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Research Article

Phytochemical composition, anti-fungal activity of *Mucuna pruriens* (L.) DC. (Fabaceae) seed extract and acute toxicity testing of formulated herbal ointment

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Abstract
The growing resistance to conventional dandruff remedies has led to the search for newer and affordable treatments from African medicinal plants. This study evaluates the anti-fungal activity of methanol extract of the seed of *Mucuna pruriens* L. (DC.) Fabaceae (MP) against *Malassezia furfur* and *Malassezia globosa* which have been implicated in dandruff etiology.
The MP seed sample was collected from Orba, Enugu State, dried, and pulverized. Macroscopic, microscopic, and phytochemical evaluations of the seed were done. Defatting was done with n-hexane extract and cold maceration with methanol for 72 h (ME extract). The anti-fungal activity of the extracts was determined using the agar dilution method with ketoconazole as standard. Two samples of ointment containing 5% and 10% MP seed methanol extract were prepared with an emulsifying base using a fusion technique. An acute skin toxicity test was carried out by applying the formulation to the skin of albino rats and observations were made. Results: Quantitative phytochemical analysis of secondary metabolites in the MP seed gave: phenolic content (8.3%), alkaloids (0.02%), flavonoids (0.2%), saponins (0.4%), tannins (0.09%). The ME extract inhibited growths of *Malassezia furfur* (MIC 3 mg/mL) and *Malassezia globosa* (MIC 2 mg/mL). The 5% and 10% samples of the herbal ointment MPO showed no toxicity or irritancy on the animals. Conclusion: Methanol extract of *Mucuna pruriens* possess antifungal activity against *Malassezia furfur* and *Malassezia globosa* and its ointment appears non-toxic.

Key Words: *Malassezia furfur*, *Malassezia* globosa, Mucuna pruriens, antifungal, ointment, dandruff.

INTRODUCTION

Dandruff which is medically known as *Pityriasis capitis* (Nengimoyo et al., 2016) is one of the fungal infections that is becoming increasingly worrisome. It usually occurs on the scalp and is characterized by itchy and flaky skin which is usually whitish. Dandruff is a reactive response of the epidermis of the scalp to various stimuli of which *Malassezia* species remains the main causative agent. This organism has also been implicated in the development and persistence of eczema in several patients with other skin disorders such as atopic dermatitis (Danuta & Urszula 2019). The existence of *Malassezia* yeasts at the boundary between commensal and pathogenic organisms makes them an unavoidable group of organisms in understudying the link between dandruff and human immunity (Hay 2011; Nengimoyo et al., 2016).

Every year, several human beings are affected by fungal diseases. The reason is due to the rise of advances in the diagnostics of fungal infections (Osman et al., 2020). However, high morbidity and mortality from fungal infections have been reported to have emanated from delays in diagnosis and treatment of infections which after some time may develop resistance and the severity of groups of illnesses (Schelenz et al., 2015). Of all causative organisms of fungal infections, *Candida spp*, *Cryptococcus neoformans*, *Aspergillus* spp, *Histoplasma capsulatum*, and *Pneumocystis jirovecii* are the commonest (Gordon et al., 2012, Felix et al., 2017). The estimated annual incidence of invasive mycoses due to some of these pathogens is on the increase. *Candida species* accounts for more than 400,000 infections with 30-95% mortality, *C. neoformans* accounts for more than 1,000,000 infections with 20-70% mortality per year> 200,000 infections per year with 30-95% mortality for *Aspergillus*

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species and *Pneumocystis jirovecii* among others accounts for more than 400,000 infections with 20-80% mortality per year (Gordon et al., 2012, Oladele et al., 2020).

With more attention being put into the eradication of skin diseases in recent times, *Malassezia* species have not been found to cause any serious disorder aside from mild discomfort. Figueredo et al., 2013; and Pedrosa et al., 2019 reported that biofilm formation and extracellular matrix generation were the major reasons for the emergence of antifungal resistance. Figueredo (2013) reported that hydrophobicity, adherence, and phospholipase production of pathogenic *M. pachydermatis* and *M. furfur* cells, may help explain the change from a commensal to a pathogenic phenotype of these organisms.

In Nigeria, there is a paucity of data on itchy scalp disease nor is there any safe and highly efficacious remedy in place; the existing ones are major of conventional origin which might pose the problems of high costs, antimicrobial resistance, long duration of use, side-effects and many adverse effects. Those who cannot afford these treatments result in random guessing and trial-and-error initiative on herbal preparations in a desperate bid to find a fast and lasting cure. The search for new compounds/agents with anti-fungal activity is accelerating due to rising yeast and fungal resistance to commonly prescribed conventional drugs. Though a lot of studies have been conducted on the plant Mucuna pruriens, very little has been explored about its anti-fungal activities against *Malassezia* species, despite being a fungus of medical importance (Klafke et al., 2012). Therefore, this study evaluated the anti-fungal activity of *Mucuna pruriens* L. (DC.) (Fabaceae) seed against selected dandruff-causing fungi and developed an alternative herbal remedy for this infection.

MATERIALS AND METHODS

PLANT MATERIALS: The seed of *Mucuna puriens* (L.) DC. (Fabaceae) was collected from Orba in Udenu Local Government Area of Enugu State in April 2020 and identified by Mr. Felix Nwafor, a taxonomist at the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka, Enugu State. The voucher specimen was deposited at the herbarium of the Department of Pharmacognosy and Environmental Medicine, UNN with a voucher specimen number (PCG/UNN/0349).

Extraction: (i) Methanol extraction: The seed of M. pruriens was defatted with n-hexane (GH-TECH) and extracted by cold maceration with methanol (GH-TECH) for 72 h and filtered using a muslin cloth and again with a filter paper. The filtrate was evaporated to dryness using a rotary evaporator at 40oC and stored at -20 oC until ready for use. (ii) Aqueous extraction: aqueous extraction was carried out using distilled water for 24 h. The filtrate was collected using a muslin cloth and again with filter paper. The filtrates were evaporated to dryness using a freeze dryer and stored at -20 oC until ready for use.

Phytochemical screening: Preliminary phytochemical screening for various classes of secondary metabolites in the methanol extract of *Mucuna pruriens* (L.) DC was carried out using standard laboratory procedures as modified by (Gavishidappa et al., 2015).

Microscopy of powdered seed: The powdered seed microscopy was carried out using standard methods as described by Nwafor et al., 2019.

Chemomicroscopic tests: Chemomicroscopic tests were carried out to determine the presence of starch, calcium oxalate crystals, and lignified vessels using methods described by Odoh et al., 2011 and Nwafor et al., 2019.

Anti-fungal assay: The Sabouraud's Dextrose agar was prepared following the manufacturer's instructions. The agar was enriched with 0.5 mg/L Chloramphenicol, 0.4 mg/L cycloheximide, and 0.5ml/L Olive oil to disallow the growth of molds and bacteria. The test microorganisms used (*Malassezia furfur, Malassezia* globosa) were obtained from the Department of Pharmaceutical Microbiology and Biotechnology Laboratory, University of Nigeria, Nsukka Enugu State, Nigeria. The pure isolate was maintained on a sterile Sabouraud's Dextrose agar slant for 2 days. The smear was prepared by placing the specimens on a slide. A drop (0.05 mL) of 20% potassium hydroxide was made on the smear. The smear was covered with a coverslip and examined immediately, then re-examined after 20 mins intervals.

Characterization of fungi isolates was carried out as described by Ogba et al., 2016 and observations were made based on the shape, chromogenesis (pigmentation), surface texture, edge, and elevation. A 24 h culture of the isolates was used to prepare the inoculum. A suspension of the organism was constituted in 5 mL of sterile normal saline. The turbidity was adjusted to 0.5 McFarland standard which is an equivalent of 1 x 106 to 5x106 CFU/mL. One mL of the McFarland-adjusted culture was inoculated on Sabouraud's Dextrose agar plate. After the surface has dried, three holes were bored with a sterile 2 mm diameter punch and filled with 5 µL of Tween 20, 40, and 80 respectively. The plates were incubated for 7 days at 37°C. The tween utilization was assessed by the degree of growth and/or precipitation of the lipid-loving yeasts around the wells as well as their rates of growth. Plates with visible growths were incubated at 40°C for 48 h, only *M. furfur* can survive this temperature. The organisms were standardized using 0.5 McFarland turbid equivalents.

Afterward, a 50 mg/mL stock concentration of the crude extract was prepared by serial dilution to achieve concentrations ranging from 10 mg/mL to 1mg/mL. The molten agar plates with different concentrations of the methanol extract were allowed to gel while the plates were divided into two equal parts with a permanent marker. The test fungi were streaked on the segments, labeled and the culture plates were incubated in an inverted position at 25oC for 1 week. After the due period of incubation, the plates were observed and the results were recorded (Vincent Okore, 2009; Mounyr et al., 2016).

The same procedure was used for different concentrations of the standard drug Ketoconazole ranging from 0.5 to 5.0

µg/mL. The n-hexane extract and the vehicle, Dimethyl sulfoxide (DMSO) at concentrations ranging from 9.0 mg/mL to 1 mg/mL. The in vitro antifungal property of M. pruriens seed extract was assessed using the agar dilution method. The Minimum Inhibitory Concentration (MIC) was determined as the minimum concentration to inhibit the growth of the organisms using the agar dilution method.

Ointment formulation: According to the findings of the antimicrobial tests, both *Malassezia furfur* and *Malassezia globosa* growth were inhibited by the methanol extract of M. pruriens. The methanol extract was utilized to make the ointment formulation after the MIC was determined, using the following emulsifying ointment formula:

Emulsifying wax – 30 g

Soft white paraffin – 50 g

Liquid paraffin – 20 g

Total – 100 g

Preparation of 5 and 10% ointments: The ointment base was prepared by melting together 28.5 g emulsifying wax, 47.5 g white soft paraffin, and 19 g liquid paraffin for the 5% ointment and 27 g emulsifying wax, 45 g white soft paraffin, 18 g liquid paraffin for the 10% ointment all at 60ºC. Each mixture was maintained at this temperature and stirred vigorously to keep it from solidifying. A preservative, 1 g of cetrimide was dissolved in 2 drops of 0.05 mL methanol and mixed with 3 g of methanol extract of M. pruriens for each preparation and using the fusion method, this was incorporated into the ointment base when the ointment base became a colorless and clear solution (showing complete dissolution) and 15 mL of each of the 5% and 10% preparation was poured into ointment containers just before it solidified and it was packaged. The preparations were labeled appropriately.

Acute toxicity testing of *Mucuna pruriens* ointment: Wistar rats of average weights between 176 g to 238 g of both sexes were used for these experiments. They were obtained from the Department of Pharmacology & Toxicology Animal House, UNN. The animals were housed in standard environmental conditions, fed with a standard rodent diet, and given water *ad libitum*. The experimental protocol was in conformity with the Ethics Committee Guidelines of the University of Ibadan with ethical approval number (UI-ACUREC/17/0105) as well as the US guidelines of internationally accepted principles for laboratory animal use and care. Animals were also treated in agreement with OECD guidelines for testing of animals as modified by Tiza et al., 2018. An evaluation was conducted using Wistar rats as described by Rasheed et al., 2003 with slight modifications (Yakubu et al., 2015). Animals were grouped into four with three animals in each group and anesthetized with 50 mg/kg body weight of ketamine hydrochloride, to restrict the mobility of animals for at least 120 mins after administration following the guidelines of the Organization for Economic Co-operation and Development test guidelines with slight modifications by (Tiza et al., 2018). The back of the animals was shaved free of fur with an electric clipper for 24 h after which the *MP ointment* (MPO) was applied. This was carefully done to avoid skin injury which could alter its permeability. A small piece of cotton wool was dipped into the methylated spirit and rubbed as an antiseptic on the shaved region to stop the colonization of organisms such as bacteria. A meter rule was used to measure an area of 40 mm x 30 mm on the shaved skin.

For the test groups, A & B 0.5 g of the MPO (5%) and MPO (10%), respectively, was applied on a free surface of the skin. The third group C received the standard drug Ketoconazole while the negative control group received the vehicle (petroleum jelly). The MPO-skin contact was maintained with a zinc oxide adhesive plaster plus a non-occlusive bandage dressing for 24 h. After the contact period, the bandage and the plaster were removed and the MPO test material cleared off while the site that received the treatment was rinsed with distilled water and after 1 h, examined for skin irritation. Observation at the sites was done 24 h after application and repeated at 48 and 72 h, 7, 14, and 21 days thereafter.

Statistical analysis: All the experiments were conducted in triplicate and the results obtained were expressed as mean ± standard errors of means (SEM). Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 10 (SPSS Inc., Chicago, USA), with one-way analysis of variance (ANOVA). Differences in the activity lower than $p < 0.05$ were considered statistically significant.

RESULTS

Microscopy of M. pruriens seed: Histological data of plant materials are very useful in taxonomic classification since they help to explore the inherent cellular characteristics for distinguishing species of different or the same genius. They also make up the vital microscopy standards that constitute parts of a medicinal plant monograph in a herbal pharmacopeia; a compendium that provides adulterant-detecting and overall quality-assurance tools for crude drugs in commerce.

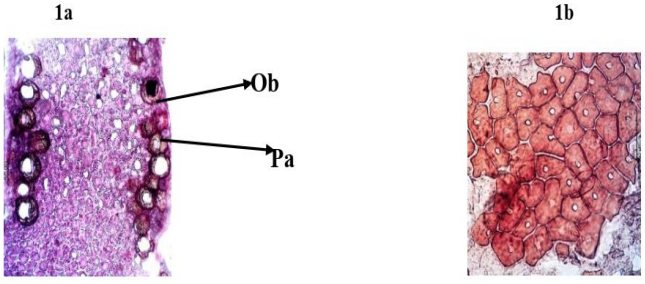

Figure 1: (a) Transverse section of M. pruriens seed showing the oil bodies (Ob) and parenchyma tissue (Pa). (b). Microscopy of M. pruriens seed showing the irregularly shaped parenchyma cells magnification X400

The seed microscopy of M. pruriens as shown in Fig 1a and 1b revealed the presence of oil bodies, parenchyma tissue, lignified tissues, fibers, and protein bodies. The parenchyma is the most diverse and versatile cell type and comprises the majority of cells. This tissue serves as one of the ground

tissues responsible for photosynthesis (chlorenchyma), protection (epidermis), storage, meristematic conduction (translocation via phloem) of nutrients, secretion and wound repair (Crang et al., 2018). In *Mucuna pruriens* seed, a very clear and distinguishing characteristic is the presence of protein bodies, fats, and oils which accord the plant a rich nutritive property confirming its use as food for both man and livestock (Lucia et al., 2012).

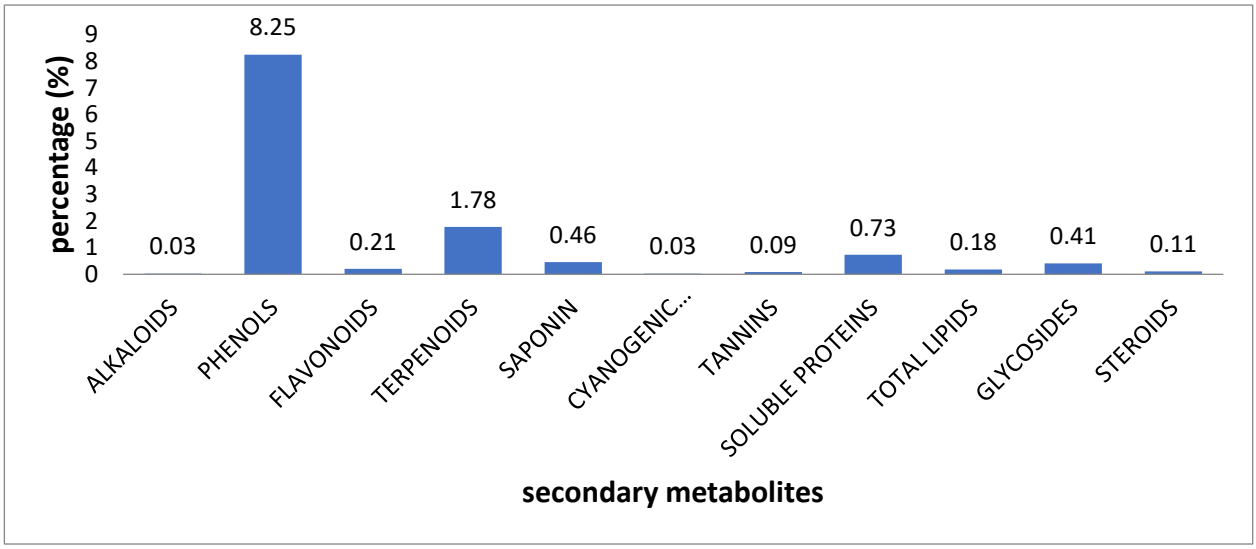

Fig. 2:
Percentage composition of secondary metabolites in *Mucuna pruriens*

Chemomicroscopy of M. pruriens seed: The result of the chemomicroscopy tests carried out on the powdered *Mucuna pruriens* seed samples showed the presence of starch grains, lignins, cellulose, fats and oils, protein bodies, and tannins while calcium oxalate crystals, gum, and mucilage were absent as presented in Table 1. The presence of starch in plants serves as a reserve food supply which is broken down into smaller sugar molecules that supply energy to plants for cell metabolism and tissue regeneration. When consumed, humans and animals benefit greatly from this energy. Starch is a vital by-product of photosynthesis that is indispensable throughout the lifespan of the plant.

Antifungal studies: Both *Malassezia* species used for this study are circular, have a raised elevation, creamy coloration with short unbranched hyphae, and spherical cells. Slight differences were observed as one had a glistening appearance (M. furfur) with entire edges while the other (M. globosa) was more wrinkled with lobular edges. Clear differentiation was made with Tween as only *M. furfur* can survive at 40ºC and in the presence of Tween 20, 40, and 80. The results of the cultural characterization and differentiation of the *Malassezia* species are presented in Tables 2 and 3 below.

Table 1:
Microchemical tests on *Mucuna pruriens* seed

Chemical	Reagent	*M. pruriens* seed powder
Starch	Iodine solution	Present
Lignins	Phloroglucinol plus Conc. HCl	Present
Cellulose	Zinc chloride; Conc. Sulphuric acid	Present
Calcium oxalate	Iodine solution Conc. Sulphuric acid	Absent
Protein bodies	Biuret reagent; Nihydrin	Present
Tannins	Ferric chloride	Present
Fats and oil	Sudan IV reagent	Present
Gum and mucilage	Ruthenium red	Absent

Table 2:
Cultural characterization of *Malassezia* species.

Organism	Shape	Chromogenesis	Surface texture	Edge	Elevation
***Malassezia species* A**	Circular	Cream to yellow	Glistening	Entire	Raised
***Malassezia species* B**	circular	Cream	Lightly wrinkled	Lobate	Raised

Table 3:
Criteria for identification and differentiation of *Malassezia* species

	Width	SDA	40ºC	Tween 80	Tween 40	Tween 20	Catalase
Malassezia furfur	Wide	+	+	+	+	+	+
Malassezia globosa	Narrow	+	-	-	-	-	+

Key: +Growth, - No growth, SDA: Saboroud's dextrose agar

Determination of Minimum Inhibitory Concentration (MIC): The n-hexane extract showed growth inhibition for *Malassezia furfur* at 6 and 7 mg/mL for *Malassezia* globosa. At 3 mg/mL, the methanol extract inhibited the growths of Malssezia furfur as shown by the absence of visible growth in the plate while that of *Malassezia globosa* was at 2 mg/mL. The reference drug Ketoconazole showed inhibition of *M. furfur* at 7µg and M. globosa at 9µg as shown in Table 4.

Table 4:
The minimum inhibitory concentration for the extracts and standard drug

Test Agents	**Minimum inhibitory Concentration (MIC) mg/mL**	
	M. furfur	***M. globosa***
n-hexane extract	6.0	7.0
Methanol extract	3.0	2.0
Ketoconazole (Standard drug)*	7.0	9.0

*MIC in µg/mL

Table 5:
Skin irritation test for *Mucuna pruriens* Ointment

s/n	Groups/Drug samples (0.5g)	Duration of observation of toxicity (irritation)					
		1h	24h	48h	72h	7days	14days with drug
1	5% MP ointment	-	-	-	-	-	-
2	10% MP ointment	-	-	-	-	-	-
3	Standard (ketoconazole)	-	-	-	-	-	-
4	Petroleum jelly (control)	-	-	-	-	-	-

Key: -No sign of irritation and closed-up wounds inflicted during shaving.

Skin irritation test: After application of the formulated MP ointment, no sign of irritation was observed for the first hour and after 24 h. The animals were further observed at 48 h, 72 h, 7, 14, and 21 days, no sign of toxicity was seen suggesting that the MPO is non-irritant to the skin as shown in Table 5 and this finding was corroborated by previous studies by (Ohadoma et al., 2015; Toppo and Pawar 2016).

DISCUSSION

A previous study in 2009 compared the activities of herbal and chemical agents against *Malassezia* species, and the following MICs were obtained for different herbal oils and extracts: coleus oil (20-25 mg/mL), pepper extract (75-80 mg/mL), tea tree oil (95-100 mg/mL), neem extract (95-100 mg/mL) and basil extract (95-100 mg/mL) while the synthetic agents' ketoconazole and zinc pyrithione gave MIC of 2.5 and 5 µg/mL respectively, hence suggesting better activity for synthetic agents (Prabhmanju et al., 2009). However, in 2013; Onlom et al., studied the antifungal activity of ethanol extract of Asparagus racemosus roots using the broth microdilution method, and both *M. furfur* and M. globosa were inhibited at MIC of 25 mg/mL but a higher potency of 0.2 mg/mL and 0.4 mg/mL respectively was reported when a saponin-enriched extract of the plant was tested (Onlom et al., 2013). The study also revealed that no antagonistic effect was observed when the extracts were used with known anti-fungal agents namely: ketoconazole and zinc pyrithione. Other researchers such as Padmalochana and Indumathi (2016) have reported MIC of 78.4 µg/mL using methanol extract from Albizia saman leaf and formulated an anti-dandruff shampoo (Padmalochana and Indumathi, 2016).

This study revealed that the methanol extract of M. pruriens possessed significant anti-microbial properties which agrees with previous researchers (Ashkok et al., 2009; Murugan and Mohan; 2011) other authors also reported activity with the n-hexane extract as well (Stephen et al., 2011; Tayade et al., 2020). The reference drug ketoconazole showed inhibition of growth at 0.7 µg/mL for *Malassezia furfur* and 0.9 µg/mL for *Malassezia globosa* which is a lower MIC than the extract (2 µg/mL for M.globosa and 3 µg/mL for M.furfur). The control vehicle showed no inhibition of growth for both organisms at all concentrations and is indispensable in imparting its pharmacological activity. This study shows the chemical composition and secondary metabolites that are linked to the antifungal activity of Mucuna pruriens.

The percentage of flavonoids present in M. pruriens seed from this study (Fig 2) is shown to be 0.2% though higher values of a 9-fold increase have been reported from the leaves Ushie et al., 2019 and a 3-fold increase by Jimoh et al., 2020; both studies showed a comparable increase when compared to the present study. These flavonoids might be responsible for the anti-oxidant properties of M. pruriens and some researchers have recommended their use in the management of sickle cell anaemia (Anosike et al., 2019). Previous studies have identified the presence of different alkaloids in M. pruriens (Lucia et al., 2012; Renata et al., 2016). These alkaloids were

found to be responsible for some pharmacological properties of the plant. On the smooth and skeletal muscles, varying degrees of spasmolytic actions were elicited against acetylcholine, histamine, and oxytocin-induced spasms.

Phenols are considered to be one of the most diverse groups of plant secondary metabolites and comprise several groups such as simple phenols, quinones, coumarins, lignins, etc (Elaine, 2008). These phenolic compounds are present in varying proportions in plant tissues and bring about the color, flavor, and astringency of some fruits. They are also shown to exhibit a wide of activities such as antioxidant and antimicrobial activities as well as plant defense mechanisms (Santosh, 2020). In this study, the high percentage of phenol suggests that M. pruriens is rich in phenols and corroborates with the 3.7% gotten by Jimoh et al (2020). It can also be linked to the use of a polar solvent in extraction which is amphiprotic and can break covalent bonds by acid-base reactions thereby facilitating the dissolution of the polar components of plant phytochemicals such as amines, phenols, and polysaccharides (Jimoh et al., 2020). Jimoh et al., 2020 reported 3.7% showing that not only is the plant rich in phenols but also that the phenol content of M. pruriens varies widely. The phenol content of M. pruriens has been linked to their cytotoxicity, anti-oxidant, and anti-parasitic activity (Anosike et al., 2019; Jimoh et al., 2020).

Plant tannins present in this study (0.09%) are similar to those documented in the literature by Jimoh et al., 2020 (1.8%). These high molecular weight polyphenols tend to bind to proteins and decrease protein digestibility, however, they are of beneficial importance due to their high anti-microbial properties. Polyphenols are strong anti-oxidants and have been associated with strong anti-bacterial activities for both gram-positive bacteria like S. aureus, B.subtilis, and L. monocytogenes and some gram-negative bacteria like E. coli and P. aeruginosa (Elansary et al., 2020). In the same study, the high concentration of these polyphenols in the stem of Ferocactus sp resulted in significant antifungal activity against Aspergillus ochraceus and Aspergillus niger. These compounds have been recommended as viable antimicrobials from natural sources. The presence of lignified tissues confers support and protection to the plant. Lignin is an important tissue for the cell wall rigidity and movement of nutrients and minerals through the vascular bundles in plants (Renata et al., 2016). The protective role of lignins helps the plant survive the attack from pathogens and pests. The tissue is very useful to man and his environment as they are used in making biodegradable plastics when processed. As a high-quality legume, M. pruriens has an abundance of proteins and fatty acids which corroborates the findings of Renata et al., 2016 (Sachchidac et al., 2020). These phytochemicals play an overlapping role in the overall activity of M. pruriens (Jimoh et al., 2020). The herbal ointment appears non-toxic on the skin of the animals tested.

CONCLUSION

The anti-fungal activities of *Mucuna pruriens* (L.) DC. (Fabaceae) seed was confirmed against *Malassezia furfur* and *Malassezia globosa* which are the causative organisms for dandruff in humans. A remedy, *Mucuna pruriens* Ointment (MPO), at concentrations of 5 and 10% M. pruriens methanol seed extract was prepared for topical application to the hair and scalp.

Further studies to isolate and chemically characterize the actual chemical constituent (s) of the seed responsible for the inhibitory activities against *Malassezia* species are recommended alongside long-term toxicity studies including the determination of the shelf-life of MPO are also recommended.

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