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Formulation and Evaluation of Anti-Dermatophyte Creams from Ethanol Extract of *Acalypha wilkesiana* Leaves

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Abstract

Dermatophytosis infections are caused by dermatophytes. Drug resistance and toxicity associated with long-term treatment with conventional antifungal drugs has necessitated search for new drugs to treat fungal infections. Natural products found in plants have been scientifically proved to avoid these side effects. The aim of this study was to formulate herbal antifungal cream containing extract of *Acalypha wilkesiana* as an anti-dermatophytic preparation and evaluate its physicochemical properties, stability and efficacy of the product. The formulated creams containing 0.5, 1 and 2% w/w of extract were subjected to stability tests using temperature variation method at -10, 4, 30, 37 and 45°C. Freeze-thaw test, Centrifuge test, pH and exposure to UV light test were also carried out using standard method. Efficacies of the cream formulations were determined using albino rats.

The percentage yield of the extract was (10.2%). Percentage ethanol phytochemical composition indicated that for Alkaloid it is 4.58 ± 0.01%, saponins (3.10 ± 0.23%), flavonoids (1.61 ± 0.04%) and tannins (0.81 ± 0.02%).The antifungal results are in the increasing order *Microsporum audounii* = *Epidermophyton floccosum* < *M.furfur* < Trichophyton *mentagrophtes*. Temperature stability tests carried out indicated that the cream was very stable. Centrifuge testing indicated that there was no separation of the cream. Light testing indicated no change in the colour and odour of the products. There was no change observed in all the test samples during the freeze-thaw testing. Animal studies evaluation of the ethanolic formulations of the cream indicated that their efficacy against the dermatophytes is concentration dependent and the efficacy is in the increasing order *M.audounii* < *E.floccosum* < *M.furfur* < *T.mentagrophyte* which shows that 2% *Acalypha wilkesiana* cream was statistically significant (P<0.05) against all the test microorganisms.

Keywords: Dermatophytosis; Keratinization; *Acalypha wilkesiana;* Alkaloid, *Malassezia furfur*

Introduction

Dermatophytosis is a skin condition caused by dermatophytes which is fungi, and they require keratin for growth. There have been reports by researchers that dermatophytes have the ability to obtain nutrients from keratinized materials such as the skin, hair and nails. Skin infection commonly called ringworm which infects the legs, arms, beard area, scalp and the groin area has been attributed to be caused by these dermatophytes [1].

There have been reports of the ability of Medicinal plants to synthesize chemical compounds that are used to defend themselves against attack from predators such as insects and fungi [2]. Developing countries often resort to the use of medicinal plants products to treat diseases because the products are more affordable than purchasing modern pharmaceuticals.

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Acalypha wilkesiana otherwise known as copper leaf is classified into the family of Euphorbiacaece. The genus is comprised of about 570 species, while some are classified as weeds; others are known to be used as ornamental plants [3]. It is a tropical plant found in America, Africa and Asia which grows everywhere and might have been introduced into West Africa [4]. The plant is an evergreen shrubs having splash of colours determined by how it is cultivated [5]. Studies carried out by Oladunmoye reported the presence of important phytochemicals present in the leaves of *A. wilkesiana*. Adesina et al., also reported that *A. wilkesiana* has antibacterial and antifungal properties. Our aim for this study was to formulate a cream with different concentrations of *A. wilkesiana* extract as antidermatophyte preparations, and evaluate their stability and physicochemical properties.

Materials and Methods

Plant materials

Leaves of *Acalypha wilkesiana* was collected at the Botanical Gardens of the University of Ibadan. It was identified by the taxonomist and sample deposited with herbarium number MPNH/2017/1252 at the Medicinal Plants of Nigeria Herbarium of NNMDA. The plants were air dried in the shade and pulverized to fine-sized particles for solvent extraction processes.

Preparation of plant extracts

200g of the pulverized plant sample was extracted with ethanol and using soxhlet extraction method. Rotary evaporator was used to recover the solvent from the mixture.

Phytochemical constituents Determination

Simple qualitative and quantitative methods of Trease and Evans (1989) and Sofowora (1993) were used to determine the presence or otherwise of phytochemical constituents.

Micro-organisms

Clinical isolates of *Microsporum audouinii, Epidermophyton floccosum, Trichophyton mentagrophyte* and *Malassezia furfur* were obtained from Spectralab Medical and Diagnostic Services, Sagamu, Ogun State.

Microbiological Assay

Zones of inhibition were determined using the method as described by Irobi and Daramola (1994). The zones of inhibition were measured in mm and recorded.

Materials for Emulsion Formulation

All oil soluble substances were placed in a stainless steel container and heated to between 70-75°C. All water-soluble substances were placed in another stainless steel container and heated to the same temperature. The oil phase was then added to the aqueous phase slowly with stirring. Heating was continued at the same temperature for about 10-15 minutes. The coarse emulsions formed were then cooled to about 35°C gradually. The emulsions were allowed to stay at room temperature for twelve hours and then homogenized with the aid of a mechanical stirrer. The samples were then poured into labeled containers. Emulsions containing 0.5%, 1% and 2%, of *Acalypha wilkesiana* ethanolic extracts were produced. The prepared herbal emulsions were then vigorously homogenized.

Stability Tests for the formulated emulsion

Stability tests were carried out on the emulsions following the methods as described by Cannel (1992) whereby the temperature variation tests include storing the samples at -10°C, 4°C, 30°C, 37°C and 45°C were carried out. All observations including pH, colour and odour were noted and recorded. The creams were made to pass through Freeze thaw cycles testing which involves making the samples pass through three cycles of temperature testing by placing the samples at -10°C for 24 hours and then at room temperature for 24 hours. The creams were made to pass through centrifuge testing whereby the samples were heated to 50°C and they were then centrifuged for thirty minutes at 2000, 2500, 3000 and 4000 rpm. They were then inspected for signs to determine if the dispersed phase of the emulsion has separated and risen to the top. The creams were made to pass through light testing whereby they were placed in test tubes and also in the actual package. They were then put in the window where direct sun rays fell on them. This method is used to determine the sensitivity of the emulsions to the Ultra Violet radiation.

In vivo antidermatophytic activity

Ethical statement: The experimental procedures complied with University of Ibadan ethics committee in line with approval number UI-ACUREC/App/12/2016/06.

Laboratory Animals: Albino rats weighing between 150-200g were kept in cages with access to water and feed. They were left in this environment for two [6] weeks to acclimatize [7].

Selection and grouping of animals: The animals were randomly allocated to six [8] groups (5 rats/group) such that the difference in average weight did not exceed 5g. Each animal was used once in the experiment. The rats were then inoculated with the dermatophytes. One week after inoculation of the animals with the dermatophytes, the inoculated skin area of $2cm^2$ were treated with the plants extracts and the formulated emulsions with plants extracts for seven days. They were euthanized at the end of seven days.

Histopathological studies: $2cm^2$ skin areas were cut and put in 10% formalin for histopathological analysis. Skin biopsy samples were examined for presence of fungal hyphae, hair follicles, sebaceous gland, inflammation and tissue destruction using light microscope [9].

Statistical analysis of Data: ANOVA was used followed by tukeys post hoc analysis. Data is reported as mean ± SEM. $P \leq 0.05$ was considered significant. Also epidermal thickness and keratin layer were obtained with the aid of calibrated Toupview® software (Table 1).

Antifungal activities

The results obtained for the antifungal activities indicated that the ethanol extract of the leaves of the plants was very active against the test microorganisms as shown in Figure 1. At 10µm/mL, *M.furfur* had the highest activity with zone of inhibition of 9mm followed by *T.mentagrophyte* (7mm) and then *M.aoudinin* (5mm).There was no activity recorded against *E.floccossum*. At 100µm/mL, the activity of *M.furfur*, *T.mentagrophyte* and *M.aoudinin* remain the same but activity was noticed for *E.floccossum* (4mm) at this concentration. There was marked

Table 1: Percentage phytochemical composition.

Medicinal plants	Alkaloid (%)	Saponin (%)	Flavonoid (%)	Tannin (%)
A.wilkesiana	4.58 ± 0.01	3.10 ± 0.23	1.61 ± 0.04	0.81 ± 0.02

Each value in the table is the average ± standard deviation of triplicate experiments.

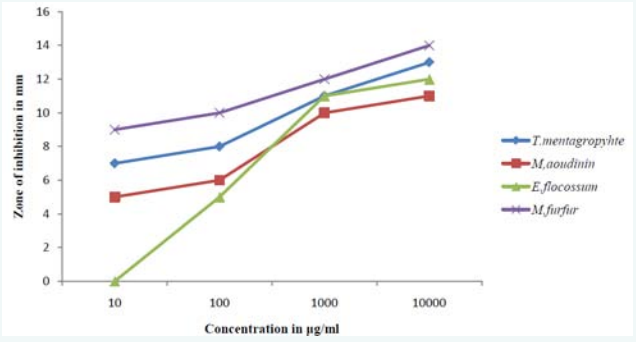

Figure 1 Antifungal Screening of *Acalypha wilkesiana* ethanol extracts.

activity of the extract against the dermatophytes at 1000µm/mL. Activity against *M.furfur* was 11mm > *T.mentagrophyte* = *E.floccossum* (9mm) > *M.aoudinin* (8mm). Activity of the extract at 10000 µm/mL was *M.furfur* (14mm) > *T.mentagrophyte (*12mm) > *E.floccossum* (10mm) > *M.aoudinin* (9mm). The results obtained indicated that the activity against the dermatophytes are in the order *M.furfur* > *T.mentagrophyte* > *E.floccossum* > *M.aoudinin.*

Temperature Stability Testing

The pH of the samples at two weeks of test was slightly lower compared to the pH at day 1 of production. Colour and odour remain stable across the test temperatures except at 45°C where there are detectable changes in the colour and odour of the samples. At four weeks of test, the pH remained stable at all the test temperatures. There was no colour or odour change. After eight weeks of test; no changes were detected in the pH, colour and odour of the test samples. At twelve weeks, there was slight decrease in the pH of the samples and also slight changes in the colour and odour. At sixteen weeks of test, there were no significant changes in pH, colour and odour compared to what was observed at twelve weeks of test.

Centrifuge Testing

No phase separation was detected in all the test samples at 2000, 2500, 3000 and 4000 rpm. Also, during light and cycle testing, there were no changes noticed (Table 2).

Histopathology of skin tissues

Tissues were examined for the presence of fungal elements; inflammation, Fungal hyphae, loss of hair follicles, absence of sebaceous gland and discontinuity/Tissue destruction as well as dekeratinisation and epidermal thickness.

Animal studies using Acalypha wilkesiana ethanol extracts against the dermatophytes

The untreated control group indicated heavy infection of the hairs by the dermatophytes (Figures 2,5 and 6). Same goes for the group treated with the emulsion alone, the group treated with 0.5% *Acalypha wilkesiana* extract alone and the group treated with 0.5% *Acalypha wilkesiana* extract emulsion was not statistically different from one another (Figure 3). The values obtained for 1% *Acalypha wilkesiana* extract alone and 1% *Acalypha wilkesiana* emulsion indicated slight efficacy by the formulations (Figure 4). Comparatively, 2% *Acalypha wilkesiana* extract alone and 2% *Acalypha wilkesiana* extract emulsion showed moderate mycological efficacy. The standard drug 1% clotrimazole cream demonstrated high mycological efficacy in preventing attack by the dermatophyte on the keratin layer of the hair. Statistically, there is significant difference in keratinization between the groups as determined by one way Anover.

Table 2: Centrifuge, Light and Cycle Testing.

Centrifugation	NPS
Light	NC
Cycle	NPS

Abbreviations: NPS = No Phase Separation; NC = No Change in colour

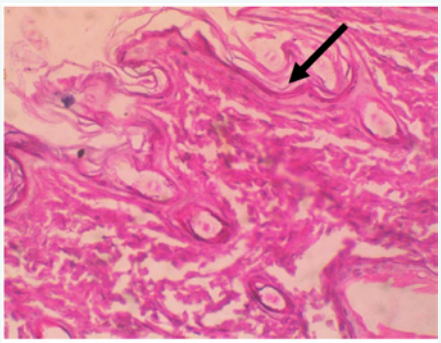

Figure 2 Tissue destruction by the dermatophyte H & E stain x100.

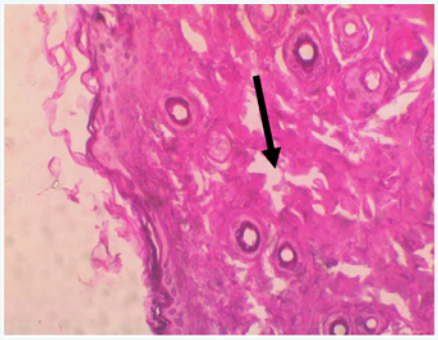

Figure 3 Discontinuity of the skin structure H & E stain x100.

There was significant infection as indicated by the result obtained for the epidermal thickness of untreated group. The values obtained for emulsion alone, 0.5% *Acalypha wilkesiana* extract, 0.5% *Acalypha wilkesiana* extract emulsion, 1%*Acalypha wilkesiana* extract and 1% *Acalypha wilkesiana* emulsion in reducing the epidermal thickness cannot be said to be significant compared with the untreated control. However, the values obtained for 2% *Acalypha wilkesiana* extract alone and 2%*Acalypha wilkesiana* emulsion with extract showed indication that there was moderate reduction in the epidermal thickness thereby suggesting that the formulations are mycologically effective against the dermatophyte compared with the values obtained for the standard drug. The statistical evaluation indicated that there is significance difference in epidermal thickness between groups.

Discussion

Angkhana Inta *et al.*, , reported that plants extracts were used in the treatment of different ailments and have been known to inhibit microorganisms. Phenolic compounds of natural origin have been reported to play an important role in the management and treatment of diseases [10].

The chemical constituents of the plant crude extract showed that it was rich in alkaloids, saponins, tannins and flavonoids which have been known to exhibit medicinal activities [11]. Our findings are in agreement with works carried out by Oladunmoye, 2006 and Ezekiel et al., [12].

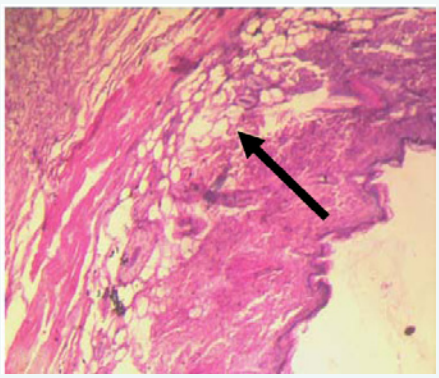

Figure 4 Inflammation of the skin tissues H & E stain x100.

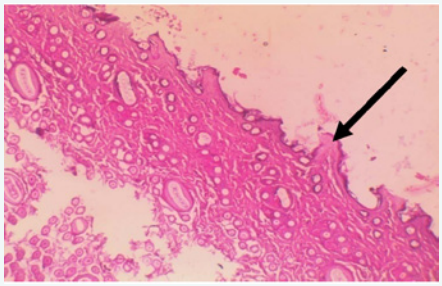

Figure 5 Increase in epidermal thickness.

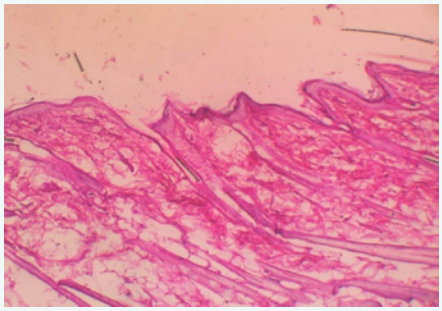

Figure 6 Invasion by dermatophyte hyphae H & E stain x100.

The results show that the crude ethanol extract of *Acalypha wilkesiana* possesses antifungal activities against the dermatophytes, thus confirming the folkloric use of the plant. This indicated that the plant extract may be effectively used in the management of dermatophytosis. The extract had activity against *T.mentagrophyte, M.aoudinin* and *M. furfur* at all concentrations with zones of inhibition in the range of 5-14 mm except against *E.flocossum* at 10µg/mL. Adesina et al., [13] confirmed this observation. This activity may be attributed to the active components which are enhanced in the presence of ethanol [14-18].

The temperature stability testing carried out on the formulation indicated that the product is very stable, though some changes were noticed at elevated temperature of 45°C. The pH was stable but there was a noticeable change in odour and colour of the product at 45°C. This could be due to the fact that the elevated temperature degenerate the components of the products. There was no significant change during cycle and centrifuge tests. Also there was no change in colour during the light test.

Evaluation of the efficacy of the antidermatophyte cream using albino rat's model indicated that 2% formulation was as effective as the control drug. Inflammatory response in the infected rats which is largely composed of neutrophilis in the early phases was reversed with treatment with the formulation (Figure 5). In most cases, few fungal elements were detected in the stratum cornea of skin from infected animals. Also it was observed that there are sebaceous glands compared to the untreated group. Our data using the epidermal thickness values showed that the cream is most active against *T.mentagrophyte* (37.01 ± 0.39) > *M.furfur* (35.83 ± 0.57) > *E.flocossum* (33.65 ± 0.74) > *M.aoudinin* (31.01 ± 0.62) which show that the formulation is statistically significant ($P<0.05$) against all the test microorganisms.

Conclusion

This study shows that *Acalypha wilkesiana* ethanol extract has high potential as an antidermatophyte agent when formulated as cream for topical application. This explains the folkloric use of the medicinal plant. Among the prepared formulations, 2% formulation showed highest activity against all the dermatophytes. The formulations showed acceptable physical properties and were stable during the accelerated stability test.

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