and potency of the preparations,[4,17] the effect of various ointment bases on the antimicrobial potency of the extracts of *Alchornea cordifolia* leaf and *Terminalia superba* stem bark were investigated. Moreover, with the problem of increasing resistance, high cost and side effects associated with topical antibiotics available in the market, it is necessary to formulate an herbal ointment which would be affordable, potent and safe to use.

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Materials and Methods

Plant material

Fresh leaves of *Alchornea cordifolia* were obtained from Ijegun, Lagos, while fresh stem barks of *Terminalia superba* were obtained from the Forestry Research Institute of Nigeria (FRIN) Ibadan, Oyo, Nigeria in 2017. *Alchornea cordifolia* was identified and authenticated by a taxonomist at the Herbarium of the Department of Botany and Microbiology, University of Lagos where Herbarium specimen assigned with Voucher number (LUH 7539) was deposited in the herbarium for future reference.

Terminalia superba was identified and authenticated by a taxonomist at the Herbarium of the FRIN, Ibadan with the voucher specimen number (FHI 111222) deposited in the herbarium for future reference.

Microorganisms

Clinical isolates of bacterial strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* and fungal strains of *Candida albicans* and *Aspergilus niger* were obtained from the Lagos University Teaching Hospital (LUTH) and the Department of Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy of the University of Lagos, Nigeria.

Laboratory animals

Swiss albino mice (20.0 – 25.0 g) were used for this study. They were obtained from the Laboratory animal Centre, College of Medicine, University of Lagos, Idi-Araba and kept under standard environmental conditions. They were kept in well spacious polypropylene cages (5 animals per cage) in full ventilated animal house with 12 h dark and light cycle and were fed on standard animal diet (Pfizer Feeds Ltd, Nigeria) and water *ad libitum*. They were acclimatized to the laboratory conditions for seven days prior to commencement of research. The use and care of the animals, and the experimental protocol were in strict compliance with the Institute of Laboratory Animal Research (ILAR) guidelines on the use and care of animals in experimental studies.[18] The animals were distributed randomly into five groups of five animals each for acute toxicity.

Preparation of plant extracts

The leaves of *A. cordifolia* were oven dried at 40℃ and milled to coarse powder with laboratory mill (Christy and Norris Ltd, Chelmsford, England). A total of 700 g of milled leaves was extracted with 7 L of 85% ethanol using cold maceration. The extract was filtered using a fine pored cloth. The maceration process was repeated after 7 days using 4 L of 85 % ethanol. The extract was concentrated in a rotary evaporator (Buchi V-801) and dried in an oven at 37ºC.[19,20]

The stem barks of *T. superba* were oven-dried at 40ºC and pulverized using the grinding machine (Christy and Norris Ltd, Chelmsford, England). A portion of 700 g of the powdered bark was extracted with 6 L of 80 % ethanol using cold maceration method. The extract was filtered using a fine pored muslin cloth. The maceration process was repeated on the residue from the fi8rst maceration after 7 days using 4 L of 80 % ethanol. The extract was filtered and concentrated using a rotary evaporator (Buchi V-801) and dried in an oven at 37ºC.[19,20]

Phytochemical screening

The phytochemical screening for the presence of cardiac glycosides, anthraquinone glycosides, flavonoids, saponins, phytosterols (steroids), tannins, alkaloids, gums and mucilages, fixed oils in both extracts was carried out using standard procedures as described by Sofowora.[21]

Evaluation of the antimicrobial activity of A. cordifolia leaf and T. superba bark extracts

The antimicrobial activities of the ethanolic extract of the leaves of *A. cordifolia* and stem barks of *T. superba* at concentrations of 50 mg/mL, 100 mg/mL and 200 mg/mL were determined using the agar diffusion method.[22] The clinical isolates of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* were used for the investigations.

The Minimal Inhibitory Concentration (MIC) was determined using the agar diffusion method.[23]

Commercial brands of antimicrobial agent "Ciprofloxacin BP" and antifungal agent "Clotrimazole BP" were used as positive control.

Acute toxicity study of *A. cordifolia* leaf and *T. superba* bark extracts

The acute toxicity screening of the ethanolic extracts of *A. cordifolia* leaves and *T. superba* stem bark were carried out using the method described by Ogbonnia *et al.*[24] Swiss albino mice (25) of both sexes weighing 20.0 - 25.0 g were used. The animals were randomly distributed into one control group and four treated groups of five animals per group. After the overnight fasting, the control group received distilled water and 2% acacia orally. The test animals were fed orally with different doses (5.0, 10.0, 15.0 and 20.0 g/kg) of 80% (w/v) of the extracts, respectively. The 80 % (w/v) solution of the extract was prepared by dispersing 16 g of the extract in 7 mL distilled water in a 100 mL beaker and transferred to a 20 mL volumetric flask. The beaker was thoroughly rinsed with distilled water; the content added to the volumetric flask and the volume made to mark. The animals were observed continuously for the first 4 h and every hour for the next 12 h, then 6 hourly for 56 h (72 h acute toxicity) to observe any death or changes in general behavior and other physiological activities.

Preparation and Evaluation of Ointments Formulations Containing Ethanolic Extracts of A. cordifolia leaf and T. superba stem bark

Different ointment bases (4): White ointment base USP, Lanolin ointment base USP, Hydrous Lanolin ointment base and Soft water washable ointment base were prepared by fusion method and their various efficacies in the formulation of herbal ointment evaluated (Table 1).

The white ointment base and the lanolin base were prepared using the fusion method described by Azubuike *et al.*[4]

The hydrous lanolin ointment base and soft water washable base were prepared by melting the oleaginous phase separately in a melting pan at 70ºC and heating the aqueous phase in another melting pan to 70ºC. The aqueous portion was then added to the oleaginous phase with continuously stirring until congealed. When ready to use, the ointment bases were melted and maintained at 70ºC.

A concentration of 1 g each of the ethanolic extract of *A. cordifolia* and *T. superba* was incorporated into the bases with continuous stirring to get an even mix. The prepared *A. cordifolia* herbal ointments and *T. superba* herbal ointments were then filled into ointment jars and stored at room temperature.

The ointment formulations (*A. cordifolia* and *T. superba* ointments) were evaluated for the following parameters: physical appearance, colour, texture, phase separation, and homogeneity spread ability and irritant effect using the method of Azubuike *et. al.*[4]

In-vitro antimicrobial efficacy of the formulated ointments was carried out using the agar diffusion method.[22]

Statistical Analysis

Data were expressed as mean ± SEM. Significant difference were analysed by one-way ANOVA and $P < 0.05$ was regarded as significant.

Results and Discussion

Preparation and Phytochemical Screening of Ethanolic Extracts A. cordifolia leaf and T. superba stem bark

The percentage yield of the ethanolic extract of *A. cordifolia* leaaf was 28.57% while that of *T. superba* stem bark was 17.83%. It must be noted that extract yields from plants are influenced by plant strain, geographical location, extraction medium and procedure among other factors.[4,25] Acording to Vogel *et al.*,[26] yields below 40% are *poor*. *A. cordifolia* and *T. superba* extracts therefore exhibited poor yield respectively which could be as a result of one or more of the factors stated above. *A. cordifolia* leaf had a better yield in comparison with *T. superba* stem bark.

The extracts were found rich in Phytochemical constituents. The ethanolic extract of the stem bark of *T. superba* revealed the presence of alkaloids, saponins, cardiac glycosides, tannins, flavonoids, gums and mucilages while the ethanolic extract of the leaves of *A. cordifolia* revealed the presence of, saponins, anthraquinone and cardiac glycosides, tannins, flavonoids, and gums and mucilage.

Antimicrobial Activity of Ethanolic Extracts of *A. cordifolia* leaf and *T. superba* stem bark.

One of the preliminary stages involved in the development and large-scale production of a new drug is the extraction of its active constituent[4]. In table 2, the *in-vitro* antimicrobial activity of the ethanolic extract of *A. cordifolia* leaf and *T. superba* stem bark are presented.

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The result of the *in-vitro* antimicrobial activity of the ethanolic extract of *A. cordifolia* leaves implies, the extract has a concentration dependent antibacterial activity but no antifungal activity. These findings are in contrast with the findings of Kra *et al*[29] which reported that T. superba was active against *Candida albicans*. The zones of inhibition of *A. cordifolia* leaf extract were remarkable for *Staphylococcus aureus* than the zones of inhibition of Ciprofloxacin. The significant ($p \leq 0.05$) antibacterial activity of the extract on *S. aureus* shows they can be employed in the treatment of skin disorder like impetigo and eczema caused by *S. aureus*. The mixture of the extracts showed reduced activity compared to each of the extracts. The mixture of *A. cordifolia* and *T. superba* extracts showed reduced zones of inhibition when compared with each of the extracts. This could be as a result of interaction between the extracts leading to the formulation of less active antimicrobial agent. These extracts therefore should not be used in combination as an antimicrobial agent. The antimicrobial activities of Ciprofloxacin BP (standard anti-bacterial) and Clotrimazole BP (standard anti-fungal) on the microorganisms are shown on Tables 3 and 4. The extracts compared favourably with the standards. The MIC values of *A. cordifolia* leaf extracts against the test organisms are: *B. sub* 1.8 mg/ mL, *E. coli* 1.8 mg/mL, *S. aureus* 12.8 mg/mL, *P. aeruginosa* 25.6 mg/mL. while that of *T. superba* against the testorganisms 26.6 mg/L for the organisms respectively (Table 5). The mixture of *A. cordifolia* and *T. superba* extracts showed reduced zones of inhibition when compared with each of the extracts. This could be as a result of interaction between the extracts leading to the formulation of less active antimicrobial agent. These extracts therefore should not be used in combination as an antimicrobial agent.

Acute Toxicity Screening of the Ethanolic Extracts of A. cordifolia leaf and T. superba stem bark

The results of the acute toxicity evaluation of *A. cordifolia, T. superba* and the mixture of both extracts are shown in Figure 1. A 100% death was recorded for the animals fed with 20.0 g/kg body weight of the single and combined extracts while 80.0 % death occurred in the group of animals fed with 15.0 g/kg body weight of *A. cordifolia*. *T. superba* and the mixture of both extracts recorded 60 % and 20 % death respectively when fed with 15.0 g/kg body weight of the extracts. There was no death recorded in the group of animals that received 5.0 and 10.0 g/kg body weight of the extracts singly and in combination. The median lethal dose (LD_{50}) for the ethanolic extracts of *A. cordifolia* leaves, *T. superba* stem barks and the mixture of these extracts were 14.79 g/kg, 15.85 g/kg and 17.78 g/kg, respectively. Each of the extracts and their combination could be classified as nontoxic, since the LD_{50} by oral route were found to be 14.8 g/kg, 15.8 g/kg and 17.8 g/kg respectively which were much higher than the toxicity index of 2.0 g/kg body weight.recommended by WHO. Acording to Ogbonnia et al, the LD_{50} of more than 15.0 g/kg body weight could be translated to 1050 g dose in an average adult man of 70 kg.

Evaluation of A. cordifolia and T. superba ointments

The formulated ointments appeared to be uniformly mixed, non- gritty, homogenous and had no phase separation after 4 weeks. The white ointment base and the lanolin base were greasy while the hydrous lanolin and the soft water washable base were non-greasy to touch.

The *in-vitro* antimicrobial screening of the formulated *A. cordifolia* and *T. superba* ointments revealed the soft water washable ointment base containing ointments possessed antibacterial activity against all the bacterial organisms (Tables 6 and 7). According to Shelke and Mahajan,[26] an ideal ointment for medicinal purposes should be non-greasy, non-sticky, non-gritty (smooth), spreadable, homogenous and have no phase-separation. From the results it can be deduced that the herbal ointments formulated using hydrous lanolin and soft water washable bases passed the organoleptic tests because they possessed the properties of an ideal ointment.

From the results of the antimicrobial potency of the herbal ointments, only the topical formulation prepared using the soft water washable base effectively released the antibacterial extracts as it had zones of inhibition similar to that of the crude extract. The *A. cordifolia* ointment formulation prepared using hydrous lanolin base was potent only against *P. aeruginosa*. White ointment base and lanolin base had no activity.

Table 1: Formulation of Ointment bases.

Formulations	Ingredients	Concentration (%w/w)	Quantity used (g)
Formulation I	White wax	5%	0.5
	White petrolatum	95%	9.5
Formulation II	Wool fat	100%	10
Formulation III	Wool fat	70%	7
	Distilled water	30%	3
Formulation IV	Stearic acid	0.07%	0.7
	Cetyl alcohol	0.02%	0.2
	Glycerin	0.10%	1
	Mineral oil	0.20%	2
	Triethanolamine	0.02%	0.2
	Purified water to:	100%	10

Formulation I: White ointment, II: Lanolin, III: Hydrous and IV: Soft water washable base.

Table 2: Zones of Inhibition (mm) of the Ethanolic Extract of *A. cordifolia*, *T.superba* and the Mixture of the Extracts.

		Organisms/Zones of Inhibition					
Extract	**Conc.**	***C. albicans***	***E. coli***	***S. aureus***	***P. aerugin***	***A. nig***	***B. subt***
	50	15.5 ± 0.5	10.0 ± 0.0	16.5 ± 0.5	16.0 ± 1.0	_	_
A. Cord	100	16.0 ± 0.0	16.5 ± 0.5	18.5 ± 1.5	20.5 ± 0.5	_	_
	200	17.0 ± 0.0	18.0 ± 0.0	20.0 ± 0.0	22.0 ± 0.0	_	_
	50	15.5 ± 0.5	10.0 ± 0.0	16.5± 0.5	16.0 ± 1.0	_	_
T. sup	100	16.0 ± 0.0	16.5 ± 0.5	18.5 ± 1.5	20.5 ± 0.5	_	_
	200	17.0± 0.0	18.0 ± 0.0	20.0 ± 0.0	22.0 ± 0.0	_	_
	50	16.5 ± 0.5	20.0± 0.0	16.5± 0.5	14.0 ± 1.0	_	_
Mixture	100	17.0 ± 1.0	20.0 ± 0.0	18.5 ± 1.5	18.5 ± 0.5	_	_
	200	20.0 ± 0.5	21.0 ± 0.0	20.0 ± 0.0	22.0 ± 0.0	_	_
D. water		_	_	_	_	_	_

-: No zone of inhibition, Conc = concentration, *P. aerugin =Pseudomonas aeruginosa*, A. nig = *Aspergilus niger, B.subt =Bacillus subtilis, A. cord = A. cordifolia,* T. sup = *T. superba,* D. water = Distilled water.

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Table 3: Zones of Inhibition (mm) Obtained with Ciprofloxacin (Positive Control for Anti-Bacterial Activity).

Microorganism	Concentration of ciprofloxacin (µg/mL)			
	2.5	5	10	20
Bacillus subtilis	20	23	26	27
Escherichia coli	15	19	21	21
Staphylococcus aureus	11.5	15	20	23
Pseudomonas aeruginosa	-	18	25	27

Table 4: Zones of Inhibition (mm) Obtained with Clotrimazole.

Concentration of Clotrimazole (µg/mL)	Microorganism/Zones of inhibition (mm)	
	Aspergillus niger	*Candida albicans*
20	28	25
40	27	23
80	25	22
160	22	21

Table 5: Minimum Inhibitory Concentration of the Ethanol Extracts of *A. cordifolia* Leaves and *T. superba* Stem Bark.

	MIC (mg/mL)	
Microorganisms	*A. cordifolia* Leaves	*T. superba* Stem barks
B. subtilis	12.8	25.6
E. coli	12.8	25.6
S. aureus	12.8	25.6
P. aeruginosa	25.6	> 25.6

This result can be attributed to the properties of the ointment bases as explained by The Pharmaceutics and Compounding Laboratory.[27] The white ointment base is an oleaginous base and oleaginous ointment bases are very occlusive and have poor drug release potential. The lanolin base is an absorption ointment base and absorption ointment bases are also occlusive, their drug release potential is also poor but it is better than that of the oleaginous ointment base. The hydrous base is a water in oil emulsion ointment base with HLB value ≤ 8, it is sometimes occlusive but has a fair drug release potential. The soft water washable base is an oil in water emulsion ointment base with HLB value 8, it is sometimes occlusive but has a fair drug release pote

Conclusion

The ethanolic extract of the leaves of *A. cordifolia* and stem barks of *T. superba* possess high safety margin with LD_{50} values of 14.8 and 15.8 g/kg, respectively, an indication that the extracts might be safe for medicinal use. Their ointment formulations with soft water washable bases could be effectively used against *Staphylococcus aureus* dermatological infections.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 6: Zone of inhibition (mm) of *A. cordifolia* Topical Formulations on the Test Bacteria.

	Bases			
Microorganism	Petroleum jelly	Lanolin base	Hydrous lanolin base	Soft water washable base
E. coli	-	-	-	14.5 ± 0.5
B. subtilis	-	-	-	16.5 ± 0.5
P. aeruginosa	-	-	10.0	18.0
S. aureus	-	-	-	18.0

- : No zone of inhibition.

Table7: Zone of inhibition (mm) of *T. superba* Topical Formulations on the Test Bacterial Organisms.

Microorganism	Bases/Zones of inhibition			
	Petroleum jelly	Lanolin base	Hydrous lanolin base	Soft water washable base
E. coli	-	-	-	14.5 ± 0.5
B. subtilis	-	-	-	19.5 ± 0.5
P. aeruginosa	-	-	-	16.0 ± 0.0
S. aureus	-	-	-	21.5 ± 0.5

- : No zone of inhibition

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