

ANTIMYCOTIC AND CYTOTOXICOLOGICAL POTENTIALS OF *Acacia mearnsii* De Wild. STEM BARK EXTRACT: IN VITRO ASSESSMENT

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ABSTRACT: Despite using antimycotic drugs in the treatment of dermatomycoses as an age long practice, the fungicidal activities of indigenous plants on spores of dermatophytes from germinating *in vitro* need to be investigated further. In this study, methanol extract of *Acacia mearnsii* was assessed for its antifungal activity against dermatophytic pathogenic fungi by agar diffusion and broth dilution methods. The cytotoxicity activity of the extract was determined by the brine shrimp lethality assay. The study showed that the mean \pm standard deviation of the inhibition zones ranged between 13 and 24 \pm 0.58 mg/ml and the activity of the extract was concentration dependent. While the minimum inhibitory concentration (MIC) was 0.3125 – 2.5 mg/ml, the minimum fungicidal concentration (MFC) values ranged between 0.3125 mg/ml and 5 mg/ml. The susceptibility of the yeast to the extract was more than those of the molds. The MIC of the yeasts ranged between 0.3125 and 1.25 mg/ml while those of the molds ranged between 0.625 and 2.5 mg/ml. The mechanism of antibiosis showed that the extract was 7.69% fungistatic and 92.31% fungicidal wielding more killing effects on the fungi than potentiating their growth. The cytotoxicity assay showed that the crude methanolic extract with the LC₅₀ being 101.59 μ g/ml was pharmacologically active and nontoxic (LC₅₀ > 100 μ g/ml). The antifungal activity of this plant can be exploited as an alternative or complementary means of treating fungal infections to eliminate fungal spread. The study further justified the folkloric use of *A. mearnsii* in the treatment of a variety of microbial infections.

Keywords: *Acacia mearnsii*, antifungal, cytotoxicity, fungicidal, mycotic infections

INTRODUCTION

Medicinal plants are a rich source of novel and powerful drugs (PONNUSAMY *et al.*, 2010; YADAV *et al.*, 2010). They manufacture an extremely disparate series of secondary metabolites which increase with commencement of drought, outbreak of fire and bacterial infection (TAIZ and ZEIGER, 2006). These secondary metabolites usually exhibit

pharmacologically essential properties including cytotoxic, antimicrobial and antioxidant (OLAJUYIGBE and AFOLAYAN, 2011; JOSEPH and SUJATHA, 2011). Consequently, many *in vitro* studies on pharmacological relevance of medicinal plants have reported antifungal effects of many plants (PRUSTI *et al.*, 2008; MOMTAZ and ABDOLLAHI, 2010). The effectiveness of these plants has been ascribed to the existence of a variety of phytochemicals.

In tropical countries, mycotic infections have become a disconcerted public health challenges (CHINELLI *et al.*, 2003). Fungal infections have outstandingly increased in both developing and developed countries with the increase in the numbers of immunocompromised individuals in epidemic human immunodeficiency viral (HIV) infections (MEENA *et al.*, 2009). While previously uncommon fungi which are difficult to manage and insidious opportunistic fungal infections caused by *Aspergillus* and *Candida* species are customarily accountable for the greater part of morbidity and mortality recorded in individuals undergoing haematopoietic stem cell transplantation (VISCOLI *et al.*, 1999; GROLL and WALSH, 2001), the rising occurrence of fungal pathogens resistant to antifungal drugs has also become a great concern (MANIKANDAN *et al.*, 2011). Consequently, while the administration of systemic drugs has become therapeutically inadequate due to increased level of toxicity and associated problems of residues in products intended for human consumption (ARAÚJO *et al.*, 2009), many aboriginal plants species widely described is now being required for treatment of infections (LEE *et al.*, 2007; VIGNESH *et al.*, 2011). Being less toxic and not detrimental to human health (SATISH *et al.*, 2009), bioactive compounds in plant extracts have become alternative to synthetic agents (KUMAR *et al.*, 2010; OLAJUYIGBE *et al.*, 2018).

Acacia mearnsii De Wild or Black Wattle, basically known as one of the world's highest yielding sources of high quality condensed tannin (YOUNG *et al.*, 1986), is a tree with 20-30 tiny pale yellow globular inflorescence flowers. The dark brown to black pods are more or less straight, 5-10 cm long, 5-8 mm wide and strongly constricted between seeds. Although hundreds of plant species have been tested for antimicrobial properties, an immeasurable majority of plants, including *A. mearnsii*, have limited information on their therapeutic effectiveness. Considering plants as sources for antimicrobial drugs with reference to antifungal agents, this study was aimed at investigating the antifungal and cytotoxicity effects of the methanolic extract of *A. mearnsii* *in vitro*.

MATERIALS AND METHODS

Collection of plant material

The stem barks of *A. mearnsii* De Wild were collected from the plant growing in Nkonkobe municipality, Eastern Cape, South Africa. The plant was authenticated in the Department of Botany, University of Fort Hare and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature, powdered in a mill (Christy Lab Mill, Christy and Norris Ltd; Process Engineers, Chelmsford, England) and stored in a sterile air-tight container for further use. One hundred gram of the powdered sample was soaked in 500 ml of methanol by shaking vigorously for 72 h on Stuart Scientific Orbital Shaker, UK at high speed. The plant material was extracted for two successive times. The solvent was removed using a rotary evaporator set at the distillation temperature of the extracting solvent (65°C) (Laborarota 4000 – efficient, Heldolph, Germany). The resulting crude extract was stored at -

20 °C until assayed. The extract was dissolved in methanol and made up with sterile distilled water to a concentration of 40 mg/ml stock solution from which serial dilutions and different concentrations were prepared.

Test organisms

The fungal isolates used in this study included *Absidia corymbifera* DSM-1144, *Aspergillus flavus* ATCC 9643, *Aspergillus terreus* DSM-826, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 2001, *Candida krusei* DSM-3433, *Candida rugosa* DSM-70761, *Cryptococcus neoformans* DSM-11959, *Fusarium sporotrichioides* DSM-62423, *Penicillium notatum*, *Trichophyton mucooides* ATCC 201382 and *Trichophyton tonsurans* ATCC 28942. These fungal isolates, maintained on potato dextrose agar and broth, were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa until they were used for the antifungal assays.

Preparation of inocula

The fungal inoculums were prepared according to the procedure of ABERKANE *et al.* (2002) with some modifications for the quantification of the fungi. Briefly, the inoculum of each isolate was prepared by first growing the fungus on potato dextrose agar for seven days. Thereafter, 1 cm² of each sporulating culture dropped in sterile distilled water were then shaken for 5 min and appropriate dilutions were made to obtain an approximate concentration of 1×10^5 spores/ml using spectrophotometer (A_{595} nm). In preparing 10^4 colony forming units (CFU) per μ l of the inoculums for the *Candida* spp., 1 ml of each of the overnight candida broth cultures was added to 9 ml of potato dextrose broth to yield.

Antifungal assay by agar diffusion method (Inhibition zones)

To determine the inhibition zones produced by the extract, 100 μ l of the adjusted fungal solutions, evenly spread with a sterile glass rod, was aseptically distributed on potato dextrose agar plates before allowing the culture to stand for 1 h on the laboratory bench. Agar wells were bored a 6 mm cork borer into the agar medium which were filled with 100 μ l of 10, 20 and 40 mg/ml of the extract while spillage of the solution onto the agar surface was prevented. The culture plates were allowed to stand on the laboratory bench for 30 min to allow suitable diffusion of the extract before incubating at 27 °C for 3-7 days. Wells in blank potato dextrose agar plates containing 5% methanol representing the final concentration of methanol in the test plates without the extract served as positive controls. After the incubation period, the antifungal effects were recorded by measuring the zones of inhibition in millimeter with a transparent meter rule. The antifungal assays were carried out in triplicates.

Macrobroth dilution for minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by serial tube dilution technique (IWAKI *et al.*, 2006; KHAN *et al.*, 2007). Here, the plant extract was serially diluted to concentrations ranging between 0.1953 mg/ml and 40 mg/ml in double strength potato dextrose broth. Each broth concentration was inoculated with 100 μ l of the prepared fungal spores' solution before incubating at 27 °C for 3-7 days. Two control tubes were included: one with spores and broth but no plant extract and one with broth and plant extract but no spores. The first tube in the series with no visible growth after incubation period was taken as the MIC.

Determination of minimum fungicidal concentrations (MFC)

The MFC assays were carried out as described by CHEESBROUGH (2006). Here, extract-free potato dextrose agar plates were inoculated with one loopful of culture taken from each of the first five broth cultures that showed no growth and the first growth-containing tube in the MIC tubes. The MFC assay plates were incubated at 27 °C for 3-5 days. After the incubation periods, the lowest concentration of the extract without visible fungal growth on agar was regarded as MFC values for this extract (IRKIN and KORUKLUOGLU, 2007). This observation was matched with the MIC test tube that did not show evidence of growth or spore germination for the fungi after five days of incubation.

Brine shrimp lethality test

The brine shrimp lethality test using the larvae of brine shrimps nauplii, *Artemia salina* L, was carried out using the standard procedure (MEYER *et al.*, 1982; McLAUGHLIN *et al.*, 1998). From 1000 µg/ml of the extract prepared as stock solution, different concentrations (0.9765-500 µg/ml) of the extract were prepared by serial tube dilution technique in different vials. Ten nauplii were transferred into each vial using Pasteur pipettes. The brine shrimps were not fed because hatched brine shrimp can survive for up to 48 h without food (LEWIS, 1995) as they still feed on their yolk-sac (PELKA *et al.*, 2000). The control vials were prepared using 5% methanol alone and the experiment was replicated three times. After 24 h of incubation, the vials were examined, the numbers of survivors in each vial were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any observable movement during several seconds of observation. The mortality percentage and LC₅₀ (lethal concentration for 50% of the population) were determined using statistical analysis and the graph of Logarithm of concentration against percent lethality.

Determination of mechanisms of antibiosis (fungicidal or fungistatic)

To know whether the observed antifungal effects were fungicidal or fungistatic, the mechanism of antibiosis of the extract was calculated using the ratio of MFC/MIC or MIC_{index} as described by SHANMUGHAPRIYA *et al.* (2008). When the ratio of MFC/MIC was ≤ 2.0 , the extract was considered fungicidal or otherwise fungistatic. If the ratio is ≥ 16.0 , the extract was considered ineffective.

RESULTS AND DISCUSSION

Since the first step towards achieving effective treatment of fungal infection is the *in vitro* antifungal activity assay (TONA *et al.*, 1998), the macrobroth dilution assay is an effective method (BARON *et al.*, 1994) permitting direct contact between the fungal cells and the test extract. In this study, all the fungal isolates were highly susceptible to different concentrations of the methanol extract. With the exception of *A. niger*, *C. krusei* and *A. corymbifera*, all the fungal strains were inhibited by the lowest concentration (10 mg/ml) of the extract. At 20 mg/ml, the mean \pm standard deviation of the inhibition zones ranged between 13 ± 0.58 and 21 ± 0.58 mg/ml. At the 40 mg/ml, the mean \pm standard deviation ranged between 15.00 ± 0.00 and 24 ± 0.58 mg/ml. Generally, the mean inhibition zones of each fungi showed that the activity of the extract was concentration dependent (Table 1).

The MICs ranged between 0.3125 and 2.5 mg/ml. With the exception of *C. krusei*, the MIC of other *Candida* strains was 0.3125 mg/ml. While the MIC of the yeasts ranged between 0.3125 and 1.25 mg/ml, those of the molds ranged between 0.625 mg/ml and 2.5 mg/ml. The MFC values, ranging between 0.3125 and 5 mg/ml, were similar or 2 – 4 folds higher than the

MIC values of the extract. The mechanism of antibiosis showed that the extract was 7.69% fungistatic and 92.31% fungicidal indicating that the extract has more killing effects on the fungi than potentiating their growth (Table 2).

Table 1. Antifungal activity of methanolic stem bark extract of *A. mearnsii*

Tested species/concentration	Mean Zone of Inhibition ± Standard deviation (± 1.00 mm)		
	10 mg/ml	20 mg/ml	40 mg/ml
<i>A. niger</i> ATCC 16404	0.00 ± 0.00	14.67 ± 0.58	17.00 ± 1.00
<i>A. terreus</i> DSM-826	12.67 ± 0.58	14.67 ± 0.58	18.33 ± 0.58
<i>A. flavus</i> ATCC 9643	12.00 ± 0.00	14.33 ± 1.00	15.00 ± 0.00
<i>C. krusei</i> DSM-3433	0.00 ± 0.00	12.67 ± 0.58	15.00 ± 1.00
<i>C. rugosa</i> DSM-70761	15.67 ± 0.58	18.67 ± 0.58	21.33 ± 0.58
<i>C. albicans</i> ATCC 10231	16.33 ± 0.58	16.33 ± 0.58	19.00 ± 1.00
<i>C. glabrata</i> ATCC 2001	12.67 ± 0.58	15.33 ± 0.58	18.00 ± 0.00
<i>P. notatum</i>	13.00 ± 0.00	15.33 ± 0.58	17.33 ± 0.58
<i>C. neoformans</i> DSM-11959	14.00 ± 1.00	15.33 ± 0.58	17.33 ± 0.58
<i>A. corymbifera</i> DSM-1144	0.00 ± 0.00	17.00 ± 0.00	20.33 ± 0.58
<i>F. sporotrichioides</i> DSM-62423	18.00 ± 0.00	20.67 ± 0.58	23.67 ± 0.58
<i>T. tonsurans</i> ATCC 28942	15.00 ± 0.00	18.00 ± 0.00	19.67 ± 0.58
<i>T. mucoides</i> ATCC 201382	17.33 ± 0.58	19.67 ± 0.58	22.00 ± 1.00

Table 2. Antifungal activity of methanolic extract of *A. mearnsii*

Tested species	Inhibitory and fungicidal activities of <i>A. mearnsii</i>		
	MIC mg/ml	MFC mg/ml	MFC/MIC
<i>A. niger</i> ATCC 16404	2.5	5	2
<i>A. terreus</i> DSM-826	2.5	5	2
<i>A. flavus</i> ATCC 9643	2.5	5	2
<i>C. krusei</i> DSM-3433	1.25	2.5	2
<i>C. rugosa</i> DSM-70761	0.3125	1.25	2
<i>C. albicans</i> ATCC 10231	0.3125	1.25	4
<i>C. glabrata</i> ATCC 2001	0.3125	0.625	2
<i>P. notatum</i>	2.5	5	2
<i>C. neoformans</i> DSM-11959	0.3125	0.3125	1
<i>A. corymbifera</i> DSM-1144	0.625	0.625	1
<i>F. sporotrichioides</i> DSM-62423	0.625	0.625	1
<i>T. tonsurans</i> ATCC 28942	0.625	2.5	2
<i>T. mucoides</i> ATCC 201382	2.5	5	2

By subjecting the brine shrimps to different concentrations of the extract, death of the brine shrimps was noticed in the experimental group but the control group remained unchanged over the same period of time. The number of surviving brine shrimps in each vial was counted and the results were noted. The percentage of death of the shrimp was calculated for every concentration of the test sample. The extract killed 6.7% of the brine shrimp at 31.25 µg/ml while 96.7% of the shrimps were killed at 500 µg/ml after 24 h incubation. The death rate of the brine shrimps increase with the increase in concentration of the extract as

shown by a graph of Logarithm of concentrations versus percentage mortality presented as Figure 1. It is evident from the results of the brine shrimp lethality testing that the crude extract with the LC₅₀ being 101.59 µg/ml had the highest (96.7%) death percentage at 500 µg/ml.

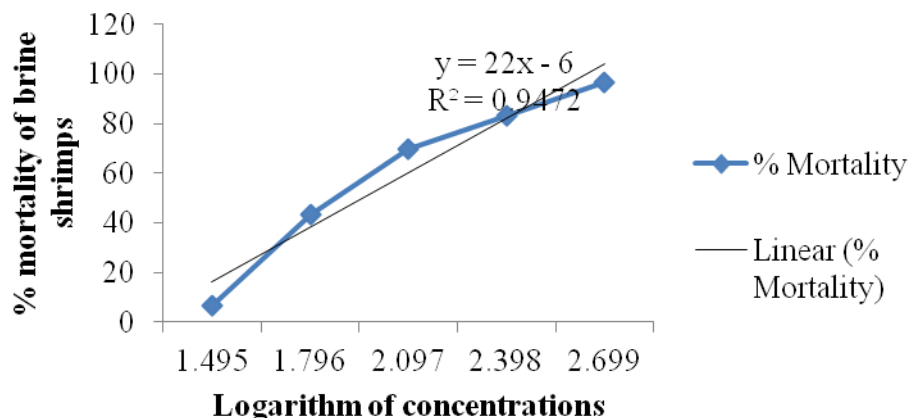


Figure 1. Cytotoxic effect of methanolic stem bark extract of *A. mearnsii* on brine shrimps naupauli

The roles of fungi in pathogenic dermatophytic and mycotic infections have been enumerated in previous studies (FAN *et al.*, 2008; DE TOLEDO *et al.*, 2011) while the use of plant extracts is indicated as an alternative form of therapy for mycosis and candidiasis (LAZAREVIĆ *et al.*, 2011). However, due to the rate of recurrence of fungal infections being on the rise (CAPOOR *et al.*, 2005), microbial resistance (SIMONS *et al.*, 2006), adamant nature of both cutaneous or superficial and systemic fungal infections and the toxicity often associated with many pharmacologically active antifungal agents, the need to explore alternative means of treating fungal infections with natural remedies having no toxicological side effects on human systems becomes extremely necessary.

Accordingly, several studies have shown the antifungal potentials vested in alcoholic extracts of several higher plants against fungal pathogens (SARFARAZ *et al.* 2002; MALABADI and KUMAR (2007). AMANLOUA *et al.* (2004) indicated that methanolic extracts of *Satureja khuzestanica* inhibited *C. albicans* and *A. niger*. MUSCHIETT *et al.* (2005) reported *in vitro* antifungal activities of 11 traditionally used Argentine medicinal plants against dermatophytes with microbroth dilution method. While dichloromethane extract of the resinous exudate from *Baccharis grisebachii* was reported active against *Epidermophyton floccosum*, *Microsporum gypseum*, *Microsporum canis*, *T. rubrum* and *T. mentagrophytes* (FERESIN *et al.*, 2003), methanol extract of *Plumbago zeylanica* bark was inhibitory against *T. mentagrophytes*, *T. simii*, *T. rubrum* (SUBRAMONEY *et al.*, 2003) and that of *Myracrodruon urundeuva* showed antifungal activity on *Fusarium* (SÁ *et al.*, 2009). In this study, the antifungal activity exhibited by the methanolic extract of *A. mearnsii* agreed with previous reports indicating antifungal activities of many traditionally used medicinal plants and corroborated its folkloric use as indicated by SOMCHIT *et al.* (2003).

Although the antifungal activities of many medicinal plants have been investigated with lacks on their cytotoxicity potentials, some of the studied medicinal plants exhibited moderate to good cytotoxicity (RHAMAN *et al.*, 2007). The brine shrimp lethality test is used to evaluate different pharmacological activities of natural remedies taking into account that pharmacology is simply toxicology at a lower dose. Since plant extracts are regarded as being mildly toxic if their lethal concentration for 50% (LC₅₀) > 30<100 µg/ml and non-toxic if their LC₅₀ is greater than 100 µg/ml in the brine shrimp lethality assay (MOSHI *et al.*, 2010), it is

evident that the methanol extract with the LC₅₀ being 101.59 µg/ml was non toxic (LC₅₀ > 100 µg/ml) and considerably active since extract with LC₅₀ lower than 250 µg/ml had earlier been considered considerably active by RIESER *et al.* (1996). Pharmacologically, the varied inhibitory and fungicidal activity exhibited by this extract against the different fungal isolates may be attributed to its toxicity level and the various phytochemicals present in the plant (OLAJUYIGBE and AFOLAYAN, 2011). Since it is important to investigate the fungicidal activity of potential chemotherapeutic agents to determine their ability to kill pathogens outright in clinical settings, the fungicidal and cytotoxicity activities of *A. mearnsii* may be valuable in treating serious dermal fungal infections especially when the host defense mechanisms are compromised.

CONCLUSION

This study indicates that *A. mearnsii* possess antifungal and cytotoxic properties *in vitro* and provides scientific basis for the use of this plant in folk medicine for the treatment of dermatophytic fungal infections. While the antifungal activity indicates that there are active compounds of significance, the cytotoxicological effect indicates that this plant is a potential source of a novel antifungal natural product that may be isolated and used in the treatment of skin infections. Further studies exploring the *in vivo* antifungal activities of this plant to establish its complete safety profile, isolate and characterize the active compounds, especially those having therapeutic indices, are required.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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