Antifungal potential and Phytochemical Screening of Combretum molle leaves and stem-bark against Macrophomina phaseolina (Tassi) Goid.

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Abstract: *Combretum molle* (Combretaceae) is reputed in folklore medicine for its anti-infective properties. In this study, the antifungal potential of *Combretum molle* leaves and stem bark extracts on the plant pathogenic fungi *Macrophomina phaseolina* was investigated. *Combretum molle* powdered plant was extracted using acetone and methanol and phytochemical screening revealed the presence of Saponins, Triterpenes, glycosides, phenols, alkaloids and anthraquinones. The stem bark extracts showed more bioactivity than the leaf extract. Agar diffusion and broth dilution techniques were used to determine the antifungal activity of the plant extracts against *Macrophomina phaseolina*. Both solvent extracts showed activity against the test organism with minimum inhibitory concentration (MIC) between 5mg/ml and 0.625mg/ml. The results justify the wide use of *Combretum molle* in African traditional medicine and also hold clues for plant derived compounds in the development of ecofriendly fungicides for better crop health and yield in agroforestry.

Key words: Antifungal potential, Combretum molle, Phytochemicals, Macrophomina phaseolina, Leaves, Stem bark, zone of inhibition

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1 INTRODUCTION

For centuries, plants have been used throughout the world as drugs and remedies for various diseases and screening of extracts of some of these plants have often yielded positive outcomes. These drugs often serve as prototypes to develop more effective and less toxic medicines (13). Many plants produce antifungal agents as secondary metabolites to protect themselves from fungal attack and a number of plant species manifest substantial antifungal activity (7). Thus, the use of plant extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides based on the availability of natural products. (12).

The disease burden of plant pathogenic fungi on crops especially in the developing world, the problem of sustainable food production , availability of land for agriculture, and other recent developments have increased the value of indigenous medicinal knowledge, which may hold clues for solving these threatening problems.

Indigenous medicinal plant knowledge is critical because chemical processes have proved inadequate for dealing with the rapid evolution of pathogens. (8).

Plant pathogenic fungi attack most crops in the field and also at post-harvest, thereby decreasing production and shelf life of many agricultural crops (1). Substantial use of chemical pesticides (fungicides) induces problems of health and environmental hazards in agricultural systems. So, for humans and plants, natural products of antimicrobial activity are best bio rational alternatives today (14).

Furthermore, knowledge of the chemical constituents of plants is very important, not only for the discovery of drugs and other therapeutic agents, but also in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances (4, 9).

In Nigeria, almost all plants are associated withsome medicinal value. So also in Africa, the use of plants to treat various ailments in humans and animals has been extensively documented by scientists. Herbalists use stems, leaves, roots and shoots of plants to prepare extracts, decoctions, concoctions, mixtures, portions, creams, infusions and pastes, which are used to cure all sorts of afflictions. The variety of plants used in a community reflects the duration of a people's presence in a certain location, their medicinal knowledge, the diversity of plants present and the availability of plants with a possible medicinal use (8).

Members of the family Combretaceae are often tanniferous and produce ellagic and gallic acids frequently and also proanthocyanins (3).Phytochemical screening revealed that species of the genus *Combretum* are particularly rich in tannins and saponins, which might be responsible for their antifungal activity (2).

Many plants produce antifungal agents by secondary metabolites to protect themselves from fungal attack, and therefore many plant species possess substantial antifungal activity, thus the use of plant extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides. This present study evaluated the antifungal potential of extracts of the leaves and stem bark of *Combretum molle* against *Macrophomina phaseolina*.

2.0 MATERIALS AND METHODS

2.1 Plant Collection

Plant sample was collected from the field (Afaka Forest Reserve) in Igabi Local government area of Kaduna state between March and April to ensure high concentration of bioactive constituents.Plants collected were identified at the Herbarium unit of the Department of Biological sciences ,Ahmadu bello university, Zaria. The parts collected were leaves and stem bark. Leaves were separated from stems, and dried between 35^0C and 37^0C. The dried plant parts were milled to a fine powder and stored at room temperature in closed containers in the dark until used.

2.2 Plant Extraction

Plant samples from each part were individually extracted by soaking 100g of finely ground plant material with 150ml of Acetone and Methanol solvents separately in conical flasks, plugged with cotton wool and vigorously shaken and then left to stand for 72hrs. The collected extract was filtered using a muslin cloth followed by whatman no.1 filter paper. The filtrate was concentrated under reduced pressure in a rotavapor at 40^0C to recover the solvents. The yielded extracts were weighed and stored in labeled tight containers for further bioassay. For the preparation of dilutions of crude extracts for antifungal assay, the extracts was reconstituted by dissolving in the extracting solvents and water and further diluted to obtain 400, 200, 100, 50, 25, 12.5mg/mL etc. and maintained at room temperature between 2-8^{o}C.

2.3 Phytochemical Analysis

Chemical constituents of the extracts were analyzed to detect the presence of Particular compounds using standard procedures according to (5)

2.4 Fungal Test Organisms

The phytopathogenic fungi selected for this study was *Macrophomina phaseolina* which was obtained from the Department of crop protection, A.B.U Zaria.

The culture was purified using the single hyphal tip method, A small segment of fungal growth in

the agar medium was transferred to the center of the petridishes containing nutrient medium using a flame-sterilized inoculation needle and incubated at 26°C for 3days. Fungus was sub-cultured at regular intervals to maintain vigor for further bioassay. Sterilized pieces of cultures were aseptically transferred to petri dishes containing PDA supplemented with streptomycin sulphate, at the rate of three pieces per plate and incubated at 26 °C that favored the pathogen development. A portion of the mycelium developing on the nutrient medium was transferred to the agar slants for purification and storage for further examination. The fungal inoculum was quantified by counting the number of spores using Haemocytometer slide and their number was adjusted to 1x106ml-1of the suspension.

2.5 ANTIFUNGAL ACTIVITY ASSAY

2.5.1 Minimum Inhibitory Concentration (MIC)

Antifungal activity was determined by disc diffusion assay. The MIC is to determine the lowest concentration of an antifungal agent that appears to inhibit growth of the fungus [16]. Residues of different extracts were re-dissolved in respected solvents to a concentration of 10mg/ml.

Potato dextrose agar (PDA) of 200ml was prepared and sterilized by autoclaving at 121°C for 15min and 10ml of PDA was poured aseptically into 10 sets of petriplates for leaf and stem-bark, and each plate mixed with 10ml of plant extracts of the two parts used (leaf and stem bark). This was homogeneously mixed and 10ml withdrawn to a fresh sterilized petriplate, the procedure was repeated for each plant part until the concentration reached 10^{-1}. The mixture was then allowed to cool and solidify. Three pairs of a 5mm sterilized paper disc were placed adjacent each other on the solidified PDA. The standardized inoculum size of 106cfu/ml in 50ul volume of test Macrophomina phaseolina were inoculated on the paper discs set on the agar medium. The inoculated plates were allowed to stand for one hour for proper diffusion of extracts and then incubated at 26°C for 48hrs and then observed for growths. Nystatin was used as reference antibiotic and positive control. Fungal growth diameter was measured to the nearest millimeter by taking an average of three diameters taken at right angles for each colony. Percentage growth inhibition was measured using the formula:

$$\text{Growth Inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}}$$

2.5.2 Test for Antifungal activity

A 20ml volume of the prepared PDA was poured into 10 sets of petridishes for each solvent extract. The standardized fungal culture was used to flood the surface of the now solidified PDA and allowed to set. Wells were bored using 6mm cup borer and their bases sealed with some quantity of PDA. A 0 .1ml volume of fungal cultures were filled into the cups and allowed to set for 2hrs. The plates were then incubated at 26oC for 48hrs. After incubation, the zones of inhibition for both the extract and standard antibiotics were measured using a pair of dividers and a clear meter rule and results recorded.(13)

2.6 Statistical Analysis

The results for the antifungal activity of the acetone and methanolic extract were expressed as mean diameter of zones of inhibition. The mean values of the control set (Nystatin was used as reference antibiotic and positive control, and appropriate solvent blanks were included.) were compared with the mean values of extract using student t-test at $p<0.05$ and 0.01(6)

Phytochemical studies carried out in the genus Combretum have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans and non-protein amino acids, among others (7). Since the 1970s, several unusual compounds have also been isolated from Combretum species, for example, 9,10dihydrophenanthrenes and a substituted benzyl from C. molle (8).

2.7 Preliminary Phytochemical Analysis

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Steroids (Salkowski's test).About 100mg of combretum molle dried extract was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface was an indicative of the presence of steroidal ring.

Cardiac glycosides (Keller killiani's test)

About 100mg of extract was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layer with 1ml of concentrated sulphuric acid. A brown ring obtained at the interface indicated the presence of a de-oxy sugar characteristic of cardenolides.

Saponins:

A drop of sodium bicarbonate was added in a test tube containing about 50ml of an aqueous extract of samples. The mixture was shaken vigorously and kept for 3minuts. A honey comb like froth was formed and it showed the presence of saponins.

Phenols (Ferric Chloride Test)

To 1ml of alcoholic solution of sample, 2ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green colour indicated the presence of phenols.

Tannins (Lead acetate test)

In a test tube containing about 5ml of an aqueous extract, a few drops of 1% solution of lead acetate was added. Formation of a yellow or red precipitate indicated the presence of tannins.

FeCl3 test

A 2ml filtrate [200mg of plant material in 10ml distilled water, filtered], and 2ml of FeCl3 were mixed. A blue or black precipitate indicated the presence of tannins.

Terpenoid

2ml of chloroform and 1ml of conc. H2SO4 was added to 1mg of extract and observed for reddish brown colour that indicated the presence of terpenoid.

Glycosides

A small amount of alcoholic extract of samples was dissolved in 1ml water and then aqueous sodium hydroxide was added. Formation of a yellow colour indicated the presence of glycosides.

Flavonoids

In a test tube containing 0.5ml of alcoholic extract of the samples, 5 to 10 drops of diluted HCl and small amount of Zn or Mg were added and the solution was boiled for few minutes. Appearance of reddish pink or dirty brown colour indicated the presence of flavonoids.

Detection of Proteins: Xanthoproteic test; 1ml of extract was added to 1ml of HNO3, and boiled in a water bath. Orange colour indicated the presence of proteins.

Alkaloids (Mayer's test)

1.36gm of Mercuric chloride and 5gm of KI were dissolved in 60ml and 10 ml of distilled water respectively. These two solvents were mixed and diluted to 100ml using distilled water. To 1ml of acidic aqueous solution of samples few drops of reagent was

added. Formation of white or pale precipitate showed the presence of alkaloids.

The selected plant (*Combretum molle*) was evaluated using Acetone and methanol as solvents and the extracts were tested against plant pathogenic fungi *Macrophomina phaseolina* which causes charcoal rot disease in over five hundred cultivated plants. The part of the plant used was leaves, and stem bark because leaves are the center of intermediary metabolism leading to biologically active secondary metabolite. Antifungal susceptibility testing was done using the disc diffusion assay and minimal inhibitory concentration. The MIC was used to determine the concentration of an antifungal agent that appeared to inhibit the growth of the fungus or that showed prominent reduction in the growth of the fungus compared to that of the drug free control.

In this study Acetone leaf extract of *Combretum molle* had promising antifungal activity with a low MIC value of 0.067mg/ml followed by the stem-bark which showed 0.623mg/ml compared to the methanolic extracts.(Table 2). The acetone leaf extract of exhibited the highest total activity

3.0 RESULTS

Table 1: Phytochemical analysis of leaf and stem bark extracts of *Combretum molle*

Bioactive compounds	Leaf extract		Stem bark extract	
	Acetone	Methanol	Acetone	Methanol
Alkaloid	+	+	+	+
Anthraquinone	+	+	+	+
Flavonoids	+	+	+	+

Cardiac glycosides	-	-	-	-
Glycosides	+	+	+	+
Phenols	+	+	+	+
Saponins	+	+	+	+
Steroids	-	-	-	-
Tanins	+	+	+	+
Terpenes	+	+	+	+

Key: + = Present, - = Absent

Table 2: Antifungal activity of *Combretum molle*

against *Macrophomina phaseolina*

	Zone of Inhibition diameter (mm)			
	Leaf extract (mg/ml)		Stem bark extract (mg/ml)	
Concentrations (mg/ml)	Acetone	Methanol	Acetone	Methanol
0	-	-	-	-
0.312	-	+	-	+
0.625	-	+	-	+
125	-	+	-	+
2.5	-	+	-	+
5	+	+	-	+

Key: + = Growth: - = No Growth

Discussion

The phytochemical screening of the leaves and stem bark of *C.molle* revealed that the plant is rich in some bioactive compounds as shown in (table 1). These bioactive compounds have some strong antimicrobial significance against some plant pathogens.

The presence of tannins and saponins in sufficient quantity may be responsible for the antifungal activity against the phyto pathogenic fungi *Macrophomina phaseolina*.

The antifungal activity of crude extract of *C. molle* were evaluated by measuring the diameter of zones of inhibition on the tested fungi species and the results are presented in table 2

It was observed from sensitivity screening that the leaf and stem bark extracts were active against the test organism at lower concentrations at 0.312,0.625,1.25(mg/ml) although the stem bark extract also showed activity against the test organism at 2.5mg/ml.

The results of the MIC indicated that the leaf and stem bark extracts exhibited selective levels of antimicrobial activity against the investigated pathogenic fungi. The variation is presumed to be due to different compounds present in these plant parts.

Table 2 showed that stem bark extracts had higher in vitro antifungal activity against *Macrophomina phaseolina* than the leaf extract. This might be due to more potency or higher concentration of the phytochemical constituents such as tannins and saponins in the stem bark extract. (10).

Tannins are found in large quantities in the bark of trees where they act as a barrier for microorganisms like bacteria and fungi. They have been found to form irreversible complexes with proline rich protein (7) (8) as well as Okigbo et al., (2005) observed that the bioactivity of a plant extract is greatly influenced by the extraction solvent, method of extraction and time of harvesting of plant materials. The micro dilution assay showed MIC values by the two extracts were in range of (0.312-5)mg/ml (Table3) This indicated that *C. molle* extract possess potential active compounds that can be used for the synthesis of eco-friendly fungicides.

The results of this study further justify the use of *C. molle* in traditional medicine possibly due to its non- selective bioactive compounds.

The acetone extract of the leaves and stem bark exhibited high activity against the test organism. At 0mg/ml which served as the control set, there was no inhibition for both acetone and methanol leaves and stem bark extracts, however at 0.312, 0.625 and 1.25mg/ml concentration, there was inhibition of growth for acetone leave extract of *Combretum molle* indicating that at low concentrations the acetone leave extract there was no growth inhibition for the methanolic extracts meaning that methanol had no effect on the tested organism.

4.0 CONCLUSION

The acetone extract of *Combretum molle* leaves and stem bark possess in vitro antifungal activity and therefore confirm the rationale behind the use of this plant in traditional medicine. This may provide starting materials for the synthesis of inexpensive and ecofriendly fungicides based on the availability of natural products.

REFERENCES:

[1] G. N. Agrios, *Plant Pathology*, 3rd. ed. Academic Press, Inc.: New York. pp. 803, 1988.

[2] F. Baba-Moussa, K. Akpagana, P. Bouchet, "Antifungal Activities of Seven West African Combretaceae used in Traditional Medicine". *Journal of Ethnopharmacology*. Vol 66, pp. 335–338 no.3, September, 1999

[3] A. Cronquist. *An Integral System of Classification of Flowering Plants*., New York: Columbia University press, pp .1215, 1981.

[4] I. K. Das, B. Fakhrudin, and D.K. Arora," RAPD cluster analysis and Chlorate sensitivity of some Indian isolates of *Macrophomina phaseolina* from sorghum and their relationships with pathogenicity". *Journal of Microbiological Research* vol 163, no.2, pp. 215-224, January, 2008.

[5] J.B. Harborne " Phytochemical methods". Chapman and Hall, Ltd., London. pp. 49-188, 1973.

[6] R. C. Michael, and H .F. Robert," Introductory Biostatistics for Health Sciences". John Wiley and Sons, Inc., Hoboken, New Jersey, pp. 144-178, 2003.

[7] P.J. Masoko, J.N. Eloff, " Antifungal activities of six South African Terminalia species" (Combretaceae), *Journal of Ethnopharmacology* Vol 99, no.2, pp. 301-308, June, 2005.

[8] P. Masoko, J. Picard and J.N. Eloff, " The Antifungal Activity of Twenty-Four Southern African *Combretum* species" (Combretaceae). *South African Journal of Botany*, Vol 73, no.2, pp. 173–183, April, 2007.

[9] F. Mojab, M. Kamalinejad, N. Ghaderi, and H. Vahidipour," Phytochemical Screening of Some Iranian Plants". *Iranian Journal of Pharmaceutical Research*.Vol 2 , no.2, pp. 77-82, May 2003.

[10] M.E. Nyenje. and R. N. Ndip "Bioactivity of the acetone extract of the stem bark of *Combretum molle* on selected bacterial pathogens: Preliminary phytochemical Screening" *Journal of Medicinal Plants Research* Vol 6, no.8, pp. 1476-1481, February, 2012.

[11] R.N. Okigbo O.U. Ogbonaya ,"Antifungal effects of two tropical plants extracts *Ocimum gratissimum* and *Aframomum melegueta* on post harvest yam *Dioscorea* spp rot". *African Journal of Biotechnology*, Vol 5, no.9, pp 727-731,12, May, 2006.

[12] S. Rashmi, H. G. Rajkumar "Preliminary Phytochemical Analysis and in Vitro Evaluation of Antifungal Activity of Five Invasive Plant Species against *Macrophomina Phaseolina* (Tassi) Goid", *International Journal of Plant Research* Vol 1, no.1 pp. 11-15, 2011.

[13] O.Y. Sanaa. E.H.A.S. Shani, A. Braaha, and Z .E .M. Asha "Antimicrobial Activity of some Medicinal plants against some Gram Positive , Gram Negative and Fungi", *African Journal of Biotechnology*, Vol 5, no. 18, pp.1663-1668, 2007.

[14] A. Sofowora, *Medicinal plants and traditional medicine in Africa*.:3rd Edition Spectrum Books Ltd., Ibadan, Nigeria, pp.23-25, 2008.

[15] R.K.S. Tiwari, A . Singh, K. Das , and A. Sinha , "Efficacy of extracts of medicinal plants against Rhizoctonia solani". *Ann. Plant Prot. Sci.* Vol 15, no.2, pp. 460-539, 2007.