Journal of Medicinal Plants Research Vol. 6(38), pp 5135-5141, 3 October, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.151
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Antifungal activity of stigmasterol, sitosterol and ergosterol from *Bulbine natalensis* Baker (Asphodelaceae)

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Accepted 17 February, 2012

Bulbine natalensis* Baker. (Asphodelaceae) is indigenous to only southern Africa and is widely used as a skin remedy. *B. natalensis* contain secondary metabolites that have antibacterial properties. Phytosterols are a group of steroid alcohols and phytochemicals that occur naturally in plants. *Aspergillus*, *Penicillium* and *Fusarium* species are considered to be the most toxigenic fungi. They produce a large consortium of mycotoxins that include aflatoxins B_1, B_2, G_1, G_2, fumonisin B_1 and ochratoxins. They are found in foodstuffs and are not destroyed by normal industrial processing or cooking since they are heat-stable. The purpose of the project was to extract and quantify phytosterols, such as ergosterol, stigmasterol and sitosterol from *B. natalensis* using phytochemical methods such as thin layer chromatography and high performance liquid chromatography. The extract was tested against *Aspergillus flavus, Penicillium digitatum* and *Fusarium verticilloides* for antifungal activities. 1 ml of saponified extract contained 0.07 µg of sitosterol, 0.16 µg of stigmasterol and 0.37 µg of ergosterol. Saponifiable, unsaponifiable and crude extracts showed minimal activity against *F. verticilloides*, whereas potent activity was exhibited against *A. flavus.

Key words: *Bulbine natalensis*, phytosterols, secondary metabolites, antifungal activity, mycotoxins.

INTRODUCTION

Plants have been one of the major development sources of medicines ever since the dawn of human civilization. Over 60% of all pharmaceuticals are plant based (Sharma et al., 2005). Herbal remedies from traditional herbs and medicinal plants are commonly use in the Philippines. Health and healing are usually in the alternative form of a hand-me-down herbal concoction in rural areas. There are thousands of herbal plants that traditionally have certified medicinal benefits. A considerable number of plants still need to be scientifically validated and much work is still needed to investigate the bioactivity and phytochemicals of plants (Apaya and Chichioco-Hernandez, 2011).

In South Africa the local people relies extensively on medicines to treat skin ailments. The scientific qualities of many of these plants used to treat wounds and burns still have to be confirmed. *Bulbine natalensis* and *Bulbine frutescens* of the Asphodelaceae family are indigenous to only southern Africa and are widely used as a skin remedy (Panter et al., 2011). Phytosterols are also called plant sterols. They are a group of steroid alcohols, phytochemicals naturally occurring in plants. Phytosterols are applied in medicine and cosmetics and are taken as food additives to lower cholesterol (Christiansen et al., 2003). Invasive fungal infections turn out to be more and more common now days in plants, animals and humans. Ergosterol is a major fungal sterol and abundant in most fungal cell membranes. It is either absent or found in small amounts in higher plants. Ergosterol is a small percentage of the sterol mixture in plants and animals (Kadakal et al., 2005; Zhao et al., 2005). Stigmasterol and β-sitosterol are well known phytosterols. They are similar in structure. B-sitostero has been shown to

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reinforce the membrane while stigmasterol is not (De-Eknamkul and Potduang, 2003). β-sitosterol and its saturated form, β-sitostanol, are known to reduce the absorption of cholesterol in the intestinal lumen (Christiansen et al., 2003). β-sitosterol was reported as an antitumor and hypoglycaemic compound (Kaufman et al., 1999).

Cholesterol is the main animal sterol in the management of heart disease. Other common sterols include the phytosterols (plant sterols) β-sitosterol and stigmasterol, which differs from β-sitosterol only by the presence of a double bond at position (C_{22} to C_{23}), which are wide spread in plants and ergosterol, which is everywhere in fungi as a cell wall component (Heinrich et al., 2004). This study was carried out with the aim of contributing to previous work and the development of a secondary metabolite library from indigenous plants. This study therefore reports on the antifungal potential of isolated phytosterols from *B. natalensis.*

MATERIALS AND METHODS

Extraction by distillation

500 g of *B. natalensis* corms was peeled, grated and crushed using a mortar and pestle. 500 ml chloroform was added to the plant material in a 1:1 (v:w) ratio, vortexed (5 min), extracted (15 min) and centrifuged (365 x g for 5 min) at room temperature. The supernatant was then removed and the extracting method repeated with the same plant material. The chloroform was evaporated in vacuo and mass of extracts determined. After mass determination the extracts were re-dissolved in chloroform until further use (Boukes et al., 2008).

Saponification

Ground corms (1 to 2 g) was accurately weighed into a 50 ml screw cap test tube, and then 10 ml of saponification reagent was prepared by freshly mixing ethanol and 33% (w/v) KOH solution at a ratio of 94:6, 0.5 ml of 20% ascorbic acid (to prevent oxidation of tocopherols during saponification), and 50 µl of 5α-cholestane solution. 1 µg/µl hexane was added immediately. The sample was then homogenized for five seconds at full speed, capped, and then incubated for 1 h at 50°C. After cooling in ice water for 10 min, 5 ml de-ionized distilled water and 5 ml hexane was added. Tubes were capped tightly and then the contents were mixed thoroughly by shaking. After 15 h for phase separation, the hexane layer containing un-saponifiables was carefully transferred to a scintillation vial and dried under nitrogen flow. To the dried sample 200 µl pyridine and 100 µl Sylon BFT [99% Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS)] was added. The sample was derivatized either at 50°C in a water bath for 1 h or overnight at room temperature, and then be analyzed using a Liquid Chromatography (Du and Ahn, 2002).

Qualitative identification using thin layer chromatography

The qualitative identification using thin layer chromatography was achieved using a modified method by Boukes et al. (2008). Ergosterol, B-sitosterol and stigmasterol standards and *B. natalensis* extracts were dissolved in chloroform and spotted onto 20 × 20 cm silica coated aluminium plates (Merck, Darmstadt) air dried. A chromatographic tank was equilibrated for 1 h using toluene-diethyl ether (40:40, v/v) as mobile phase for sterol identification. TLC plate was developed for ±20 min or until the solvent front was ±1 cm from the top of the plate. TLC plate was dried at room temperature and developed by firstly dipping into a solution containing 5% sulphuric acid in 96% ethanol for 15 s followed by a solution containing 1% vanillin in 95% ethanol for 15 s and dried at room temperature. Once dried, the plate was heated at 80 to 100°C for 5 min.

Quantitation using high performance liquid chromatography

Samples were dissolved in ethanol. Preparation of well dissolved samples was achieved by ultra-sonic treatment. All samples were filtered by membrane filtration before HPLC analysis. HPLC equipment with UV detector at 210 nm. A C18 column was used. The column temperature at 30°C. Mobile phase was HPLC grade methanol. The flow rate was 1.0 ml/min. Sample injection volume was 30 µl (Sheng and Chen, 2009).

Antifungal activity- Agar diffusion method

Antifungal activity of the crude extract was tested using agar diffusion method. An antifungal drug, Amphotericin B was used as a standard. The fungal isolate was grown on Sabouraud dextrose agar (SDA) (Oxoid) at 25°C until they sporulated. Fungal spores of *Aspergillus flavus, Penicillium digitatum* and *Fusarium verticilloides* were harvested by pouring a mixture of sterile glycerol and distilled water to the surface of the plate and the spores were scraped with a sterile glass rod. The spores collected were standardized to an optical density of 600 nm of 0.1 before use. 100 ml of the standardized fungal spore suspension was evenly spread on the SDA (Oxoid) using a glass spreader. Wells were bored into the agar media using a sterile 6 ml cork borer and the wells filled with the solution of the extract being careful not to allow spilling of the solution to the surface of the agar medium. The plates were placed on the laboratory bench for 1 h to allow for proper diffusion of the extract into the media. 200 mg of the Ketoconazole drug was dissolved in 100 ml of distilled water. 1 mg of the solution was dispensed into the wells using sterile pipettes. Plates was incubated at 25°C for 96 h and then observed for zones of inhibition. The effect of the extract on fungal isolates was compared with Amphotericin B at a concentration of 250 µg/ml (Sule et al., 2011).

Thin layer chromatography- bioautography

Crude extracts was dissolved to a concentration of 10 mg/ml in the solvent of extraction. 10 µl of the solutions was applied on silica gel TLC plates. TLC plates were developed in appropriate solvent systems and thoroughly dried for complete removal of the solvents. Afterwards, the chromatograms were sprayed with a spore suspension in a nutritive medium and incubated for 2 to 3 days at room temperature in a moisture chamber. Clear inhibition zones appeared against a dark greenish background. Ketoconazole was used as positive control in the bioautography (Scher et al., 2004).

RESULTS

Identification of phytosterols by thin layer chromatography

Plant extracts, saponified and unsaponified were spotted on a TLC plate. Standards dissolved in chloroform were

Table 1. Resolution factor values of phytosterols on the thin layer chromatography plate.

Parameter	Compound	R_f value
1 - Unsaponified	Sitosterol	0.84
	Stigmasterol	0.85
	Ergosterol	0.89
2 - Saponified	Sitosterol	0.83
	Stigmasterol	0.85
	Ergosterol	0.9
3 –Standard	Sitosterol	0.84
4 – Standard	Ergosterol	0.89
5 – Standard	Stigmasterol	0.86

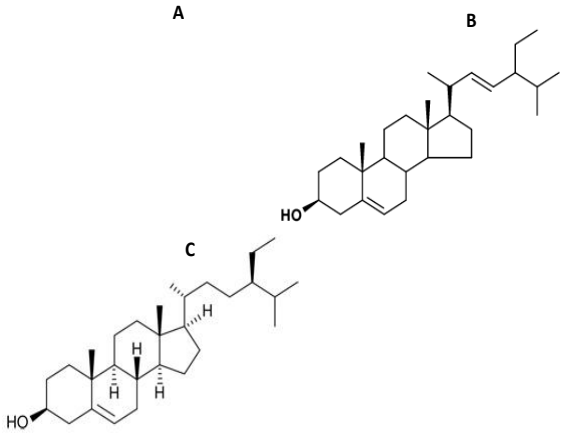

Figure 1. Structure of phytosterols A – Ergosterol, B- Stigmasterol and C- Beta-Sitoterol.

also spotted against the extract to help identify the presences of phytosterols in the extract. The phytosterol standards used were β-sitosterol, stigmasterol and ergosterol. Modified mobile phase, toluene-diethyl ether (20:80, v:v), was as the mobile phase. It was observed that saponified extract had a high concentration of the phytosterols when comparing to the spot from the unsaponified extract. This can be seen in Figure 2. The phytosterols were also identified by calculating the retention factor (Rf value) which is the distance travelled by the compound divided by the distance travelled by the solvent. The Rf values for each lane are presented on Table 1.

Quantitation of phytosterols by high performance liquid chromatography

The standards were each diluted in ethanol to five different concentrations and run on the HPLC. Standard

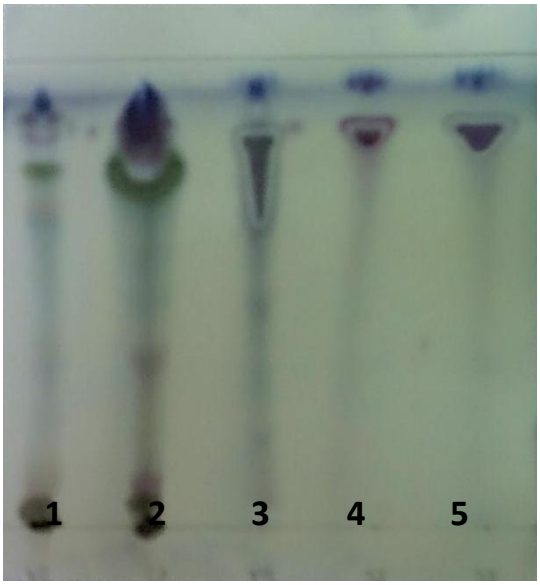

Figure 2. Thin layer chromatography plate showing the distinct differences between the saponified and unsaponified extract. In lane (1) was the unsaponified part of the plant extract, (2) saponified plant extract, (3) sitosterol standard, (4) ergosterol standard and (5) was the stigmasterol standard.

curves of sitosterol, stigmasterol and ergosterol (Figures 1, 3, 4 and 5) were constructed from the chromatograms, using the area of the peaks. The standards were identified by the retention time. Tables 2, 3 and 4, show the concentrations, area and retention times of sitosterol, stigmasterol and ergosterol, respectively. Saponified extract was run after the standards to quantify the amount of phytosterols in the extract. The retention time was used to identify the phytosterols (Figure 6) and the concentrations were extrapolated using the area of the peak against the standard curves (Table 5).

Antifungal activity - Agar diffusion

Sabouraud dextrose agar plates were prepared and 9 mm wells bored into the agar. 200 µl of extracts were suspended in the wells. The same volume was used for the three standards and Dimethyl sulfoxide was used as the negative control. Plates were left to grow for 3 to 4 days. Zone of inhibition around the well was measured in millimeters. The diameter measurements are tabulated in Table 6. Saponified extracts had bigger zones of inhibition when compared to the unsaponified extract. Ergosterol showed larger zones of inhibition when compared to the other standards.

Thin layer chromatography – bioautography

A TLC plates was prepared and dried, it was not sprayed.

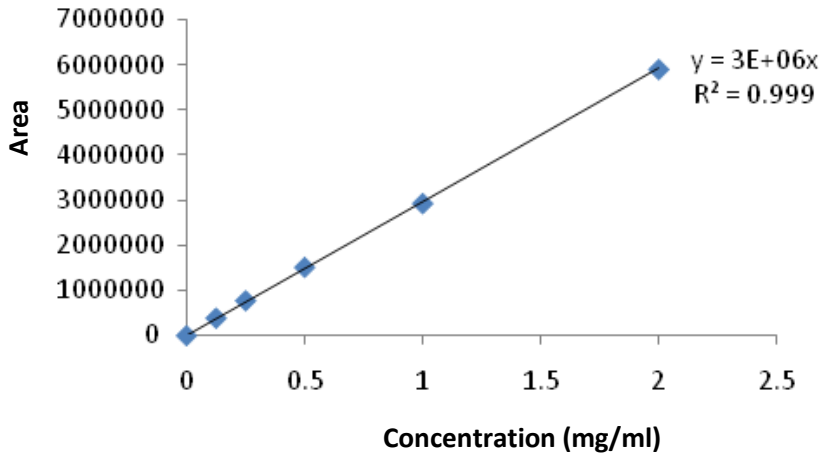

Figure 3. Sitosterol standard curve.

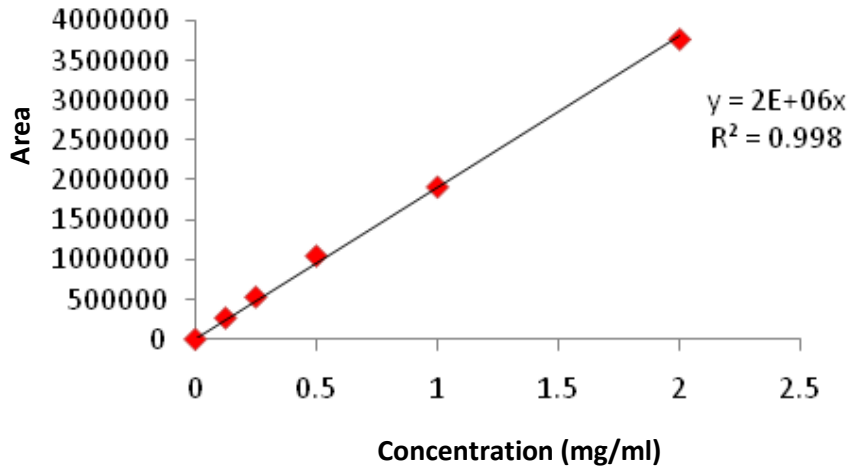

Figure 4. Stigmasterol standard curve.

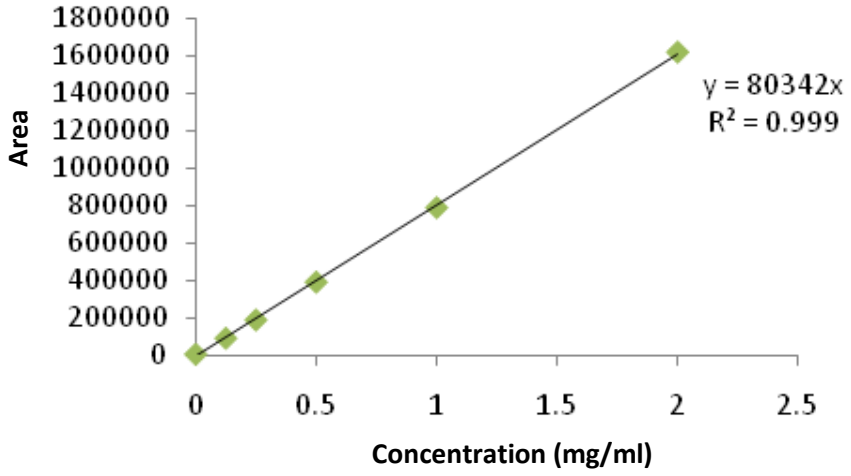

Figure 5. Ergosterol standard curve.

Table 2. Area and retention time of sitosterol standard.

Sitosterol		
Concentration (mg/ml)	**Area**	**Retention time (min)**
0.125	388864	4.98
0.25	776120	4.91
0.5	1519792	4.93
1	2935775	4.95
2	5913670	5.00

Table 3. Area and retention time of stigmasterol standard.

Stigmasterol		
Concentration (mg/ml)	**Area**	**Retention time (min)**
0.125	268794	6.11
0.25	531645	6.12
0.5	1047092	6.12
1	1913757	6.12
2	3771387	6.12

Table 4. Area and retention time of ergosterol standard.

Ergosterol		
Concentration (mg/ml)	**Area**	**Retention time (min)**
0.125	86570	6.70
0.25	184262	6.71
0.5	387451	6.75
1	787060	6.73
2	1621526	6.78

Table 5. Concentration of phytosterols in 30 g of dried *B. natalensis.*

Phytosterol	**Concentration (µg/ml)**
Sitosterol	70
Stigmasterol	160
Ergosterol	370

Extracts, standards were spotted on the TLC plate. Fungal spores of Aspergillus, Fusarium and Penicillium were harvested and standardized to an optimal density of less than 0.1. The spores were suspended in Potato Dextrose Broth, separately. The broth medium with suspended spores was sprayed onto the TLC plates. After 2 to 3 days of growth, no fungal growth was observed where the phytosterols were on the plate.

DISCUSSION

Extraction of the phytosterols using chloroform gave a dark yellowish brown extract. The extracton process was repeated on the same plant material so that as much as possible extract could be obtained from the material. 20 g of dried plant material gave about 0.35 g of extract. Saponfication was done to separate the phytosterols in the extract from other compounds in the extract. Saponification is simply the alkaline hydrolysis of fats/oils to make soap Glycerol is the other end product of saponification where the phytosterols are concentrated. Saponification resulted in higher yields of the phytosterol as can be seen in Figure 7, the TLC plate.

TLC showed the presence of phytosterols in the saponified, unsaponified and crude extract, which is the extract that has not underwent saponification. More

Table 6. Zones of inhibition of fungal growth measured on agar plates.

Parameter		Diameter (mm)		
		Aspergillus flavus	***Penicillium digitatum***	***Fusarium verticilloides***
Negative control	$DMSO_4$	0	0	0
Positive control	Amphotericin B	13	14	13
Standards	Ergosterol	19	18	18
	Stigmasterol	18	17	14
	Sitosterol	18	18	15
Samples	Unsaponified	14	15	16
	Saponified	17	20	18
	Crude	18	22	20

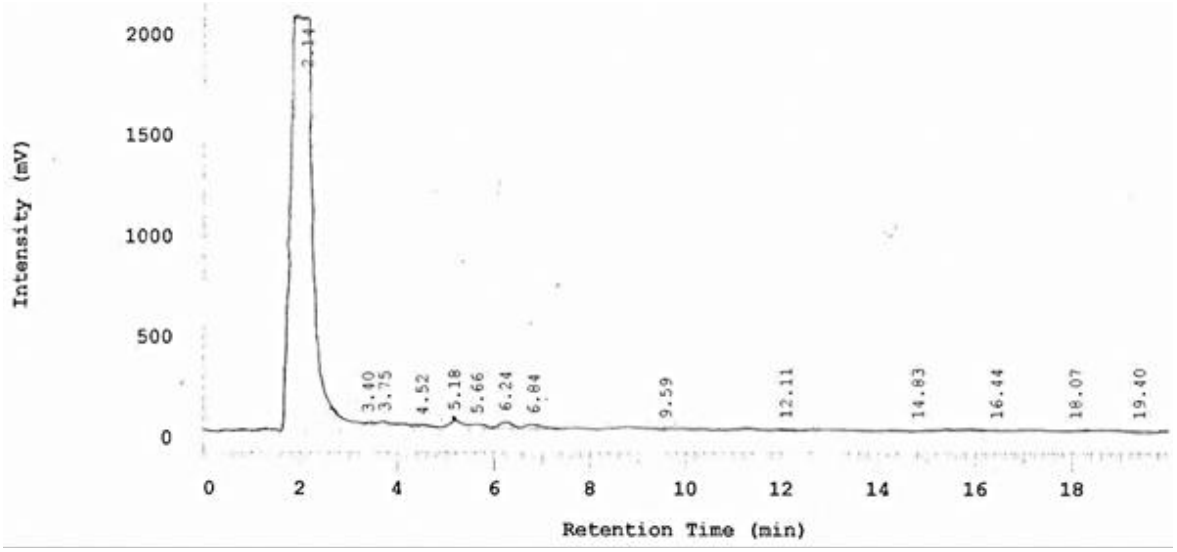

Figure 6. Chromatogram of saponified extract. Retention times of the phytosterols of interest – Sitosterol: 5.18, Stigmasterol: 6.24 and Ergosterol: 6.80.

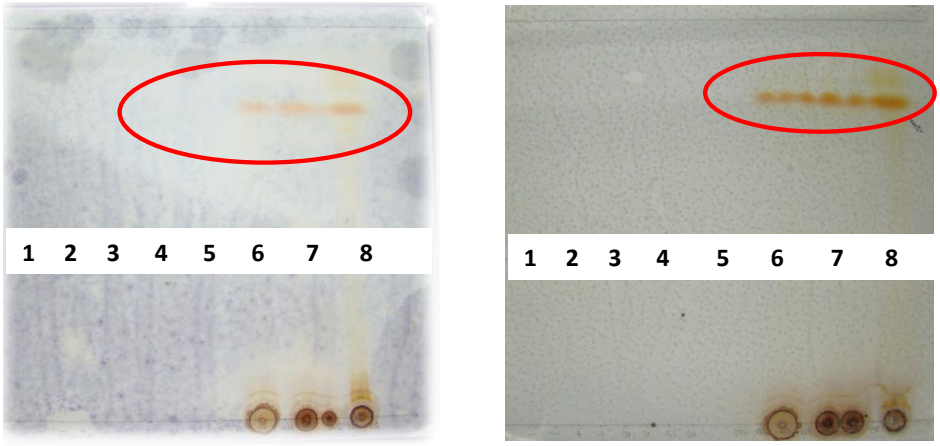

Figure 7. TLC plate sprayed with *Fusarium verticilloides* (A) and *Aspergillus flavus (B)* Lane 1 - DMSO, the negative control, lane 2 Amphotericin B, positive control, lane 3 - sitosterol standard, lane 4 - stigmasterol standard, lane 5 - ergosterol standard, lane 6 - unsaponified extract, lane 7 - saponified extract and lane 8- crude extract.

phytosterols were found in the saponified extract because saponification allows for maximum recovery of the phytosterols.

The phytosterols were futher identified and quantified by HPLC. As can be seen in Tables 2, 3 and 4, the area under the peaks almost doubled as the concentration of the phytosterols increase. This made easier to identify the compounds of interest. In the antifungal test, it could be noted that phytosterols do have an antifungal potential but it seem to be more fungiststic rather than fungicidal because growth of the fungi could be seen if the plates were incubated longer. Phytosterols extracted for *B. natalensis* do have an antifungal potential with ergosterol being the most effective, then stigmasterol and sitosterol being the least effective. This study reveals the usefulness of phytosterols in the control of disease caused by pathogenic fungal species. Plant sterols have assumed an increasingly important role in medicine as well as the healthcare industry and further work has to be undertaken. Thus, it can be very useful and seems to be a potential source for arresting the growth and metabolite activities of pathogenic fungi.

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