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Antifungal activity of *Plumbagin* & *Isodiospyrin* from *Diospyros kaki* root bark

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ABSTRACT

The chloroform extract of root bark of D. kaki exhibited remarkably good antifungal activity at against all the fungi. The bioassay guided fractionation of the crude extract was the done using benzene-chloroform step gradient by silica gel column chromatography. The active compounds A & B obtained were analysed by EI-MS, FTIR and ^{1}H and ^{13}C NMR spectroscopy. On comparision of their spectra with literature they were identified as napthoquinones, Plumbagin (5-hydroxyl, 2-methyl 1,4 -Napthoquinone) and Isodiospyrin (5-hydroxy-6-(1-hydroxy-6-methyl-5,8-dioxo-naphthalen-2-yl)-2-methyl-naphthalene-1,4-dione). The MIC values of the crude extract and compounds A & B were (160-2500 µg/ml), (1.25-20 µg/ml) and (5-160 µg/ml) & the MFC values were (630-5000), (5-160 µg/ml) and (40-160 µg/ml) respectively.

INTRODUCTION

An increasing prevalence of infections caused by newer emerging fungal pathogens has been detected in humans, particularly immunocompromised patients in the recent past and the population of patients at risk has expanded to include those with a broad list of medical conditions, such as solid organ and hematopoietic stem cell transplantation (HSCT), cancer, receipt of immunosuppressive therapy, AIDS, premature birth, advanced age and major surgery [1,2]. The presently available antifungal drugs are often unsatisfactory due to elevated toxicity and inability to eradicate infections [3,4,5,6,7]. Moreover the emergence of these pathogens has given rise to both primary and secondary drug resistance [8,9,10]. Thus there is an increased need for the development of alternative therapeutic agents. One possible answer is to use plants as a source for the development of newer antimicrobial agents [11,12,13]. Diospyros genus is well known for its medicinal use and chemical diversity. *Diospyros kaki* is a small deciduous tree valued for its delicious fruits which is antitussive, astringent, laxative, nutritive and stomachic. Stem bark is astringent and styptic. The peduncle is used to treat coughs and hiccups. The calyx is used to treat hiccups. Fruits showed strong detoxifying activity on various snake venoms. It inactivates various bacterial toxins such as *Clostridium tetani* and *Staphylococcus alpha* and *Bordetella pertusis*. Leaves are shown to increase lifespan and decrease brain haemorrhage and infarction in stroke prone spontaneously hypersensitive rats. It showed scavenging action towards active oxygen free radicals, inhibited lipid peroxidation and showed hypotensive activity against urethane anaesthatised rats [14]. Although phytochemical constituents of *D. kaki* have been studied earlier [15], no studies have been done on antifungal activity of rootbark of *D. kaki* extract. Therefore an attempt have been made in the present study to evaluate its antifungal property *in vitro*.

MATERIALS AND METHODS

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

2.2 Preparation of chloroform extract of roots :
The preparation of the extract was done as per the method of Tadhani,[16]. The bark of the roots were peeled (1 kg) and were dried at 40ºC in oven for 1-2 days. Dry powder was extracted with chloroform (Analytical grade) using environmental shaker at 250 rpm for 24 h. The procedure was repeated three times and than the extract was filtered using whatman filter paper. The filtrate was evaporated to dryness under reduced pressure. Residue was stored in labeled and sterile screw capped bottle at 4ºC.

2.3 Test fungi :
All the fungi used in the study were purchased from Indian Agriculture Research Institute, New Delhi and Post graduate institute of medical education and research, Chandigarh. The fungi 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.

2.5 Antifungal assay :
Preparation of inocula is done as per the protocol of NCCLS M38-P, 1998 [17]. 3-4 days old PDA slants freshly subcultured were scraped with 0.85 % saline and transferred to 5ml distill water after hyphal fragments deposited at the bottom. 1-2 drop of 1 % Tween 80 was added. The suspension was vortexed for about 5 min and the density of suspension ($0.4x10^4$-$5x10^4$ cfu/ml) was determined using haemocytometre. In case of yeast overnight cultures in PDB were adjusted to an optical density of ~2 at 600 nm and than diluted to the desired count. Antifungal assay was done as per the technique of Pepeljnjak (2003). Fungal inoculum prepared earlier was spread on SDA plates (100 µl). Discs impregnated with 1, 2.5, 5 and 7.5 mg of extract (in DMSO) were made and transferred to the plates. Amphotericin-B (100 µg) was used as positive control and DMSO as negative control. Plates were kept at 4 ºC for 2-3 h and then incubated at 28-30 ºC. Zone of inhibition were examined after 48 h. After incubation the zone of inhibition was measured using Hi antibiotic zone scale (HiMedia, Mumbai, India).

2.6 Isolation of active constituents :
The active compounds were purified using gravity column employing the method of Iroegbu and Nkere, 2005 [19]. The residue weighing 10 gm was subjected to column chromatography over silica gel (60-120 mesh) on a 1m x 40 mm long column. The extract was eluted with Benzene-Chloroform gradient starting with 100 % benzene to 100 % chloroform. A total of 132 fractions of 25 ml each were collected and dried in waterbath at 45˚C to evaporate the solvent. All the eluted fractions were than monitored individually by TLC and the fractions with same TLC profile were pooled. All the major fractions so obtained were assayed for antifungal activity. The fraction three to nine (F3-F9) eluted with 50:50 ratio was found to be active and was named A1 (210mg). It is purified again using the same solvent combination on a smaller column (25cm). F7-F39 was obtained as orange needles (A, 170 mg). Another active fraction was obtained with 10:90, Benzene-Chloroform (F7-F12) and 100 % chloroform (F1-F8). The fraction was named B1 (3220mg) which was then purified further using 100 % chloroform by repeated column chromatography. 50 fractions of 5 ml were collected and monitered by TLC. Again the fractions with similar TLC profile were pooled and checked for presence of desired bioactivity. The resulting fraction (F15-F44), B2 gave two very close bands on TLC. These bands were separated using P-TLC with Chloroform-methanol 99:1 resulting in another active compound (B,1900 mg) as red needles.

2.7 Instrumentation:
Silica gel plates 60 F254+366, 20 x 20 cm (Merck) were used for analytical TLC, whereas preparative TLC was performed on Silica gel PLC plates 60 F254+366, 20 x 20 cm, 2 mm (Merck). The MS using electron impact techqnique were obtained by direct insertion at an ionization voltage of 70 ev using EI-MS Micromass autospec Q

(Waters Micromass UK ltd) spectrometer. The IR spectra were obtained using a Perkin-Elmer FTIR Spectrophotometre in the range 4000–667 cm-1 as KBr pellets using Omniq software. The ^{1}H NMR spectra were recorded by using Bruker DRX500, using DMSO-d6 as solvent at 500 MHZ and ^{13}C at 250 MHZ.

2. 8 MIC & MFC :

MIC of the crude extract and pure compounds A and B were performed using micro broth dilution method of NCCLS M38 P, 1998[17]. Two fold serial dilution of the extract and compounds was done in RPMI starting with 10mg/ml-0.005mg/ml for crude extract and 32 µg/ml - 0.03 µg/ml for Am-B. Each well of the 96 well plates was inoculated on the day of test with 100 µl of 2 x conidial inoculum suspension ($0.5x10^3$- $2.5x10^3$ cfu/ml) in case of yeast and ($0.4x10^4$- $5x10^4$ cfu/ml) for filamentous fungi. 100 µl of the test compound 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 *Candida tropicalis* and *Candida krusei* were also included in the study. The micro dilution trays were incubated at 35ºC 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. MFC was determined by the method of Ingroff, 2001[20]. 20 µl aliquots were sub cultured from each well that showed complete inhibition (100 % or an optically clear well) from the last positive well (growth similar to that for the growth control (drug free medium) onto sabouraud dextrose agar plates. The plates were incubated at 28-30 ºC until growth was seen in the growth control subculture (usually 48 h). The MFC was the lowest drug concentration that resulted in either no growth or fewer than 3-5 colonies.

RESULTS AND DISCUSSION

The result of the antifungal activity of the root is presented in Table 1. The crude extract showed remarkably good antifungal activity against all the tested fungi with the inhibition zone diameter ranging from 11mm-23.67mm. Bioassay guided purification of the crude extract using silica gel column chromatography led to the isolation of two active principles A and B. On comparision of the Physical characteristics and spectral data (A) was identified to be a known 1,4 napthoquinone plumbagin. (orangish yellow needles (daylight), reddish (UV 254nm), M.P 76˚-79˚C, Soluble in alcohol, acetone, chloroform and benzene. EI-MS spectrum *m/z* (intensity %)- 188 (100), 173 (26), 160(27), 131 (44), 120 (30), 92 (39), and 63 (43). IR spectrum (KBr) – 2925, 1735, 1650, 1610 and 1450. ^{1}H and ^{13}C NMR (DMSO) - δ 2.19(Me-2), 7.06(H-3), 7.40(H-6), 7.42(H-7), 7.82(H-8), 11.97(-OH). 114.9(C-10), 119.3(C-8), 124.23(C-6), 132.0(C-9), 135.66(C-3), 136.98(C-7), 149.8 (C-2), 161.2(C-5), 184.8(C1), 191.0(C-4), 16.9(C-11). The physical characteristics of compound (B) and spectral data was obtained as (red needles (day light), dark brown colour (UV 254 nm), M.P. 231-234˚c, Soluble in alcohol, acetone, chloroform and benzene. EI-MS *m/z* (intensity, %) 374(100), 359(60), 375(28), 79(20), 331(13), 41(11), 187(11), 319(10), 376(10), 57(57), 187(11), 345(10), 187(7.5), 189(5), 383(4.5), 152(4). The IR spectrum (KBr) – 3405, 2920, 1660, 1640, 1600 and 1585. ^{1}H and ^{13}C NMR (DMSO) Spectra : δ 2.09(7-Me), 6.77(H-2), 6.89(H-3), 6.90(H-2, H-3), 7.30(H-6), 7.60(H-8), 12.02(OH-5'), 12.40(OH-5) δ 20.40(C-11), 113.31(C-10), 128.65(C-9), 121.37(C-8), 125.71(C6'), 135.22(C-6), 137.56(C-3), 130.40(C-8'), 140.14(C-2), 158.79(C-5), 184.54(C-1), 190.16(C-4). This spectral data matches with the binapthoquinone *Isodiospyrin* [21,22,23,24,25,26]. The yield of *Plumbagin* and *Isodiospyrin* obtained in this study is quite in accordance with the study of Lee, 2008 [27] and is higher than yield of other *spp.* of Diospyros [28,29]. *Plumbagin* had earlier been found in few species of *Diospyros* (*D. canaliculata*, *D. ebenum*, *D. elliptifolia*, *D. gracilipses*, *D. hebecarpa*, *D. maritima*, *D. siamang*, *D. siderophylla*, *D. walkeri*, *D. wallichi*) [14,15,30] and its antifungal activity against *Rhizopus nigricans*, *Epidermatophyton floccosum*, *Microsporon nanum*, *Penicillium notatum*, *Penicillium funiculosum*, *Penicillium canadense*, *Candida albicans*, *Aspergillus niger* and *Colletotrichum gloesporioides*, *Alternaria alternata*, *Aspergillus niger*, *Bipolaris oryzae*, *Fusarium oxysporum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Rhizopus stolonifer* var. stolonifer and *Sclerotinia sclerotiorum* [24,31,32,33,34,35] is reported. *Isodiospyrin* is also known to be present in *D. abyssinica*, *D. alboflavescena*, *D. bipindensis*, *D. chloroxylon*, *D. dendo*, *D. ebenaster*, *D. ferrea*, *D. gilleli*, *D. gracilescens*, *D. hoyleana*, *D. kakisylvestris*, *D. lotus*, *D. maaingayi*, *D. mespiliformis*, *D. montana*, *D. morrisiana*, *D. nicaraguensis*, *D. texana*, *D. usambarensis*, *D. verrucosa*, *D. virginiana*, *D. whyteana* and *D. zombensis* [14,15] but not much is known about its antifungal property [36]. MIC of the crude extract and active compounds are shown in Table 2. There is a significant difference in the MIC values of crude extract (160-2500 µg/ml) and pure compounds, *Plumbagin* (1.25-20 µg/ml) and *Isodiospyrin* (5-160 µg/ml). This indicates the fact that some components of the crude extract were interfering with the active principle as the MIC values reduce drastically on purification. The susceptibility of different groups of

fungi is varied towards both the compounds. The activity is more pronounced on yeast and dermatophytes followed by the hyaline fungi and dematacеious fungi i.e., *Rhizomucor*, *Phialophora*, *Curvularia* and *Pseudaresheria* were least susceptible. The MIC values of *Plumbagin* obtained in the present study are quite in the range with those of earlier studies [24,31,32,33,34,35]. In some cases MIC values of *Plumbagin* was found to be excellent and quite comparable to Amp-B while the MIC value of *Isodiospyrin* is higher than that of *Plumbagin*. MFC value of the extract and compounds were one to three dilutions higher than the MIC values.

Table 1. MIC & MFC of crude extract, *Plumbagin*, *Isodiospyrin* and Am-B

Fungus	Crude extract µg/ml		*Plumbagin* µg/ml		*Isodiospyrin* µg/ml		Am-B µg/ml	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans (MTCC 227)	160	630	1.25	5	5	40	0.5	1
Candida tropicalis (ATCC 6258)	160	630	1.25	5	5	40	0.5	1
Candida krusei (ATCC 750)	160	630	1.25	5	5	40	0.5	2
Cryptococcus neoformans (ITCC 1672)	320	1250	2.5	10	10	40	1	2
Soprothrix shenkii (ITCC 2317)	630	1250	5	10	10	60	2	4
Fusarium oxysporum (ITCC 4998)	630	2500	5	20	20	160	-	-
Trichophyton mentegrophytes (ITCC3572)	320	1250	2.5	5	5	60	0.5	1
Microsporon gypseum (ITCC 5277)	320	1250	2.5	5	5	60	1	2
Aspergillus flavus (ITCC 5290)	630	2500	10	40	40	160	1	2
Aspergillus fumigatus (ITCC 4880)	630	2500	10	40	40	160	1	2
Rhizomucor pussilus (ITCC W-14)	1250	5000	10	40	40	320	4	16
Phialophora verrucosa (MCCL 32006)	1250	5000	20	80	80	630	8	16
Curvularia lunata (ITCC 5248)	1250	5000	20	80	80	320	4	16
Pseudaresheria boydii (MCCL W-48)	2500	5000	20	160	160	630	-	-

CONCLUSION

Spectrum of activity of both the compounds and their MIC values confirms their broad spectrum and fungicidal nature. Moreover these compounds were found to be active against the known Am-B resistant fungi. Although their MIC values are high as compared to the standard but considering the high toxicity and rise in resistance of some fungal species to Am-B [3,37,38,39,40]; these compounds could be considered worthy of further investigation as potential leads for the development of antifungal agents.

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