

Full Length Research Paper

## ***In vitro* evaluation of the antifungal activity of *Sclerocarya birrea* extracts against pathogenic yeasts**

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The antifungal activity of *Sclerocarya birrea* which is used in South African traditional medicine for the treatment of skin diseases was evaluated against three yeasts; *Candida parapsilosis*, *Cryptococcus albidus* and *Rhodoturula mucilaginosa*. Barks of *S. birrea* were extracted with hexane, dichloromethane (DCM), chloroform, ethyl acetate, acetone, methanol and ethanol and tested against these three yeasts. The antifungal assay was performed by the microdilution technique and bioautography. Thin layer chromatography was used to analyze the phytochemicals of the extracts as well as to assay the plant for antioxidant compounds. More compounds with antioxidant activity were observed in polar separation system, ethyl acetate:methanol:water (EMW). All test organisms were resistant against all non-polar extracts. Acetone, ethanol and methanol *S. birrea* extracts had average MIC values of 0.39, 0.22 and 0.27 mg/ml, respectively. *C. albidus* was the most sensitive organism with an average MIC value of 0.17 mg/ml. Average total activity was highest for methanol (387 ml/g) followed by ethanol (363 ml/g) and acetone (299 ml/g) bark extracts. Acetone and methanolic bark extracts were more active in EMW system at  $R_f$  values of 0.07, 0.32 and 0.70 against *C. parapsilosis*. The results showed that the plant could be further explored for possible antifungal agents and provides preliminary scientific validation of the traditional medicinal use of this plant.

**Key words:** Antifungal activity, *Sclerocarya birrea*, minimum inhibitory concentration, bioautography, antioxidant.

### INTRODUCTION

The incidence of dermatophytoses has increased worldwide in recent years, especially in immunocompromised patients with atypical manifestations and more severe lesions (Carrillo-Muñoz et al., 2008). The current situation of HIV infection and immunosuppression induced in organ transplants or by cancer chemotherapy lead to increased predisposition to fungal infections (Walsh et al., 2004). Many known antifungal drugs have been used clinically for topical treatment of dermatophytoses but the prolonged duration of treatment, drug toxicity and interactions, fungal resistance and high costs are problems (Bennett et al., 2000) and hence the search and development of new, more efficient and safe antifungal drugs is warranted.

The vast majority of people worldwide still rely on traditional medicine for their everyday healthcare needs. It is also a known fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs. According to the WHO, 80% of the world's population, primarily those of developing countries rely on plant-derived medicines for their healthcare (Balick et al., 1994).

The quest for development of new antifungal agents with powerful and wide range of fungicidal activity led us to investigate *Sclerocarya birrea* for potential antifungal compounds. The selection of this plant is based on its ethnopharmacological use in traditional medicine.

*Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro, commonly known as marula (English), morula (Northern Sotho), mufula (Tshivenda), ukanyi (Tsonga), umganu (Zulu) and maroela (Afrikaans) in the family Anacardiaceae which encompasses 73 genera and

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600 species (Pretorius et al., 1985), is widespread in Africa from Ethiopia in the north to KwaZulu-Natal in the south. In South Africa it is more dominant in the Baphalaborwa area in the Limpopo province. It occurs naturally in various types of woodland, on sandy soil or occasionally on sandy loam (van Wyk et al., 1997).

Ripe marula fruit can be consumed by biting or cutting through the thick leathery skin and sucking the juice or chewing the mucilaginous flesh after removal of the skin. The ripe fruit has an average vitamin C content of 168 mg/100 g which is approximately three times that of oranges and comparable to the amounts present in guavas (Wilson, 1980). Marula can be fermented to make alcoholic drinks; fruit can also be processed to make jam, jelly and juice. Kernels are eaten directly or cooking oil can be extracted from them. Leaves are browsed by livestock and have a variety of medicinal uses (Muok et al., 2007).

In South Africa and in some other African countries, the stem-bark, roots and leaves of *S. birrea* are used for an array of human ailments, including: malaria and fevers, diarrhea and dysentery, stomach ailments, headaches, toothache, backache and body pains, infertility, schistosomiasis, epilepsy, diabetes mellitus, etc. (Watt and Breyer-Brandwiik, 1962; Hutchings et al., 1996; Van Wyk et al., 1997).

Several researchers have reported biological activities of *S. birrea* extracts, but no comprehensive antifungal activities of *S. birrea* have been reported, although it is widely used by traditional healers. Eloff (2001) reported antibacterial activity of acetone extracts of *S. birrea* against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*. MacGaw et al. (2007) have also reported its antibacterial, antihelminthic and cytotoxic effects. Anticonvulsant effect of *S. birrea* stem-bark aqueous extract in mice was reported by Ojewole (2007). Runyoro et al (2006) have investigated 34 medicinal plants used by Tanzanian traditional healers in the management of *Candida* infections and they reported that the ethanolic extract of dried stem bark of *S. birrea* showed antifungal activity against *C. albicans*.

Nevertheless, there is no information about the antifungal property of *S. birrea* barks. Thus, and as part of our ongoing study of South African medicinal plants used in Limpopo Province, we report here the antifungal activity of extracts of *S. birrea* barks.

## MATERIAL AND METHODS

### Plant material collection and storage

The *S. birrea* (UNIN9041) plant was verified by University of Limpopo herbarium. Different plant parts (leaves, bark and the roots) were collected in winter from a tree in the University of Limpopo (Turloop campus) grounds in the Limpopo Province. The plant materials were air-dried on the laboratory bench at room temperature until well dried. The dried materials were ground with an electric grinder into fine powder and stored in air-tight containers at room temperature in the dark until required.

### Extraction procedure

To determine the efficiency of different extractants, 1 g samples of finely ground leaves and rhizomes were extracted in 10 ml in each of hexane, dichloromethane (DCM), chloroform, ethyl acetate, acetone, methanol and ethanol (technical grade-Merck), respectively in centrifuge tubes. These tubes were vigorously shaken for 3 - 5 min in a Labotec model 20.2 shaking machine. After centrifugation at 959 x g for 10 min, the supernatant was decanted into labeled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were later combined. The solvent was removed under a stream of air in a fume cupboard at room temperature before dissolving the resultant residues in acetone to a concentration of 10 mg/ml.

### Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F<sub>254</sub>). The TLC plates were developed with one of the three eluent systems, i.e., ethylacetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour by lining the tank with filter paper wetted with the eluent.

### TLC analysis of the extracts

Visible bands were marked under daylight and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600) before spraying with freshly prepared *p*-anisaldehyde (1 ml *p*-anisaldehyde, 18 ml ethanol, 1 ml sulphuric acid) or vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagents (Stahl, 1969). The plates were carefully heated at 105°C for optimal colour development.

### Qualitative 2, 2 -diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

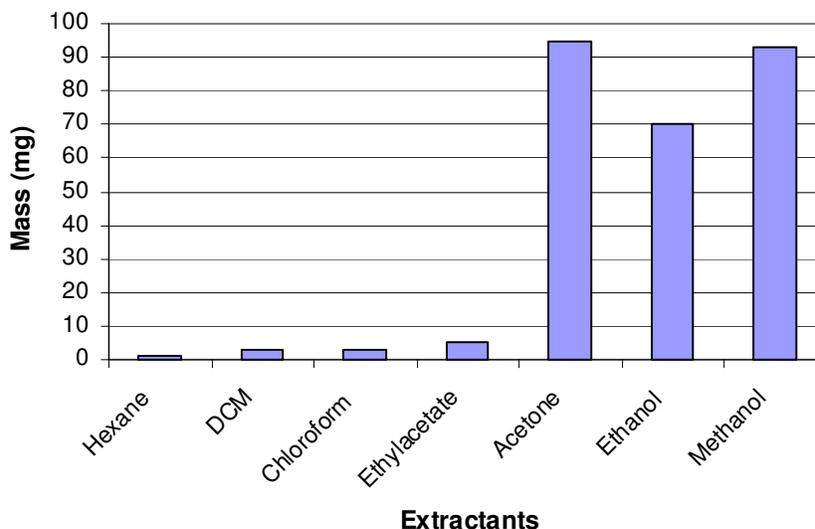
TLCs were used to separate extracts as described earlier. The plates were dried in the fume hood. To detect antioxidant activity, chromatograms were sprayed with 0.2% 2, 2 -diphenyl-2-picrylhydrazyl (DPPH) (Sigma®) in methanol, as an indicator (Deby and Margotteaux, 1970). The presence of antioxidant compounds were detected by yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol.

### Fungal strains

Microorganisms used in the determination of antifungal activities were *Candida parapsilosis* Y0225, *Cryptococcus albidus* Y0821 and *Rhodoturula mucilaginosa* Y0478 obtained from the Central University of Technology, Free State. The fungal cultures were maintained on Yeast-Malt Extract (YM) media and subcultures were freshly prepared before use.

### Quantitative antifungal activity assay by minimum inhibitory concentration (MIC)

The microplate serial dilution method (Eloff, 1998) modified by Masoko et al. (2005) was used to determine the minimum inhibitory concentration (MIC) of extracts against the three yeast pathogens. The three fungal strains were all standardized to colony forming unit of 10<sup>7</sup> cfu/ml. Extracts (10 mg/ml) were dissolved in acetone and



**Figure 1.** Mass of samples extracted by different solvents with varying polarity from 1 g of *S. birrea*.

serially diluted with sterile water in microplates in a laminar flow cabinet. The same volume of an actively growing culture of the test fungi was added to the different wells and cultures were grown overnight in 100% relative humidity at 25°C. The next morning 40 µl of 0.2 mg/ml of tetrazolium violet was added to all the wells. Growth was indicated by the development of a violet colour. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control acetone had no influence on the growth at the highest concentration used (25%).

#### Total activity of the extracts

The total activity in ml/g was calculated by dividing the MIC value with the quantity extracted from 1 g of plant material. The resultant value indicates the volume to which the extract can be diluted and still inhibit the growth of a microorganism (Eloff, 2004).

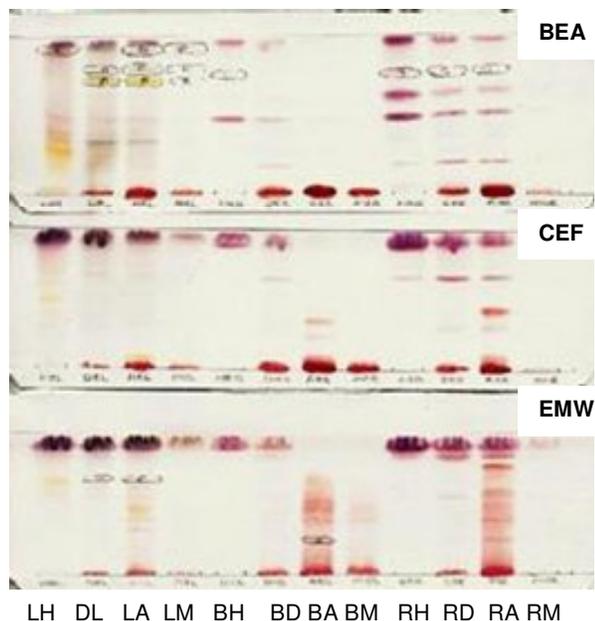
#### Qualitative antifungal activity assay by Bioautography

The bioautography procedure was done according to the Begue and Kline (1972) method modified by Masoko and Eloff (2005). TLC plates (10 x 10 cm) were loaded with 100 µg (5 µl of 20 mg/ml) of each of the extracts. The prepared plates were developed in the three different mobile systems (CEF, BEA and EMW) separately. The chromatograms were dried for up to a week at room temperature under a stream of air to remove the remaining solvent. Cultures were grown on Yeast-Malt Extract media agar for 3 to 5 days. Sabouraud broth was prepared in 250 ml bottles. Cultures were transferred into the broth from agar plates with sterile swabs. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately  $10^9$  of actively growing yeasts per ml in a Biosafety Class II cabinet (Labotec, SA). The plates were sprayed until they were just wet, incubated overnight and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) (Sigma®) and further incubated for 2 to 3 h at 35°C in a clean chamber at 100% relative humidity in the dark. White areas indicate the spots where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the tested yeasts.

## RESULTS AND DISCUSSION

*Sclerocarya birrea* was selected for antifungal activity testing based on its use in traditional medicinal treatments for humans in southern Africa. The majority of traditional healers use water to extract active compounds from these plants, because water is not harmful to domestic animals and humans and is generally the only extractant available. Successful isolation of compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Use of water alone leads to difficulties in extracting non-polar active compounds. The mass sample extracted from *S. birrea* using different solvents [hexane, dichloromethane (DCM), chloroform, ethyl acetate, acetone, methanol and ethanol] are shown in Figure 1. Methanol was quantitatively the best extractant, extracting a greater quantity of plant material than any of the other solvents used. Non-polar solvents were more selective extractants for *S. birrea*, because all the samples extracted with non-polar solvents were below 10 mg (Figure 1).

After evaporation of extracting solvents (hexane, DCM, chloroform, ethyl acetate, methanol and ethanol) extracts were redissolved in acetone because this solvent was found not to be harmful towards fungi (Eloff et al., 2007). The separated compounds on TLC plates were made visible by spraying with vanillin-sulphuric acid. Of the seven solvents used, methanol extracted more chemical compounds from the roots of *S. birrea* (Figure 2); however, the extract probably contained highly polar compounds and tannins that may not be significant for clinical application. More bands were separated with the non-polar BEA followed by polar EMW but intermediate polarity CEF separated less bands. Of all the mobile systems used, acetone root extracts displayed more bands. There



**Figure 2.** Chromatograms of *S. birrea* developed in BEA (top), CEF (centre) and EMW (bottom) solvent systems and sprayed with vanillin-sulphuric acid reagent. LH, Leaves extracted with hexane; LD, Leaves – dichloromethane; LA, leaves – acetone; LM, leaves – methanol; BH, barks – hexane; BD, barks – dichloromethane; BA, barks – acetone; BM, barks – methanol; RH, roots – hexane; RD, roots – dichloromethane; RA, roots – acetone; RM, roots – methanol.

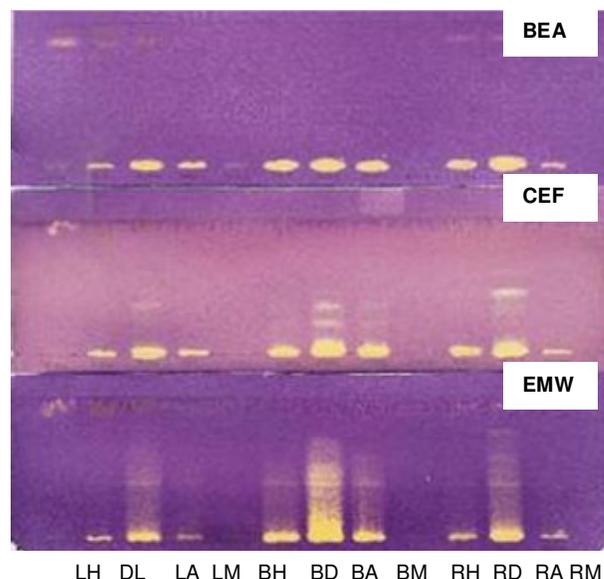
were similarities between compounds separated with polar solvents but methanol was different, especially with BEA, the non-polar solvent system; furthermore, substantial differences between the other extracts were visible, but the differences were apparent with CEF and EMW solvent systems (Figure 2).

The dichloromethane, hexane, and acetone extracts showed antioxidant activity after spraying chromatogram with 0.2% DPPH (Figure 3). Methanol extracts apparently did not have any antioxidant activity. More compounds with antioxidant activity were observed in polar separation system (EMW).

There have been reports on the antioxidant activity of *S. birrea* extracts and their potential for commercial development. Ndhlala et al. (2007) have reported that the pulp of *S. birrea* possesses high total phenolics, flavanoids and condensed tannins, i.e., 2262 µg GAE/g, 202 µg catechin/g and 6.0% condensed tannins, respectively. Mdluli (2005) has also investigated the antioxidant potential of marula fruits and observed the antioxidant activity of the juice.

Antioxidant activity was observed on the fruits and was never investigated on the bark. In this study we confirm that *S. birrea* has antioxidant compounds on the bark. Traditionally, the bark of *S. birrea* is used for different diseases.

Amphotericin B used as a positive control inhibited the growth of *C. albicans* and its MIC value was below 0.02 mg/ml. Amphotericin B had an MIC of 0.02 mg/ml against



**Figure 3.** Chromatograms of *S. birrea* extracts separated by BEA (top), CEF (centre) and EMW (bottom) solvent systems and sprayed with 0.2% DPPH, clear zones indicate antioxidant activity. Lanes from left to right in each group were: hexane, dichloromethane, acetone, and methanol. LH, Leaves extracted with hexane; LD, Leaves – dichloromethane; LA, leaves – acetone; LM, leaves – methanol; BH, barks – hexane; BD, barks – dichloromethane; BA, barks – acetone; BM, barks – methanol; RH, roots – hexane; RD, roots – dichloromethane; RA, roots – acetone; RM, roots – methanol.

both *R. mucilaginosa* and *C. parapsilosis*. All test organisms were resistant against all non-polar extracts (hexane, DCM and ethyl acetate). Acetone, ethanol and methanol *S. birrea* extracts had the average MIC values of 0.39, 0.22 and 0.27 mg/ml, respectively (Table 1). The ethanolic extract of *S. birrea* was very active against all the tested pathogens. *C. albicans* was the most sensitive organism with an average MIC value of 0.17 mg/ml, followed by *C. parapsilosis* with average MIC of 0.28 mg/ml; the least sensitive organism was *R. mucilaginosa* with 0.43 mg/ml (Table 1).

Other researchers have reported antifungal activity of *S. birrea*. Hamza et al. (2006) have reported that methanolic extracts from *S. birrea* roots inhibited the growth of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Cryptococcus neoformans* with the MIC of 0.5 mg/ml. Eloff (2001) reported antibacterial activity of *S. birrea* leaves and bark extracts against Gram-positive and -negative bacteria such as *S. aureus*, *P. aeruginosa*, *E. coli* and *E. faecalis* with MIC values ranging from 0.15 to 3 mg/ml, and further reported that based on minimum inhibitory concentration values, inner bark extracts tended to be the most potent followed by outer bark and leaf extracts.

The MIC values of *S. birrea* root against several *Candida* species was approximately 1.8 higher than the value for *C. parapsilosis* obtained in this study and 2.9 times higher for *C. neoformans* compared to *C. albicans*,

**Table 1.** Minimum Inhibitory Concentration (MIC) of *S. birrea* extracts after 24 h incubation at 25°C.

Microorganisms	Minimum Inhibitory Concentration (MIC) mg/ml							Amphotericin B (µg/ml)
	Hexane	DCM	Ethyl acetate	Acetone	Ethanol	Methanol	Average	
<i>C. albidus</i>	NA	NA	NA	0.21	0.13	0.16	<b>0.17</b>	0.02
<i>R. mucilaginosa</i>	NA	NA	NA	0.64	0.32	0.32	<b>0.43</b>	0.02
<i>C. parapsilosis</i>	NA	NA	NA	0.32	0.21	0.32	<b>0.28</b>	0.02
<b>Average</b>				<b>0.39</b>	<b>0.22</b>	<b>0.27</b>		

NA = No activity.

**Table 2.** Total activity in ml/g of *S. birrea* extracts after 24 h incubation at 25°C.

Microorganism	Total activity ml/g						
	Hexane	DCM	Ethyl acetate	Acetone	Ethanol	Methanol	Average
<i>C. albidus</i>	NA	NA	NA	452	538	581	<b>524</b>
<i>R. mucilaginosa</i>	NA	NA	NA	148	218	290	<b>219</b>
<i>C. parapsilosis</i>	NA	NA	NA	29	333	290	<b>306</b>
<b>Average</b>				<b>299</b>	<b>363</b>	<b>387</b>	

NA = No activity

which may suggest that the bark may contain higher antifungal compounds. This observation is consistent with Eloff's (2001) findings which also found lower MICs in bark extracts against bacteria

The quantity of antifungal compounds present was also determined (Table 2). To determine which plant parts can be used for further testing and isolation, not only the MIC value is important, but also the total activity. Since the MIC value is inversely related to the quantity of antifungal compounds present, an arbitrary measure of the quantity of antifungal compounds present was calculated by dividing the quantity extracted in milligrams from 1 g leaves by the MIC value in mg/ml. This value indicates the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the fungi (Eloff, 2004). Extracts with higher values were considered the best to work with. From Table 2, three extracts displayed substantial total activity against *C. albidus* followed by *C. parapsilosis* after 48 h; *R. mucilaginosa* was relatively resistant. Average total activity, a measure of potency, was highest for methanol (387 ml/g) followed by ethanol (363 ml/g) and acetone (299 ml/g) bark extracts.

Bioautography was used to screen for antifungal compounds to obtain more information on the diversity of antifungal compounds present in different extracts. Inhibition zones of antifungals were observed as white spots on a purple-red background (results not shown). The scoring was used to determine relative activity of fungal compounds (Table 3).

In some cases the organisms did not grow too well and it was difficult to detect inhibition zones, such cases were

not included in this report. In other cases there were no fungal growth possibly due to traces of formic acid left on the chromatogram that inhibited fungal growth.

Most of the active compounds against *C. parapsilosis* were observed in BEA and EMW mobile systems. In BEA system active compounds were at  $R_f$  values of 0.08 and 0.32 in both the leaves and the bark, but the bark extracts were found to be more active. We have also observed that the acetone and methanolic bark extracts are more active in EMW system at  $R_f$  values of 0.07, 0.32 and 0.70 (Table 3). Caffeic acid, vanillic acid, *p*-hydroxybenzaldehyde, ferulic acid, *p*-hydroxybenzoic acid and *p*-coumaric acid were identified in the peel while caffeic acid, ferulic acid and *p*-coumaric acid in the pulp of *S. birrea* (Ndhlala et al., 2007). Therefore, it is likely that the bark of *S. birrea* might also contain similar compounds; future work will seek to verify this. Attempts are under way to isolate and characterize these compounds.

The study demonstrated high antifungal activity due to polar compounds extractable with polar solvents. Additional investigations are required to determine whether similar activities occur in extracts from different plant parts (e.g., stems, branches, roots, and leaves), and what the optimum extraction and storage conditions are to obtain the highest quality yields of desired functionality. Future experiments must also aim at investigate the effectiveness of water extracts, since water extracts from medicinal plants are commonly used by traditional healers.

In conclusion this study validates and documents, in a systematic way, the antifungal properties of *S. birrea*. The study also provides valuable information for further phyto-

**Table 3.** The inhibition of fungal growth by bioautography of *S. birrea* extracts separated by TLC with BEA and EMW as eluents. The  $R_f$  values of active components and relative degree of inhibition are shown.

$R_f$	BEA											
	LH	LD	LA	LM	BH	BD	BA	BM	RH	RD	RA	RM
0.31			XXX	XXX		XXX	XXXX	XXXX			XX	
0.08			XX	XX		XX	XXXX	XXXX				
$R_f$	EMW											
0.70			XX	XX	XX		XX	XX				
0.37							XX	XX				
0.07							XXX	XXX				

Relative degree of inhibition: X = slight inhibition, XXXX = very high inhibition.

BEA = benzene:ethyl acetate:formic acid, EMW = ethyl acetate:methanol:water.

LH, Leaves extracted with hexane; LD, Leaves – dichloromethane; LA, leaves – acetone; LM, leaves – methanol; BH, barks – hexane; BD, barks – dichloromethane; BA, barks – acetone; BM, barks – methanol; RH, roots – hexane; RD, roots – dichloromethane; RA, roots – acetone; RM, roots – methanol.

chemical isolation and characterization of biologically active compounds, which maybe developed into new antifungal drugs.

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