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Antifungal potential of root bark of *Diospyros kaki* against some human pathogenic fungi

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ABSTRACT

The antifungal activity of petroleum ether, ethyl acetate, chloroform, methanol and aqueous extracts of the root bark of Diospyros kaki (Ebenaceae) were evaluated, against 14 human pathogenic fungi. The antifungal assay was done using agar disc diffusion method. The result showed that all the extracts except aqueous extract showed varying degree of inhibitory activity against various human pathogenic fungi. The chloroform extract showed best activity with the MIC value in the range 160-2500µg/ml. The results obtained in the present study authenticate and support the use of Diospyros kaki in folklore medicine for treatment of various skin diseases.

Key Words: *Diospyros kaki*, Ebenaceae, antifungal activity.

INTRODUCTION

Diospyros kaki is a deciduous tree belonging to family Ebenaceae, measuring to 12 m in height and 7 m in diameter. The tree needs a subtropical to mild-temperate climate and is native to China, India, Japan and Myanmar. It is a handsome ornamental tree known for its delicious fruits. Its timber is used for making furniture's and fruit and seeds are used in food industry [1].The tree possesses many medicinal properties like the stem bark is astringent and styptic. The fruit is generally antitussive, astringent, laxative, nutritive and stomachic. The peduncle is used to treat coughs and hiccups and the calyx is used to treat hiccups. Leaves possess antithrombotic activity, polyphenols in leaves have ant wrinkle effect [2] The antimicrobial activity has been reported in the peels and leaves against various pathogens. The active compounds in persimmon leaves i.e presence of volatile oil, total flavonoid, coumarins, and organic acid are responsible for antimicrobial activities against seven food spoilage and food-borne pathogens. *D. Kaki* tannins with purity of 30%, and oligosaccharides (non-fermentable tetra- or less- saccharides) are

claimed as human intraoral pathogenic bactericidal agent. The isolation and identification of an antimicrobial compound kaempferol (kaempferol is an aglycon of astragalin (kaempferol 3-O-β-D-glucopyranoside) against *Streptococcus mutans* from the leaves of *D. kaki* has also been reported. In this study we investigated the crude extracts as well as the various fractions of etanolic extract of the root bark of *D. kaki* using agar disc diffusion assay, against pathogenic fungi.

MATERIALS AND METHODS

Plant material:
The root bark of *D. kaki* were collected from company garden, Saharanpur, India and authenticated by Department of Botany, M.S College Saharanpur, where the voucher specimens have been deposited.

Extraction
The shade dried root bark of *D. kaki* was extracted with different solvents petroleum ether, ethyl acetate, chloroform, methanol and water using successive extraction with soxhlet apparatus. Each extract was concentrated under reduced pressure at 40-50ºC to a dark viscous mass.

Test fungi
The fungi used in the study were obtained from Indian Agriculture Research Institute, New Delhi and Post graduate Institute of Medical Education & Research, Chandigarh and were maintained by monthly subculturing on SDA. They were: *Candida albicans* MTCC 227, *Candida tropicalis* ATCC 6258, *Candida krusei* ATCC 750, *Cryptococcus neoformans* ITCC 1672, *Sporothrix shenkii* ITCC 2317, *Fusarium oxysporum* ITCC 4998, *Trichophyton mentegrophytes* ITCC 3572, *Microsporon gypseum* ITCC 5277, *Aspergillus flavus* ITCC 5290, *Aspergillus fumigatus* ITCC 4880, *Curvularia lunata* ITCC 5248, *Rhizomucor pussilus* ITCC W-14, *Phialophora verrucosa* MCCL 32006 and *Pseudaresheria boydii* MCCL W-14.

Preparation of inocula
Inocula were prepared according to the Protocol of NCCLS M38-P. 3-4 days old PDA slants freshly subcultured were scraped with 0.85% saline and transferred to 5 ml distill water after hyphen fragments deposited at the bottom. 1-2 drop of 10% Tween 80 was added. The suspension was vortexed for about 5 min and the density of suspension (0.4 x 104- 5 x 104 CFU/ml) was determined using haemocytometre. In case of yeast overnight cultures in Potato dextrose broth were adjusted to an optical density of ~2 at 600 nm and then diluted to the desired count.

Agar disc diffusion assay Preliminary antifungal assay was done through Agar Disc diffusion method. Fungal inoculum prepared earlier was spread on SDA plates (100µl). Disc impregnated with 1, 2.5, 5 and 7.5 mg of extract (in DMSO) were made and transferred to the plates. AmB (100µg) was used as positive control and DMSO as negative control. Plates were kept at 4 ºC for 2-3 hours and then incubated at 28-30 ºC. Zones of inhibition examined after 48 h.

MIC determination

MIC of the chloroform extract was performed using micro broth dilution method of NCCLS M38 P (1998) with some modification. Two fold serial dilution of the extract was done in RPMI starting with 10 mg/ml-0.005 mg/ml. Each well of the 96 well plates was inoculated on the day of test with 100 µl of 2 x conidial inoculum suspension (0.5 x 10^3-2.5 x 10^3 cfu/ml) in case of yeast and (0.4 x 10^4- 5 x 10^4 cfu/ml) for filamentous fungi. 100 µl of the extract was then added in the desired concentration to each well of the microtitre plate. The growth control wells contained 100 µl of the corresponding diluted inoculum suspension and 100 µl of 2 x sterile drug free medium and DMSO. Both quality control isolates *C. tropicalis* and *C. krusei* were also included in the study. The micro dilution trays were incubated at 35 ºC and examined after 21-26, 46-50 and 70-74 hours of incubation. The growth in each MIC well was compared with that of growth control with the aid of reading mirror. The MIC was defined as the lowest concentration able to inhibit any visible growth.

RESULTS AND DISCUSSION

Five different extraction solvents covering the wide polarity range from nonpolar to polar i.e. petroleum ether, ethyl acetate chloroform, methanol and water were used to make petroleum ether, ethyl acetate chloroform, methanol and aqueous extract respectively. The all five extracts were screened for antifungal activity by disc diffusion assay against fourteen human pathogenic fungi. The antifungal activity of different extracts against various fungi is shown in Table 1. All the extracts except aqueous extract showed varying degree of inhibitory activity against various human pathogenic fungi. Aqueous extract was not found to be active against any of the fungi used in the present study. The chloroform extract was found to have significant antifungal activity with maximum diameter range (5mm-25mm) and is active against all the fungi used at 5 mg/ml while at lower concentrations it showed no effect on few dematacieous fungi. The results of antifungal assay of chloroform extract are shown in Fig 1. *Candida spp.* along with dermatophytes was more susceptible to the extract followed by other hyaline fungi. *Candida spp.* along with dermatophytes was more susceptible to the extract followed by other hyaline fungi. Inhibition zone diameter of petroleum ether extract varies from 10.67-23 mm at 7.5 mg/ml but it showed no activity against *C. lunata, R. pussilus, P. verrucosa and P. boydii* at this concentration. Inhibition zone diameter range of ethyl acetate and methanol extracts were relatively low i.e. 9.34-18.34 mm and 8.67-18 mm respectively at the highest concentration used in the study i.e. 7.5 mg/ml. Both these extracts were inactive against *C. lunata, R. pussilus, P. verrucosa* and *P. boydii* at 7.5 mg/ml. The susceptibility of different groups of fungi is varied towards the *extract*. The activity is more pronounced on yeast and dermatophytes followed by the hyaline and dematacеious fungi i.e. *Rhizomucor, Phialophora, Curvularia* and *Pseudaresheria* being least susceptible. Also the MIC values of the extract were determined and are given in Table 2.

The MIC values towards *Candida spp.* was significantly low (160µg/ml and) , so these results are relevant since *C. albicans* is the leading primary agent causing superficial and often disseminated infection in immunocompromised patients[3-6] while in some patients (neutropenic patients) non albicans *spp.* are detected more frequently[6]. The MIC values was also appreciably low for *C. neoformans* (320µg/ml) and dermatophytes , *T. mentegrophytes* and *M. gypseum* (320µg/ml) These results hold significance as *Cryptococcus* is the cause of most

common life threatening meningitis in HIV positive patients [7] and infection caused by dermatophytes affects 2-13 % of population worldwide and upto 30 % of groups at high risk such as elderly and the people with diabetes [8]. Extract also exhibited substantial activity against *Aspergillus spp.* (630µg/ml) but the low susceptibility (1250-2500µg/ml) of *Rhizomucor, Phialophora, Curvularia and Pseudaresheria* could be due to the presence of melanin in the cell which is an important factor of high virulence.

D. kaki is chosen for the present study because *Diospyros spp.* have been reported in the literature to exhibit interesting biological and pharmacological properties i.e. treatment of whooping cough, leprosy, snakebite, scabies, skin eruptions, dysentery, eye infection, menstrual troubles etc [9]. Although there are reports of antimicrobial activity in several species of *Diospyros* genus including *D. crassiflora*, *D. anisandra*, *D. pregrina*, *D. maritima*, *D. novoguinensis*, *D. tricolor, D. nigra* and *D. rubra*[10-14] this is the first report of antifungal activity from the root bark of *D. kaki*. Moreover in this study, a very broad range of potentially human pathogenic fungi have been taken. Therefore this study further confirms the traditional use of *D. kaki* in the treatment of skin ailments.

This study therefore justify the use of *D. kaki* in traditional medicine for treating skin diseases and underline the importance of ethnobotanical approach for the selection of plants in the discovery of new bioactive substance. Purification of the crude extract is needed in order to locate the active principal responsible for antifungal activity of this plant.

Table 2: MIC (µg /ml) of chloroform extract of *D.kaki* root bark.

S.No	Fungus	MIC
1	*Candida albicans* (MTCC 227)	160
2	*Candida tropicalis* (ATCC 6258)	160
3	*Candida krusei* (ATCC 750)	160
4	*Cryptococcus neoformans* (ITCC 1672)	320
5	*Soprothrix shenkii* (ITCC 2317)	630
6	*Fusarium oxysporum* (ITCC 4998)	630
7	*Trichophyton mentegrophytes* (ITCC3572)	320
8	*Microsporon gypseum* (ITCC 5277)	320
9	*Aspergillus flavus* (ITCC 5290)	630
10	*Aspergillus fumigatus* (ITCC 4880)	630
11	*Rhizomucor pussilus* (ITCC W-14)	1250
12	*Phialophora verrucosa* (MCCL 32006)	1250
13	*Curvularia lunata* (ITCC 5248)	1250
14	*Pseudaresheria boydii* (MCCL W-48)	2500

Table 1. Inhibition zone diameter (mm) of various extract of *D. kaki* root bark

s.no	Petroleum ether extract (mg/disc)				Methanol extract (mg/disc)				Ethylacetate extract (mg/disc)				Chloroform extract (mg/disc			
	1	2.5	5	7.5	1	2.5	5	7.5	1	2.5	5	7.5	1	2.5	5	7.5
1	12.67±0.34	15.34±0.34	18.67±0.34	23.34±0.34	8.67± 0.34	12± 0.34	15± 0.58	18±0	8.67± 0.34	11.67±0.34	15±0	18.34±0.34	11.67 ± 0.34	15.34± 0.34	19.67±0.34	25± 0
2	9±0.58	13±0	17±0	20.34±0.34	8.34± 0.34	12±0	14.67±0.34	18.67± 0.34	8.34± 0.34	12±0	14.67±0.34	19±0	13±0	16.34± 0.34	20.34±0.34	24.67± 0.34
3	8.67± 0.34	11.67±0.34	16.67±0.34	21± 0.34	8±0	11.67±0.34	14.67±0.34	18.34± 0.34	8.34± 0.34	11.67±0.34	14.67±0.34	18± 0.58	11.67±0.34	16±0	20±0	23.67± 0.67
4	8±0	11±0	15.67±0.34	18.67±0.34	6.34± 0.67	8.67± 0.34	11.34±0.34	16± 0.58	7±0	8± 0.58	11.34±0.34	17±0	9.0± 0.58	14.34±0.58	17.34±0.34	20.34± 0.67
5	5.34± 0.34	6.34± 0.89	10.67±0.34	14.34±0.67	4.67± 0.58	6.0± 0.58	7.61± 0.67	12± 0.58	4.67± 0.34	6±0	8± 0.34	12.34±0.34	9.34± 0.67	14±0	17±0	21.67± 0.67
6	5.67± 0.34	9.67±0.34	12.67±0.89	16± 0.58	5.34± 0.34	9±0	12.34±0.34	16.67± 0.34	5.0± 0.58	8.34± 0.34	11± 0.34	11± 0.58	7.67± 0.34	11.34±0.34	14.34±0.34	17± 0
7	8±0	11.67±0.34	14.67±0.34	18.67±0.34	-	5.0± 0.58	9.34± 0.58	13.34± 0.34	-	5.67± 0.34	9.34± 0.34	14.67±0.34	7±0	11.67± 0.67	17± 0.67	20.34± 0.34
8	4.67± 0.34	8±0	11± 0.58	17.34±0.89	-	4.67± 0.67	9±0	14±0	-	4.67± 0.67	8.67± 0.58	14.67±0.34	9±0.58	14.34± 0.34	16.34±0.34	20.67± 0.67
9	-	4.67±0.34	9±0	12± 0.58	-	-	6.0± 0.58	10.67± 0.34	-	-	6±0.58	11±0	7.67± 0.34	13.34± 0.34	13.34±0.58	21.67± 0.34
10	-	-	6.67± 0.34	13.34±0.58	-	-	5.34± 0.34	8.0± 0.67	-	-	5±0	9.34± 0.34	5±0.58	10± 0.58	12.67±0.34	16± 0
11	-	-	5.67± 0.34	11±0	-	-	-	-	-	-	6±0	-	-	5.67± 0.34	8±0.58	13.34± 0.58
12	-	-	-	-	-	-	-	-	-	-	5±0	-	-	-	7.34± 0.34	11.67± 0.34
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.34± 0.67	9.34± 0.34
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5±0.58	11±0

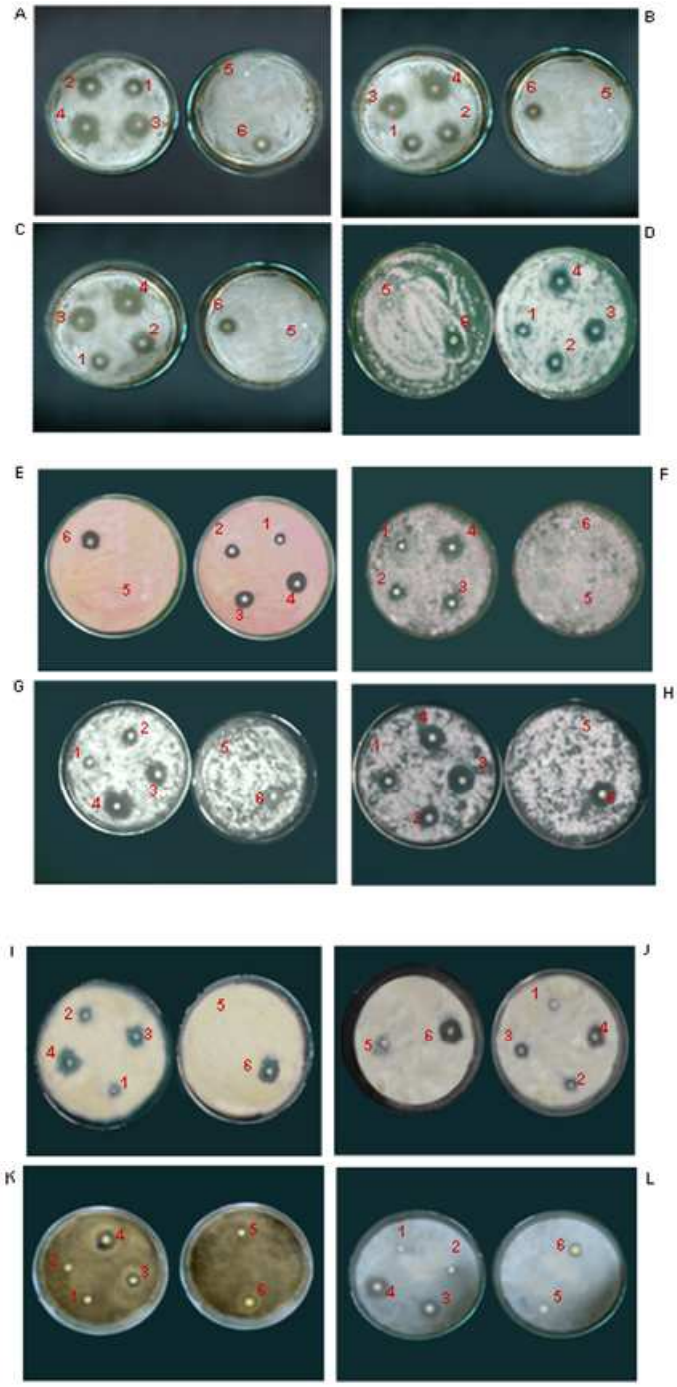

Fig.1. Disc diffusion assay of chloroform extract of *D. kaki* root bark. 1, 2, 3 & 4 are 1, 2.5 ,5 & 7.5 mg/ disc of extract ; 5) DMSO; 6) 100 µg Am-B. A.) *C. albicans* ; B) *C. tropicalis* ; C) *C. krusei* ; D) *C. neoformans ;) S. Shenkii ; F) F. oxysporum ; G) T. mentegrophytes ; H) M. gypseum) A. fumigatus ; J) A. flavus ; K) C. lunata ; L) P. boydii. R. pussilus ; N) P .verrucosa*

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