Antifungal Activities of *Anogeissus leiocarpus* (African birch) Against *Aspergillus niger* and *Rhizoctonia species*

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Abstract
Methanol and aqueous extracts from the stem bark of Anogeissus leiocarpus, well reputed for its treatment of diarrhea, dysentery, cough, and African trypanosomiasis, was phytochemically screened and tested in vitro for its biological activity against Aspergillus niger and Rhizoctonia species. All fractions were subjected to phytochemical screening and antifungal activity using the agar well diffusion method. The concentrations of extracts that showed minimum diameter of zone of inhibition against the test organisms were then evaluated to determine Minimum Inhibitory Concentrations (MIC) and then Minimum Fungicidal Concentrations (MFC). Mancozeb, and Dimethylsulphoxide (DMSO) were used as positive and negative control, respectively. Methanol extract was positive for all bioactive components tested except for flavonoids while aqueous extract was negative for flavonoids and glycosides. The methanol extract was active on all the pathogens especially at concentrations of 1,000,000 µg/ml which shows greater activity against Rhizoctonia species (21mm). However, Aspergillus niger developed resistance against the aqueous extract at all concentrations. Similarly, activity was greater against R. species in response to all fractions (21mm, and 20mm) at the highest concentrations than A. niger (18mm, and 0) thereby justifying the fact that antifungal activity of the extract was dose-dependent. The study succinctly reaffirms the fact that A. leiocarpus contains some bioactive compounds of

potential therapeutic and prophylactic significance and could be promissory candidate for antifungal drug development. Thus, validating its folkloric claims.

Key words: *Anogeissus leiocarpus, Antifungal activities*

1. Introduction

Infectious diseases with increasing trends of drug resistant microorganisms have been common global problem posing enormous public health concerns (Iwu *et al.*, 1999). The global emergence of antimicrobial resistant fungal strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Hancock, 2005). In many poor countries, the available drugs are costly and beyond the reach of the common man (WHO, 2002). Strategies to improve the current situation include research in finding new and innovative antimicrobials (Freeman, 1997), thus warranting the need to develop new antimicrobial compounds of botanical origin.

The interest in medicinal plants has increased together with the number of investigations into their biological effects on human beings and animals. Medicinal plants were believed to be an important source of new chemical substances with potential therapeutic effects. It has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Sofowora, 1993; Fansworth, 1985).

Indeed, Nigeria is naturally endowed with both savannah and tropical rain forest vegetation. These diverse floras offer a wide spectrum of unique medicinal plants which continue to play an essential role in health care delivery. Mann *et al.* (2008) reported that, the indigenous people in Nigeria are exploiting a variety of herbs for effective curing of various ailments. Similarly, Maigari *et al.* (2005) reasoned that increase awareness and application of preventive measures (which entails the use of ethno - therapy) might be the reason for low infection rates encountered in Animal Trypanosomosis. Several ethno-botanical studies of Nigerian plants used in the traditional management of fungal diseases indicated both significant *in vitro* and/ or *in vivo* fungicidal activities. Notable among these plants include *Anogeissus leiocarpus* and *Terminalia avicennoides* (Mann *et al.*, 2008).

A. leiocarpus, locally known as ***'Marke'*** in Hausa language which is commonly called African birch or axle-wood (Victor, 2013), is a typical tree of woodland and savannah of the Sudanian region and center of endemism (Andary *et al.*, 2005). It has a large ecological distribution ranging from the boarders of Sahara up to the out layer humid tropical forest (Hennenberg, 2013; Ouederago, 2013).

A. leiocarpus has been reported to have a wide range of pharmacological properties including; trypanocidal, antimicrobial, anthelmintic, antiplasmodial, leishmanicidal, antidioxidant and hepatoprotective effects (Ahmad, 2014). A study conducted in Togo to investigate antifungal activity of *A. leiocarpus* against 20 pathogenic fungi demonstrated that hydro-ethanolic extract possess in *vitro* antifungal properties (Batawila *et al.*, 2005). Also in a similar investigation conducted on the antifungal activity of crude extracts of *A. leiocarpus* and *T. avicennoides*, all the plant extracts inhibited the growth of all the test organisms, but, *A. leiocarpus* appears to be more effective as an antifungal agent than *T. avicennioides.* Ethanolic extracts of the two plant roots were more effective than the methanol, chloroform, or aqueous extracts against all the test fungi (Mann *et al.*, 2008).

Hennenberg (2013) contends that, *A. leiocarpus* is widely distributed in northern Nigeria possibly because of its wide range of values ranging from the medicinal properties, which is largely dependent upon the phytochemicals constituents of the plant, or economic importance and traditional uses. The crude extract of the plant has promising potent fungicidal activity (Mann *et al.*, 2008). Thus, the main objective of the present study was to

determine the presence of bio-active components and evaluate antifungal activities of aqueous and methanol stem bark extracts of *A. leiocarpus*.

2. Methods

2.1. Study Area and *plant material*

The study was conducted at the Plant Pathology Laboratory, Department of Plant Biology, Bayero University, Kano.

Fresh stem barks of *A. leiocarpus* were collected from Madobi Local Government Area of Kano state during the month of August, 2015. The plant material was properly verified and authenticated by a curator at the Herbarium of Plant Biology Department, Bayero University, Kano; in accordance with the criteria stipulated by International Committee for Botanical Nomenclature. A voucher specimen was deposited in the Herbarium with the Herbarium Accession Number BUKHAN 0029.

2.2. Preparation and *extraction of plant material*

The plant part was thoroughly washed, rinsed with distilled water and shade-dried under an ambient temperature for 2 weeks to a constant weight. The dried part was then pulverized using mortar and pestle. The powdered material was also sieved and fine powder was then stored in a sterile and tight container until needed (Bukar *et al.*, 2009).

Exactly 50g of the powdered plant part was weighed and macerated separately in 500ml of distilled water and methanol (0.1g/ml) and allowed to stand for five days with intermittent stirring. Each preparation of the stem barks of the plant was filtered through a Whatman No 1 filter paper and Buckner funnel, and the filtrates obtained were evaporated to dryness using a water bath at 39^0C. All extracts were then stored in refrigerator at 4^0C until required (Aliyu and Sani, 2011).

2.3. Phytochemical screening

Phytochemical screening for major bioactive constituents was undertaken using standard qualitative methods as described by Abalaka *et al.* (2010) and Scott (1989). Saponins, alkaloids, tannins, glycosides, and flavonoids were the major bioactive components tested in all the fractions.

2.3.1. Test for Saponins

This was carried out by frothing test. 2ml of the extract was vigorously shaken in the test tube for 2 minutes. Presence of frothing in the test tube confirmed positive test.

2.3.2. Test for Alkaloids

A 1ml hydrochloric acid was added to the test solution in a test tube. About 2 drops of Mayer's reagent to 1ml of the extract was added and observed for creamy and turbid precipitate as an indication of the presence of alkaloids.

2.3.3. Test for Tannins

To 1ml of the test solution in a test tube, few drops of Ferric chloride were added. Presence of blue-black and blue-green precipitate was a positive test for tannins.

2.3.4. Test for Flavonoids

To 2ml of the test solution, a few magnesium ribbons and a few drops of concentrated hydrochloric acid was added. Appearance of magenta coloration indicates the presence of flavonoids.

2.3.5. Test for Glycosides

To 1ml of the test solution, 10ml of H_2SO_4 was added and the mixture was heated in boiling water for 15 minutes. This is followed by addition of 10ml of Fehling's solution. The mixture was then boiled. A brick red precipitate was confirmatory for the presence of glycosides.

2.4. Test Organisms and *Inoculum*

The test organisms used in the research were fungal isolates procured from the Laboratory of Plant Pathology, Bayero University, Kano. These strains were *Aspergillus niger* and *Rhizoctonia spp*.

The fungal strains were inoculated separately in a potato dextrose agar and incubated at 37^0C for 4 days. The suspensions/stock cultures were put in an agar slant and maintained in a refrigerator at 4^0C in accordance with the procedure by (Scott, 1989) until required for the screening.

2.5. Preparation of concentration for sensitivity test

This was achieved using serial dilution method as described by (Aliyu and Sani, 2011). Two grams of each plant extract was dissolved in 2ml of appropriate diluent (DMSO) to arrive at 1.0g/ml (1,000,000µg/ml) which serves as stock solution (Abhishek *et al.*, 2011).

From the stock, 1ml was added to another bottle containing 1ml of the diluent to serve as the second concentration, 1ml from the second was added to another bottle containing 1ml of the diluent to serve as the third concentration, and 1ml from the third was added to another bottle containing 1ml of the diluent to serve as the fourth and final concentration. Finally, four different concentrations were prepared; 1,000,000µg/ml, 500,000µg/ml, 250,000µg/ml, and 125,000µg/ml (1.0g/ml, 0.5g/ml, 0.25g/ml, and 0.125g/ml), respectively.

2.6. Antifungal activity assay/ Sensitivity test

The agar well diffusion method (Stock and Ridgeway, 1980) was modified. Potato dextrose agar (PDA) was used for fungal cultures. The culture medium was inoculated with 1ml of the fungal strains separately suspended in potato dextrose agar. Each petri plate was partitioned in two using a permanent marker to accommodate two concentrations. A total of 6mm diameter wells were punched into the agar using a sterile cork borer and filled with plant extracts of different concentrations. A standard antibiotic (Mancozeb, concentration 1g/ml) was used as positive control and DimethylSulphoxide (DMSO) as negative control. The fungal plates were incubated for 3 days at 37^0C. The degree of sensitivity was determined by observing and measuring the diameter in millimeter (mm) of the visible zone of inhibition of the microbial growth produced by the diffusion of the extracts (Abdolhassan *et al.*, 2013).

2.7. Minimum Inhibitory Concentration (MIC)

The concentrations of antifungal plant extracts that showed minimum diameter of zone of inhibition against the test organisms were then evaluated to determine MIC. The broth dilution method was adopted as described by (Banso *et al.*, 1999). Three concentrations; 250,000µg/ml and 125,000µg/ml aqueous and methanol concentrations of *R. spp*., and 250,000µg/ml concentration of *A. niger* were then assayed for their MIC as *A. niger* proved to be resistant and showed no any sign of susceptibility against all the concentrations of the aqueous plant extract.

For aqueous and methanol extracts concentrations of 250,000µg/ml of *R. spp*., and *A. niger*; serial dilutions of 500,000µg/ml concentrations were incorporated into 1ml of sterile potato dextrose broth (PDB) in 4 glass tubes in order to adjust to the concentrations of 250,000µg/ml, 125,000µg/ml, 62,500µg/ml, and 31,250µg/ml respectively. The same procedure was adopted for the methanol extract concentration of 125,000µg/ml of *R. spp.* by

serial dilution of 250,000µg/ml to arrive at 125,000µg/ml, 62,500µg/ml, 31,250µg/ml, and 15,625µg/ml concentrations respectively. All tubes were inoculated with 0.5ml of standardized inoculums of each two species and incubated at 37^0C for 24 hours.
Positive control experiments glass tubes were also inoculated with 0.5ml of fungal strains and performed without plant extract. Meanwhile, the negative control tubes were treated with plant extract but without inoculation for the purpose of comparison. The least concentration of the plant extracts that does not permit any visible growth of the inoculated test organism in the broth medium was regarded as the MIC in each case (Mann *et al.*, 2008). Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the plant extract with no growth after incubation.

2.8. Minimum Fungicidal Concentration (MFC)

Minimum Fungicidal Concentration was used to determine if the crude extracts were fungistatic or fungicidal in their effect using (Mann *et al.*, 2008) method. The concentration of the tubes that showed no visible fungal growth or turbidity in the minimum inhibitory concentration experiment (i.e. 250,000µg/ml and 62,500µg/ml aqueous and methanol concentrations of *R. spp*., and 250,000µg/ml methanol concentration of *A. niger*) were cultured into fleshy prepared potato dextrose agar plate to assay for fungicidal effect of the extracts. The plates containing the test organisms were incubated at 37^0C for 5 days. The minimum fungicidal concentration was regarded as the lowest concentration that did not yield any fungal growth on the solid medium used.

3.0. Results and Discussion

The phytochemical screening of the stem bark extracts of *A. leiocarpus* indicated the presence of alkaloids, tannins, saponins, and glycosides respectively. Meanwhile flavonoid was negative in all the fractions (**Table 1**). These secondary metabolites have been established to be frequently responsible for the antimicrobial properties of most medicinal plants (Khaig, 2011).
The result shows that the extract exhibited significant antifungal activity against *R. spp.*, only (20mm, 16mm, and 13mm) with the exception of lower concentrations. *A. niger* developed resistance and showed no any sign of susceptibility against the extract at all concentrations (**Table 2**). The extract exhibited significant antifungal activity against all the test organisms, *R. spp.* (21mm, 17mm, 12mm, and 9mm), and *A. niger* (18mm, 15mm, and 11m) respectively with the exception of *A. niger* at lowest concentrations. Methanol extract showed greater activity against *R. spp.* (21mm) at 1,000,000 µg/ml than *A. niger* (18mm). Activity was greater against *R. spp*. in all the fractions (21mm and 20mm) at the highest concentrations than *A. niger* (18mm and 0) (**Table 3**). From these results one could deduced that, antifungal activity of the extracts was enhanced by an increase in the concentration of the extracts (**Tables 2 and 3**), that is, the higher the concentrations of the plant extracts, the greater the zone of inhibition. Mancozeb was the antibiotics used as the control plate against the test organisms, and showed greater activity than the crude extracts (32mm and 30mm).
This finding agrees with the report of (Banso *et al.*, 1999), that higher concentrations of antimicrobial substance showed appreciations in growth inhibition. The fact that methanol fraction showed greater activity than the water extract is an indication of better solubility of the active components in methanol than in aqueous (Aliyu and Sani, 2011). Similar observation was reported by (Banso *et al.*, 1999), who noted that higher antifungal activity exhibited by the methanol portion may be due to the presence of substantial amounts of polar constituents in the plant material. The climatic conditions, percentages humidity of the harvested material, the solvent used, and the methods of extraction are other possible sources

of variation for the chemical composition, toxicity, and bioactivity of the extracts (Khaig, 2011).

Table 4 shows the minimum inhibitory concentration of water extract against *R. spp.*, only. Turbidity which indicates fungal growth was found in all the tubes at different concentrations with the exception of 250,000µg/ml being the only least concentration of the extract with no visible growth after incubation.

Table 5 also shows the minimum inhibitory concentration of methanol extract against the test organisms. For *R. spp*. 125,000µg/ml and 62,500µg/ml were the concentrates that showed no visible growth in the glass tube after incubation, while turbidity was observed in the remaining tubes containing the other concentrates (31,250µg/ml and 15,625µg/ml), respectively. Also from **Table 5**, 250,000 µg/ml was the only concentrate that inhibited growth against *A. niger* after incubation which is contrary to the other tubes in which fungal growth/turbidity was observed.

The concentrations of the tubes that showed no visible growth or turbidity in the MIC experiment were found to be fungistatic (i.e. inhibits fungal growth temporarily) in their action when cultured on the solid medium with the exception of 250,000µg/ml methanol extract of *A. niger* which was found to be fungicidal (i.e. inhibits fungal growth permanently).

The fact that the results of this study showed that stem bark extracts of *A. leiocarpus* exhibit antifungal properties justify its traditional use as medicinal plant. This may be due to the presence of active principles in the plant materials. Plants generally produce many secondary metabolites which constitutes an important source of microbicides, pesticides and many pharmaceutical drugs (Ibrahim, 1997; Ogundipe *et al.*, 1998). Plant products still remain the principal source of pharmaceutical agents used in orthodox medicine (Ibrahim, 1997; Ogundipe *et al.*, 1998).

The zone of inhibition produced by the test organisms indicated their susceptibility to the plant extracts. It was observed that the zone of inhibition observed by the fungal isolates varied. Similar result was reported by (Ogundipe *et al.*, 1998) with the methanol and butanol extracts of *Cordia dichotoma* stem bark. The growth inhibition zone ranges from 12-21mm for fungal strains (*A. niger, A. clavatus* and *Candida alloicans*). The antifungal activity of the extracts increased linearly with increase in concentrations of the extracts as compared with standard drugs. According to (Prescott, 2002) the effect of bioactive agent varies with target species. Mann *et al.* (2008) also reported that, the position of the zone edge (diameter of the zone of inhibition) is determined by the initial population density of the organisms, their growth rates and diffusion of the antimicrobial agents, which clearly explains the difference in the zone of inhibition observed in the present study. Mancozeb was the antibiotics used as the control plate against the test organisms. This antibiotic showed greater activity than the crude extracts, this is not surprising because standard antibiotics are well refined industrial products so there is no doubt its activity will be more compared to crude extracts.

The minimum inhibitory concentration values of the plant extracts against the test organisms showed that fungi vary widely in the degree of their susceptibility to antifungal agents. When the broth culture of the extract and the test organisms used in the minimum inhibitory concentration test were sub-cultured on a solid medium for the assessment of the minimum fungicidal concentration of the extracts, the result suggests that the antifungal substances contained in the extracts were fungistatic at lower concentrations while becoming fungicidal at higher concentrations of the extracts. Similar observations have been reported by Mann *et al.* (2008) and Banso *et al.* (1999), respectively.

4.0. Conclusion and Recommendations

The present study confirms the earlier claims that stem bark of *A. leiocarpus* contains some active pharmacological constituents comprising of tannins, alkaloids, saponins, and cardiac glycosides. Moreover, the extract also shows good inhibitory activities against all the test organisms compared with standard antibiotics such as mancozeb which justifies further that *A. leiocarpus* contains some bioactive compounds.

Therefore, considering the current global upsurge of fungal resistance to antifungal drugs and the growing interests in obtaining plant materials with potent medicinal value, extracts used in this study could be further explored to isolate and characterized the active phytoconstituents with novel efficacy, evaluate its safety through toxicity studies, and conduct multiple investigations using different solvents and model organisms for the purpose of evaluating its efficacious phyto-therapy against fungal infections.

Competing interest:

The authors declared no any conflict of interest.

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Table 1: Phytochemical screening of aqueous and methanol stem bark extracts of *A. leiocarpus*

Rxtract	**Bioactive Compounds**				
	Saponins	**Tannins**	**Flavonoids**	**Alkaloids**	**Glycosides**
Aqueous	+	+	-	+	-
Methanol	+	-	-	+	+

+ indicates presence
- indicates absence

Table 2: Antifungal Activity of Aqueous stem bark extract of *A. leiocarpus*

Isolates	**Concentration of Extracts (µ/ml)**					
	1,000,000	500,000	250,000	125,000	+ve control	-ve control
	Zones of inhibition					
R. spp.	20	16	13	0	32	0
A. niger	0	0	0	0	30	0

Positive control = (1g/ml)
Negative control = DMSO

Table 3: Antifungal activity of methanol stem bark extract of *A. leiocarpus*

Isolates	**Concentration of extract (µg/ml)**					
	1000,000	500,000	250,000	125,000	+ve control	-ve control
	Zones of inhibition (mm)					
R. *spp.*	21	17	12	9	32	0
A. niger	18	15	11	0	30	0

Positive control = Mancozeb (1g/ml)
Negative control = DMSO

Table 4: Minimum Inhibitory Concentrations of aqueous extract against *R. spp*.

Isolate	**Concentration (µg/ml)**					
	250,000	125,000	62,500	31,250	-ve control	+ve control
R. spp.	-	+	+	+	+	-

+ indicates turbidity (growth)
- indicates growth inhibition

Table 5: Minimum Inhibitory Concentrations of methanol extract against *R. spp.* and *A. niger*

Isolates	**Concentrations (µg/ml)**					
	125,000	62,500	31,250	15,625	+ve control	-ve control
R. spp.	-	-	+	+	+	-
	250,000	125,000	62,500	31,250	+ve control	-ve control
A. niger	-	+	+	+	+	-

+ indicates turbidity (growth)
- indicates growth inhibition