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Effectiveness of *Zornia diphylla* (L.) Pers. against fungal diseases

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Abstract

The traditional medicinal plant, *Zornia diphylla* (L.) Pers., was studied to determine its likely utility to develop a medicine for the treatment of fungal infections. The *in vitro* antifungal activity was determined by disc diffusion and tube dilution methods. The *n*-hexane extract of the whole plant powder showed substantial antifungal activity against the test fungi (*Candida albicans, Aspergillus niger, A. fumigatus, Fusarium oxysporum* and *Trichophyton rubrum)* compared to ethanol and water extracts. An active fraction (AF) was separated from the n-hexane extract by column chromatography. *A. fumigatus* and *C. albicans* were more susceptible to the AF compared to the other three fungi. In mice model for aspergillosis and candidiasis, AF (100 mg/kg) protected all the immune-compromised mice challenged with lethal quantity of *A. fumigatus* and the same dose of AF protected 83% of animals challenged with *C. albicans*. AF was devoid of any conspicuous toxicity to mice in the short term (30 days) limited toxicity evaluation. Thus, Z. *diphylla* (AF) showed promising *in vitro* and *in vivo* antifungal activity against *A. fumigatus* and *C. albicans*. It is a promising candidate for the development of valuable antifungal medicine.

Key words: *Zornia diphylla* (L.) Pers., antifungal activity, aspergillosis, candidiasis

Introduction

Fungal diseases are one of the major health problem in the present century and are on the rise (Anassie and Bodey, 1989; Singh, 2001). Due to many intrinsic and extrinsic characters of fungus, systemic fungal infections are difficult to cure. Eventhough there are many antifungal drugs in the market, it is almost impossible to cure systemic fungal infections completely without any adverse side effects or toxicity. Amphotericin B, fluconazole, ketoconazole, voriconazole, echinocandins *etc*. are potent antifungal agents, but their prolonged use in high doses causes harmful side effects. Moreover, these types of conventional antifungal drugs are relatively expensive or not easily available to common man (Jeannina and David, 2008; Walsh *et al*., 2002). In this context, search for new generation antifungal compounds are warranted. In traditional medicinal practices, a large number of plants are used to treat fungal diseases but most of them are not scientifically validated. Antifungal drug discovery based on traditional medicinal plants may provide economically viable medications or lead molecules for drug development. Recent studies contribute a good number of plants to the list of antifungal plants (Vera and Joullie 2002, Subhisha and Subramoniam, 2005; Subhisha and Subramoniam, 2006; Subhisha and Subramoniam, 2008), but a fully safer and effective antifungal phytomedicine remains to be developed for treatment. This warrants search for novel antifungal agents from plant sources. A survey conducted by the authors in Western Ghats area of Thiruvananthapuram District, Kerala State, India, revealed the use of *Zornia diphylla* (L.) Pers. (Family: Fabaceae) in the treatment of fungal diseases in folklore medical practices. Based on this ethnomedical knowledge, study was carried out to determine the likely utility of *Z. diphylla* for the development of an antifungal medicine.

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Materials and Methods

Plant materials

Z. diphylla (L.) Pers. (whole plant) was collected from Neyyar Dam tribal settlement area, Thiruvananthapuram District, Kerala State, India and was identified by the taxonomists of Tropical Botanic Garden and Research Institute (TBGRI). A voucher specimen, No 50976 was deposited in the Herbarium of TBGRI. The whole plant was dried at 26-29°C, powdered and used for the experiments.

Chemicals

Morpholinopropane sulphonic acid (MOPS), dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, USA), RPMI-1640 culture media, agar, dextrose, ketoconazole, fluconazole (Himedia, India), cortisone (GSK, Mumbai, India), penicillin (AHPL, Mumbai, India) were used. All other chemicals and reagents used were of analytical grade and purchased from E. Merck India Ltd. Mumbai, India

Microorganisms

Candida albicans (Robin) Berkhout [MTCC 227], *Aspergillus niger* Tieghem [MTCC 1344], *A. fumigatus* Fresenius [MTCC 343], and *Fusarium oxysporum* Schlechtendahl [MTCC 284] and *Trichophyton rubrum* (*A. castellani*) Sabourand [MTCC 296] were obtained from Institute of Microbial Technology, Chandigarh as pure cultures. These cultures were sub-cultured and maintained in Sabouraud agar.

Animals

Swiss albino mice (25 -30 g body weight) were used for the experiments. Animals were maintained under standard laboratory conditions in TBGRI animal house as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals. Animal experiments were approved by the Institute Animal Ethics Committee.

Preparation of plant extracts

The water extract was prepared by extracting the plant powder with distilled water (5 g/100 ml) with constant stirring for 4 h and then filtered through a filter paper. Residue was again extracted as above with water. The combined filtrate was freeze-dried in a lyophilizer. The alcohol extract of the powder was prepared similarly using ethyl alcohol instead of distilled water. However, in this case the combined extract was dried free of solvent in a rotary evaporator at 40°C under reduced pressure. The *n*-hexane extract was prepared as above using n-hexane instead of ethanol. However, to ensure complete extraction, 2 g powder was extracted with 100 ml *n*-hexane and the process was repeated three times. The filtrates were combined and dried in a rotary evaporator at 40°C under reduced pressure. The yield of the extracts was determined.

Separation of an active fraction (AF) *from n-hexane extract*

The hexane extract was fractionated using column chromatography. Briefly, 4 g of extract was loaded in to a packed column with silica gel of 60-120 mesh size and seven fractions each of 500 ml were eluted with pure *n*-hexane, *n*-hexane: chloroform different ratios (9:1, 3:1, 1:1, 1:3, v/v), pure chloroform and pure ethyl acetate. Each fraction was dried free of solvent using a rotary evaporator at 40°C under reduced pressure. These fractions were tested for antifungal activity. The most active fraction was subjected to phytochemical analysis (Wagner *et al*., 1984). Silica gel 60 F_{254} TLC and HPTLC of AF were carried out using *n*-hexane: chloroform (4:1 v/v) solvent system. The plate was derivatised with anisaldehyde-sulphuric acid reagent and heated to 80°C for visualization.

Assay for antifungal activities

Disc diffusion method

Preliminary screening of the extracts was done by disc diffusion method. Precisely the spores of fungal species, *C. albicans, A. niger, A. fumigatus, F. oxysporum* and *T. rubrum* were plated in sterile Sabouraud Dextrose Agar (SDA) medium in petridishes and the extracts in different concentrations (10 to 1000 µg/disc) were taken in 6 mm (diameter) paper discs (free of solvents) and placed in the fungus inoculated plates. Along with the test discs, control discs with vehicles (5% Tween 80 for n-hexane extract and 5% DMSO for alcohol extract) were also placed and kept in a bacteriological incubator at 35°C. Antifungal activity of the extracts was recorded as zone of inhibition after 24 h in the case of *C. albicans*, *A. fumigatus, A. niger, F. oxysporum* and after 72 h in the case of *T. rubrum* (Subhisha and Subramoniam, 2005).

Tube dilution method

Spores (2.5 x 10^3/ml) of *A. fumigatus, A. niger* and *F. oxysporum* were inoculated in 1 ml sterile Sabouraud Dextrose Broth, separately in test tubes and added with various concentrations (50 to 1000 µg/ml) of extracts in the respective inoculated tubes. The tubes were kept in a bacteriological incubator at 35°C. Antifungal activity of the extracts or active fraction was recorded as the weight of fungal mycelia after 24 h (Subhisha and Subramoniam 2005). Each test was carried out in triplicate.

The 50% inhibitory concentration (IC_{50}) of the active fraction was determined by the broth dilution method. Briefly, sterile RPMI 1640 medium, buffered to pH 7.0 with morpholinopropane sulphonic acid (0.165 M) was inoculated with 2.5 x 10^3 /ml spores from 48 h old cultures of *C. albicans, A. niger, A. fumigatus, F. oxysporum* or *T. rubrum*. Various concentrations of active fraction (0 to 250 µg/ml) were prepared in sterile buffered RPMI 1640 medium along with control or positive

controls and incubated at 35°C. IC_{50} of *C. albicans*, *A. fumigates, A. niger* and *F. oxysporum* was calculated from the absorbance at 530 nm (spectrophotometrically) 24 h after inoculation while in the case of *T. rubrum* the absorbance was recorded 72 h after inoculation. Each test was carried out in triplicate (Cuenca-Estrella, 2002; Arendrup *et al*., 2010).

In vivo antifungal efficacy evaluation in immune-compromised mice models for aspergillosis and candidiasis

Mice are normally resistant to infection with *A. fumigatus* and *C. albicans* when administered orally. The resistance was reduced by subcutaneous cortisone treatment (5 mg/ animal) which suppresses immune function. Penicillin was administered intramuscularly (long acting penicillin, 30000 units/animal) for the prevention of bacterial infections.

To determine *in vivo* anti-*A. fumigatus* activity, 48 male mice were divided in to 8 groups of 6 mice in each group. One group was kept as normal control without any treatment. Seven groups were treated with cortisone and penicillin. 48 h after cortisone treatment, 6 groups were challenged with 1 million spores orally and one group was kept as cortisone penicillin control without spore challenge. Three groups of spore challenged mice received 25, 50 and 100 mg/kg doses, respectively of active fraction daily for 10 days (p.o.). Group 4 and group 5 received 50 and 100 mg/kg doses, respectively of ketoconazole daily for 10 days (p.o.) as standard and the 6th spore challenged group (control) received the vehicle (5 % Tween 80; p.o.). Mortality was observed daily for 30 days (Subhisha and Subramoniam 2005). Similarly, the *in vivo* anti-*C. albicans* activity was determined. However, in this case, 0.1 million spores/mouse was used to challenge the mice and fluconazole was used as a standard drug (Subhisha and Subramoniam, 2005; Andes *et al.,* 2001).

Subacute toxicity evaluation of active fraction in mice

To study preliminary subacute toxicity (30 days) of the active fraction, 24 male mice of 20-25 g body weight were divided into four groups of six each. One group was kept as control and groups 2, 3 and 4 received 100, 250, and 500 mg/kg active fraction (AF), respectively. The drug was administered daily (p.o) for 29 days. Control group received the vehicle in an identical manner. The behavior of the animals was observed daily for 1 h for 29 days. Initial and final body weights, water and food intake, state of stool and body temperature were observed. The animals were killed on the 30th day. Hematological and serum biochemical parameters were determined. Important organs were dissected out, weighed and observed for pathological and morphological changes. Hemoglobin was measured using hemoglobinometer. Glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and alkaline phosphatase were measured following standard methods (Reitman and frankel, 1957; Kind *and King*, 1954). Urea and cholesterol were determined by conventional methods (Jam, 1986). The peritoneal macrophages and total leukocytes were counted (Subramoniam *et al*., 1998).

Statistical analysis

Statistical comparisons were done, using one way ANOVA followed by Dunnet's post hoc comparison when more than two groups were involved. p values < 0.05 were considered as significant.

Results and Discussion

As shown in Table 1, in disc diffusion assay, *n*-hexane extract was found to be more active than ethanol and water extracts. At 50 µg/ disc, *n*-hexane extract was active against *A. fumigatus*, *C. albicans* and *A. niger* whereas at this concentration, water and alcohol extracts did not cause any inhibition zone. At 100 µg/disc, *n*-hexane extract was active against all the five test fungi. Water and alcohol extracts showed activity against all test fungi only at the highest concentration (1000 µg/disc) studied (Table 1). The *n*-hexane extract did not show a concentration dependent inhibition zone in the disc diffusion assay. This could be due to the fact that the water insoluble *n*-hexane extract may have limited diffusion into the aqueous agar surface.

The inhibitory effect of *n*-hexane extract on *A. fumigatus*, *A. niger* and *F. oxysporum*, when directly diluted in liquid medium, is given in Table 2. Here, the extract showed concentration dependent inhibition of fungal growth as seen from the weight reduction of fungal mass.

The *n*-hexane extract was fractionated into 7 fractions by column chromatography. The third fraction (eluted with n-hexane: chloroform, 3:1 v/v) was the active fraction (hereafter designated as AF). AF, when subjected to chemical class test, showed positive reaction to coumarins and triterpenes. It was resolved into 5 components on silica gel TLC (Figure 2). AF showed inhibition zone at 10 µg/disc against *A. fumigatus* and *C. albicans* (Table 3).

When assayed by broth dilution method, AF showed maximum activity against *A. fumigatus* and *C. albicans*. IC_{50} of AF against *A. fumigatus*, *C. albicans*, *A. niger*, *T. rubrum* and *F. oxysporum* were approximately 50, 65, 90, 150 and 200 µg/ml, respectively. The growth inhibition was found to be concentration dependent in all the five test fungi (Figures 1a and 1b).

The *in vivo* efficacy test was done only for the most susceptible organisms based on *in vitro* study. As shown in Table 4, AF at a dose of 100 mg/kg protected all the *A. fumigatus* challenged mice whereas 50 mg/kg protected five out of six mice. Thus, protection of *A. fumigatus* challenged mice by AF at 50 and 100 mg/kg doses was 83 and 100%, respectively. As shown in Table 5, 100 mg/kg of AF protected 5 out of 6 *C. albicans*-challenged mice whereas all the fungus

challenged animals died in the untreated control group. However, lower doses (25 and 50 mg/kg) could not protect *C. albicans*-challenged mice. The body weight of the surviving animals was found to be almost the same to that of normal control on the 30th day. *In vivo* antifungal studies on medicinal plants for systemic antifungal infection are limited in the literature. In the present study, the locally available likely non toxic medicinal plant protected immune-compromised mice from challenge with lethal dose of *A. fumigatus* or *C. albicans*.

In the sub-acute toxicity study, AF administration (as high as 500 mg/kg) did not significantly influence body weight, weight of major organs, food and water intake, rectal temperature and general behavior of the animals (data not shown). Further, AF did not influence most of the serum biochemical and hematological parameters studied (Table 6). However, at the highest dose (500 mg/kg), WBC count marginally decreased. At all doses studied, AF decreased the levels of serum triglyceride, without any influence on the levels of total serum cholesterol (Table 6).

The study shows for the first time the antifungal properties of the plant *Z. diphylla*. Although the standard drugs used (ketoconazole and fluconazole) showed better activity compared to the plant extract and active fraction, it should be noted that the standard compounds are pure chemical entity drugs whereas the herbal drug is an active fraction containing at least 5 compounds. The active principle, when isolated in a pure form, is likely to act at low concentrations. Further, the herbal drug has comparatively broad spectrum activity. The plant extract showed varying levels of activity against all the 5 test fungi. It is of interest to note that the extract and active fraction are active against phylogenetically different fungi such as *C. albicans* and *A. fumigatus.*

In the present studies, *A. fumigatus* as well as *C. albicans* were found to be highly susceptible to the active fraction. Invasive aspergillosis is a clinical problem in immune compromised patients and patients under post operation-care with a high mortality rate (Garcia-Vidal *et al.,* 2011). Aspergilli, mainly *A. fumigatus*, less susceptible or resistant to antifungal agents have been reported as a clinical problem from many countries (Garcia-Vidal *et al.*, 2011; Mayr and Lass-Flori, 2011; Mortensen *et al*., 2011). *C. albicans* is the leading fungal pathogen causing invasive disease in immune-compromised patients. In severely ill, immune-compromised patients, *Candida* sp particularly *C. albicans*, cause systemic candidiasis with significant mortality despite amphotericin B and other therapy (Szabo and Mac Callum, 2011). Development of resistance to commonly used antifungal agents by *Candida* sp has been reported recently (Rambach *et al.,* 2011).

In the recent past, many antifungal extracts and chemical isolates from plants have been reported. These include a potent antifungal agent with an MIC (Minimum Inhibition Concentration) of 2 µg/ml against *A. niger* from *Turbinaria conoides* Kutzing (Kumar *et al.,* 2010) and anti-*C. albicans* activity (MIC: 1.56 mg/ml) of methanol extract of *Vernonia cinerea* Less (Latha *et al.,* 2011). The indole compound indirubin from *Wrightia tinctoria* R. Br. has been reported to have activity against *Trichophyton* sp. with an MIC range of 6 to 50 µg/ml (Ponnusamy *et al.,* 2010). However, in these cases, *in vivo* activity and safety remain to be studied.

In the present studies, as judged from the limited toxicity study in male mice and the traditional use of the plant without any known or recorded toxicity, the herbal drug (active fraction) is likely to be safe even at 5 times higher than the therapeutic dose. Therefore, the drug can be used as a systemic fungicide at relatively higher concentrations without toxic symptoms. In this connection, it should be noted that most of the currently used antifungal agents have considerable toxicity.

Literature search did not reveal any phyto-chemical as well as pharmacological studies on this medicinal plant. It is a herb which can be cultivated by conventional propagation techniques and the bio-mass can be produced as per requirement. This plant grows as a weed; it can be cultivated without much expense. Thus, this plant is an attractive material for the development of an inexpensive, but safe and effective, therapeutic agent for a wide variety of fungal infections.

In conclusion, the present study provides scientific basis to support the traditional use of *Z. diphylla* to treat certain fungal infections. Furthermore, for the first time, a coumarin and triterpene rich antifungal fraction was isolated from Z. *diphylla* which showed promising *in vitro* and *in vivo* antifungal activity against *A. fumigatus* and *C. albicans*. It is devoid of any toxicity to mice in short term toxicity evaluation. It is a promising candidate for the development of a valuable antifungal medicine.

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Table 1. Antifungal activities of *Z. diphylla* extracts

Groups	Test fungi	Inhibition zone (in mm)						
		Concentration of extracts or standard drugs (µg/disc)						
		0	10	25	50	100	500	1000
Water extract	*A. fumigatus*	0	0	0	0	7	8	10
	A. niger	0	0	0	0	0	7	7
	F. oxysporum	0	0	0	0	0	0	7
	C. albicans	0	0	0	0	7	7	8
	T. rubrum	0	0	0	0	7	9	11
Alcohol extract	*A. fumigatus*	0	0	0	0	0	8	9
	A. niger	0	0	0	0	0	7	7
	F. oxysporum	0	0	0	0	0	7	8
	C. albicans	0	0	0	0	7	8	9
	T. rubrum	0	0	0	0	0	8	10
n-Hexane extract	*A. fumigatus*	0	0	7	9	10	12	14
	A. niger	0	0	0	7	8	10	11
	F. oxysporum	0	0	0	0	7	8	10
	C. albicans	0	0	0	8	9	11	13
	T. rubrum	0	0	0	0	8	9	11
Ketoconazole	*A. fumigatus*	0	12	ND	ND	ND	ND	ND
	A. niger	0	12					
	F. oxysporum	0	11					
Fluconazole	*C. albicans*	0	12	ND	ND	ND	ND	ND
	T. rubrum	0	14					

Values are mean of 3 separate determinations. The anti-fungal activity was assayed by disc diffusion method; 20 µl/disc (diameter of disc was 6 mm); concentration 0 refers to control; ND, not done. Alcohol extract was dissolved in 5 % DMSO and n-hexane extract was suspended in 5 % Tween 80 for the addition on the disc. Vehicle control discs were also used. The yield obtained for *n*-hexane, ethyl alcohol and water extracts were 2 %, 7 % and 12 % respectively of the plant powder.

Table 2. Antifungal activity of n-hexane extract of *Z. diphylla* against *A. fumigatus*

Test Material	Concentration (mg/ml)	Dry mycelia Weight of *A. fumigatus* (mg)	Dry weight of *C. albicans* (mg)
Control	0	72.3 ± 4.2 (0)	58.3 ± 2.8 (0)
n-Hexane extract	50	63.3 ± 1.5 (12.4)	52.6 ± 2.1 (9.8)
Ketoconazole	100	55.7 ± 2.5 (23.0)	45.4 ± 2.3 (22.1)
	500	40.7 ± 2.5 (43.7)	33.9 ± 1.9 (41.9)
	1000	26.3 ± 2.2 (63.6)	24.3 ± 1.6 (58.3)
	50	7.7 ± 2.5 (89.3)	ND
Fluconazole	50	ND	(84.6)

The extract was directly added into the liquid medium. Values are mean ± S.D of 3 different experiments. Values in brackets represent % growth inhibition compared to control. All the values are statistically significant compared to control, *p* d" 0.05. ND, Not done

Table 3. Antifungal activity of different fractions from *n*-hexane extract of *Z. diphylla*

Fractions of n-hexane extract of Z. diphylla	Test fungi	Inhibition zone (in mm)				
		Concentration of fractions (mg/disc)				
		0	10	25	50	100
H.F-1	*A. fumigatus*	0	0	0	0	7
	C. albicans	0	0	0	0	0
H.F-2	*A. fumigatus*	0	0	7	8	10
	C. albicans	0	0	0	7	9
H.F-3	*A. fumigatus*	0	7	9	12	14
	C. albicans	0	7	8	10	12
H.F-4	*A. fumigatus*	0	0	7	8	9
	C. albicans	0	0	7	7	8
H.F-5	*A. fumigatus*	0	0	0	7	7
	C. albicans	0	0	0	0	7
H.F-6	*A. fumigatus*	0	0	0	0	7
	C. albicans	0	0	0	0	0
H.F-7	*A. fumigatus*	0	0	0	0	0
	C. albicans	0	0	0	0	0
Ketoconazole	*A. fumigatus*	0	12	14	ND	ND
Fluconazole	*C. albicans*	0	12	13	ND	ND

Values are mean of 3 separate determinations. The anti-fungal activity was assayed by disc diffusion method; 20 µl/disc (diameter of disc was 6 mm); ND- not done, concentration 0 refers to control, H.F 1 to H.F 7 are the column chromatographic fractions (Details under materials and methods).

Table 4. Protection of *A. fumigatus* challenged hydrocortisone treated male mice with an active fraction isolated from *n*-hexane extract of *Z. diphylla*

Animal groups	Number of mice survived after *A. fumigatus* challenge					
	1st Day	6th Day	12th Day	18th Day	24th Day	30th Day
Normal control	6	6	6	6	6	6
Cortisone + pencillin	6	6	6	6	6	6
Cortisone+ pencillin + fungal spore ($1x10^6$/ml)	6	0	0	0	0	0
Cortisone+ pencillin + fungal spore ($1x10^6$/ml) + 25 mg/kg active fraction	6	6	5	3	1	0
Cortisone+ pencillin + fungal spore ($1x10^6$/ml) + 50 mg/kg active fraction	6	6	6	5	5	5
Cortisone+ pencillin + fungal spore ($1x10^6$/ml)+ 100 mg/kg active fraction	6	6	6	6	6	6
Cortisone+ Pencillin + fungal spore ($1x10^6$/ml) +50 mg/kg ketoconazole	6	6	6	6	6	6
Cortisone+ pencillin + fungal spore ($1x10^6$/ml) +100 mg/kg ketoconazole	6	6	6	6	6	6

The active fraction was dissolved in 5 % Tween 80 and administered orally, daily for 10 days. Ketoconazole was dissolved in 5 % DMSO and administered similarly. (The body weight of the surviving animals on the 30th day was found to be almost same to that of normal control).

Table 5. Protection of *C. albicans* challenged hydrocortisone treated male mice with an active fraction isolated from *n*-hexane extract of *Z. diphylla*

Animal groups	Number of mice survived after *C. albicans* challenge					
	1st Day	6th Day	12th Day	18th Day	24th Day	30th Day
Normal control	6	6	6	6	6	6
Cortisone + pencillin	6	6	6	6	6	6
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml)	6	0	0	0	0	0
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml) + 25 mg/kg active fraction	6	3	1	0	0	0
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml) + 50 mg/kg active fraction	6	6	4	3	1	0
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml) + 100 mg/kg active fraction	6	6	6	6	5	5
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml) + 50 mg/kg active fraction	6	6	6	6	6	6
Cortisone + pencillin + fungal spore ($0.1x10^6$/ml) + 100 mg/kg Fluconazole	6	6	6	6	6	6

The active fraction was dissolved in 5 % Tween 80 and administered orally, daily for 10 days. Ketoconazole was dissolved in 5 % DMSO and administered similarly. (The body weight of the surviving animals on the 30th day was found to be almost same to that of normal control).

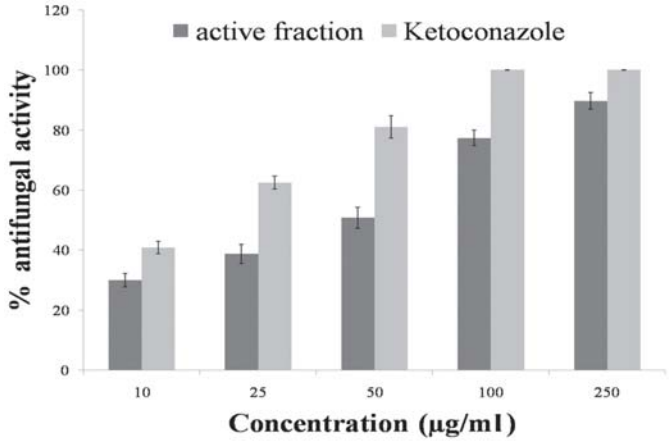
Table 6. Effect of short term (29 days) active fraction (AF) administration to mice on hematological and serum biochemical parameters

	Control mice	AF treated mice (100 mg/kg)	AF treated mice (250 mg/kg)	AF treated mice (500 mg/kg)
Haemoglobin (mg/dl)	13.1 ± 0.6	14.0 ± 0.4	12.2 ± 0.9	13.5 ± 0.8
WBC (cells/ml $x10^{-6}$)	14.2 ± 1.2	12.8 ± 0.8	12.2 ± 1.1	10.2 ± 0.9*
Peritonial macrophage (cells/ml $x10^{-6}$)	7.6 ± 0.9	8.5± 0.7	8.7± 0.8	8.6± 0.8
Serum glucose (mg/dl)	90 ± 3.4	87 ± 4.5	90 ± 3.9	88 ± 5.1
Serum urea (mg/dl)	42 ± 3.0	39.3 ± 2.8	41.6 ± 3.1	38.9 ± 2.2
Serum cholesterol (mg/dl)	124 ± 5.4	119 ± 5.9	131 ±6.2	128 ± 4.3
Serum triglyceride (mg/dl)	184 ± 6.8	143 ± 5.9**	134 ±5.1**	163 ±5.3*
Serum protein (g/dl)	8.4 ± 1.4	7.1 ±1.6	7.8 ±1.8	7.9 ± 1.6
Serum Albumin (g/dl)	4.2 ± 0.9	4.5 ± 0.6	4.8 ± 1.0	4.2 ±0.8
Bilirubin direct (mg/dl)	0.11 ± 0.01	0.09 ± 0.02	0.10 ± 0.01	0.11 ±0.02
SGOT (IU/l)	76 ± 4.1	77 ± 3.5	69 ± 3.2	70 ± 3.7
SGPT (IU/l)	32 ± 2.1	37 ± 2.5	32 ± 1.8	35 ± 1.9
SALP (KAU)	14 ± 2.2	13 ± 2.1	13 ± 2.8	12.5 ± 2.1
Serum Creatinine (mg/dl)	0.17 ± 0. 02	0.20 ±0.03	0.17 ±0.01	0.21 ±0.02

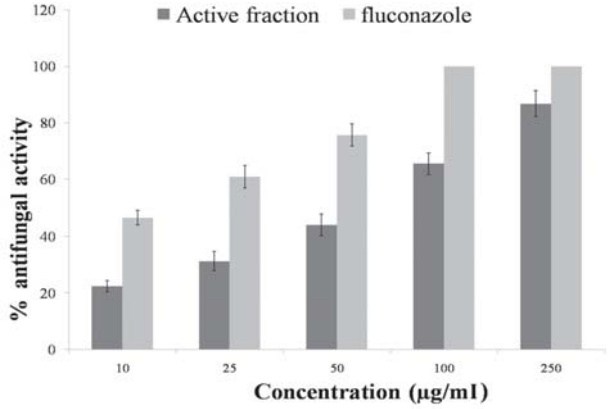
The active fraction was dissolved in 5 % Tween 80 and administered orally for 29 days and the animals were sacrificed on the 30th day. Values marked with ostrich significantly different from control values, * p d" 0.5, ** p d" 0.001. AF, active fraction separated from n-hexane extract of *Z. diphylla;* SGOT, serum glutamate oxalate transaminase; SGPT, serum glutamate pyruvate transaminase; SALP, serum alkaline phosphatase

Figure 1. Antifungal activity of active fraction isolated from *n*-hexane extract of *Z. diphylla*

(a) Anti-*A. fumigatus* activity of the active fraction

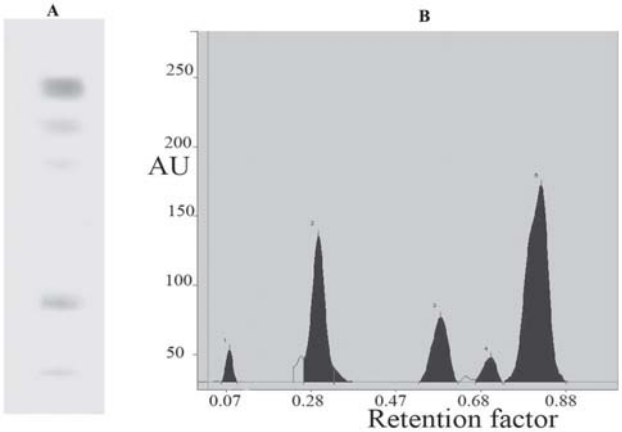


(b) Anti-*C. albicans* activity of the active fraction



Active fraction was suspended in 5 % Tween 80 and used for the test. Ketoconazole was taken in 5 % DMSO and fluconazole was taken in 1% DMSO. All the values are statistically significant compared to control (without drug), *p* d" 0.05 .

Figure 2. HPTLC profile of the active fraction isolated from n-hexane extract of *Z. diphylla*



A. TLC plate after derivatization with anisaldehyde-sulphuric acid reagent under visible light

TLC was done on precoated silica gel 60 F_{254} plates using n-hexane: chloroform (4:1 v/v) as solvent system. Five components are seen.

B. HPTLC profile of the active fraction

The gel was scanned densitometrically at 580 nm after derivatisation (details under materials and methods)

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