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Research article

IN VITRO AND IN VIVO ANTIFUNGAL ACTIVITY OF ALKALOID 3,5-bis(4,4''-dimethoxy-[1,1':2',1''-terphenyl]-4'-yl)-4H-pyrazole-4,4-diol FROM *DERRIS INDICA* (LAM) BENNETT SEEDS

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ABSTRACT

Objectives: The aim of the present study is to isolate an antifungal compound from *Derris indica* (Lam) Bennett seed oil with various solvents and evaluation of its antifungal activity against the clinical species of *Candida.*

Methods: *D. indica* seed hexane extract was tested against *Trichophyton rubrum*, *Trichophyton tonsurans* and *Candida albicans.* Hexane extract was fractioned using different solvents through column chromatography (CC). Isolated compound D1 was identified and characterized using ultraviolet, Fourier-transform infrared, ^{1}HNMR, and mass spectroscopy. *In vitro* evaluation of D1 carried out against 12 *Candida* strains. *In vivo* evaluation of D1 carried out against *T. rubrum*, *T. tonsurans,* and *C. albicans* using an excision wound healing model on male Wistar rats.

Results: Different concentrations of hexane extract showed antimicrobial activity against tested microorganism with varying minimum inhibitory concentration values. On fractionation with hexane-petroleum ether through CC, it yielded a crystalline fraction. Compound D1 characterized as a 3,5-bis (4,4''-dimethoxy-[1,1': 2',1''-terphenyl]-4'-yl)-4H-pyrazole-4,4-diol. A novel alkaloid compound from *D. indica* is a new report and proved to be inhibitory against *C. albicans* MTCC 3017 (14.83±0.28), MTCC 1637 (16.0±0.0), *Candida glabrata* MTCC 3814 (16.83±0.28) and MTCC 3014 (16.66±0.57), *Candida tropicalis* MTCC 230 (20.0±0.0), MTCC 1406 (12.33±0.57). *C. glabrata* MTCC 3981 was found to be resistant to the compound. *In vivo* studies showed no visual symptoms at the end of treatment indicating the therapeutic property of the compound.

Conclusion: The D1 was found to be effective against human fungal pathogens and can be used as a base molecule in designing new antifungal drugs.

Keywords: *Derris indica*, Seed oil, Alkaloid compound, *In vitro*, *In vivo* studies.

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INTRODUCTION

Derris indica belonging to the family Fabaceae (Papilionaceae) is a medium-sized evergreen, fast-growing, glabrous, and deciduous tree widely distributed in the region of Southeast Asia and Pacific Islands [1]. It is one of the nitrogen fixing tree producing seeds containing 30–40% oil [2]. This versatile medicinal plant is the unique source of various types of chemical compounds [3]. The plant is reported to produce a wide range of flavonoids with antimicrobial activity [4-16]. The seeds contain 13.5% mucilage, traces of essential oil and complex amino acids, termed glabrin. Four furanoflavones karanjin, pongapin ($C_{19}H_{12}O_6$), kanjone ($C_{18}H_{12}O_4$), and pongaglabrone ($C_{18}H_{10}O_5$), have been isolated from Indian Karanja seed [17]. Three furanoflavonoids (Pongamosides A, B, and C) and a flavonol, glucoside, and pongamoside D have been reported from n-butanol-soluble fraction of ethanolic extract of *D. indica* fruit [7]. Pongaglabol, hydroxyfuranoflavone, aurantiamide acetate, four furanoflavones (karanjin, lancheolatin B, kanjone, and pinnatin) and a rarely occurring modified phenylalanine dipeptide have been isolated [18]. Conventionally, the plant has been used in the treatment of several ailments. The bark is used in pile, leave in medicated bath and rheumatic pains, seeds in hypertension, bronchitis, whooping cough, skin diseases and rheumatic arthritis, roots in cleaning gums, teeth, ulcers and gonorrhea, and flowers are used in the treatment of diabetes[19].

Nowadays, antibiotics have created an enormous clinical problem in the treatment of infectious diseases [20]. Skin fungal infections are predictably grouped into superficial, subcutaneous and systemic infections [21]. According to the World Health Organization survey on the incidence of dermatophytic infection, about 20% worldwide populations have suffered with cutaneous infections [22]. Unlike other fungi, dermatophytes are communicable and cause infections in healthy, immune-competent individuals as well as in those with immune dysfunction [23]. Herbal drugs have great need and play a key role in health-care systems [24]. Therapeutic property of herbal drugs is due to the presence of active constituents such as alkaloids, glycosides, saponins, terpenoids, lactones, phenols, and flavonoids present in different parts of plants [25,26]. *D. indica* is a unique source of various phytoconstituents belonging to alkaloids, glycosides, flavonoids, fixed oils, and carbohydrate responsible for various activities [27]. Previously, numerous authors reported the presence of various phytocompounds in seeds [28-31]. These compounds have many interesting pharmacological and industrial applications. The aim of the present study is to isolate an antifungal compound from *D. indica* seeds.

METHODS

Collection of plant material

D. indica mature seeds collected from Gulbarga University campus and local areas of Kalaburagi district, Karnataka, India, and authentically identified using herbarium deposited in the Department of Botany, Gulbarga University, Kalaburagi, Karnataka, India, with voucher specimen number HGUG-206 and literatures [32-34].

Preparation of extract

Seeds were washed with water, dried for 4–5 days at room temperature and pulverized to fine powder (particle size 2 mm) and extracted

with Soxhlet extractor (40°C) with hexane for 12 h. The extracted oil obtained was condensed and filtered using Whatman No. 1 filter paper, evaporated in vacuo, percent of the extract was recorded and stored in an airtight screw-capped bottle and stored at 4°C in the refrigerator for further use.

Fractionation and identification

Hexane extract (10 mg) was dissolved in 100 ml of hexane and used as a test solution. It was subjected to standard methods of phytochemical analysis to detect the presence of phytoconstituents [35-38] and also physicochemical analysis, namely, color, boiling point, solubility, estimation, iodine value, acid value, and saponification value. The column chromatography (CC) over silica gel-H of mesh size 60–120 μ (HiMedia, Mumbai) using eluent mixtures of hexane, pet ether, chloroform, ethyl acetate, and methanol (Sd-fine, Mumbai) of increasing polarity at a flow rate of 1 ml/min was employed. Each 5 ml of 16 fractions were collected into a plate. Active fraction from antifungal assay, fraction-1 using solvent system hexane-petroleum ether (6:4, v/v) was checked by the thin-layer chromatography (TLC) coated silica gel- G, to know the purity of fractions. TLC plates were prepared as formulated [39] TLC plates were visualized by spraying H_2SO_4 (50%), heating for 8–10 min at 80°C.

Fraction 1 was purified by recrystallization to give pure compound. The melting point was determined by Thiele tube melting point apparatus. The obtained pure compound was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis to determine the corresponding molecular ions present in the compound and analyzed in a Shimadzu LC-MS model 2010 A, in JEOl Model GS×400 spectrophotometer with $CDCL_3$ (denaturated chloroform, DMSO) as the solvent. An ultraviolet (UV)/visible spectrum was obtained using a 5704SS ELICO spectrophotometer in the range of 200–800 nm wavelengths. Fourier-transform infrared (FTIR) spectrum was measured using KBr discs on Perkin-Elmer R×1 spectrophotometer in the wave numbers (cm^{-1}) in the range of 4000–450 cm^{-1}. NMR was performed to determine the structure of an isolated pure compound. ^{1}HNMR was recorded using Bruker AM×400 NMR spectrometer using TMS (Tetramethyl saline) as an internal reference at 400.137 MHz in $CDCL_3$ at 300 K. The chemical shifts were recorded in σ (ppm) and J in hertz and compared with literature data.

Biological assay

Test organisms

Trichophyton rubrum and *Trichophyton tonsurans* were collected from Mahadevappa Rampure Medical College, Kalaburagi and 12 *Candida* species, namely, *Candida albicans* MTCC 183, MTCC 3017, and MTCC 1637, *Candida glabrata* MTCC 3814, MTCC 3014, and MTCC 3981, *Candida haemulonii* MTCC 1966, MTCC 2766, and MTCC 8303, and *Candida tropicalis* MTCC 230, MTCC 1406, and MTCC 2795 were obtained from Institute of Microbial Technology, Chandigarh, India. The fungal cultures were maintained on Sabouraud Dextrose Agar (SDA) medium supplemented with Chloramphenicol and Streptomycin sulfate and subcultured on Potato Dextrose Agar every 15 days to prevent pleomorphic transformations. Yeast peptone dextrose (YPD) agar media were used for the assay.

Drug preparation

The isolated compound D1 diluted in 1% (v/v) Dimethyl formamide (DMF) at a concentration of 5 mg/ml. Stock solution of the drug powder, Ketoconazole was prepared in 1% (v/v) DMF at a concentration of 1mg/ml and stored at −20°C and used for the assay.

Agar well diffusion assay

Antifungal activity of the *D. indica* hexane extract was evaluated against *T. rubrum* and *C. albicans*. The fungal cell suspensions were prepared according to the following procedure. Fungal lawn was prepared using 5 days old culture strain. The fungal strains were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards (108 CFU/ml) and used for the assay. The determination of minimum inhibitory concentration (MIC) of extract was performed by serial dilution (0.6, 1.2, 2.5, 5, 10, 20, and 40 mg/ml) in DMF (v/v) against tested organisms by agar well diffusion method [40]. The petri dishes were prepared in triplicates and maintained at 37°C for 48 h. The diameter of zones of inhibition was measured in mm and statistically analyzed and expressed as mean (n = 3) ± standard deviation (SD). DMF used as negative control and Ketoconazole (1000 μg/ml) as positive control. Similarly *in vitro* antifungal testing of isolated compound D1 was carried out against *Candida* species by above mentioned method on YPD agar plates.

Excision wound healing from isolated compound D1

Animals

Wistar male rats weighing about 250 g were used for wound healing study. Animals were maintained in propylene cages at room temperature 22±2°C; relative humidity ~60% in a 12-h light–dark cycle. Rats were given pelleted diet and tap water *ad libitum*. Animal experiment procedures were carried out in accordance with the guidelines of the Public Health Service Policy on Human Care and use of laboratory animals (Matoshree Taradevi Rampure Institute of Pharmaceutical Sciences, Kalaburagi) and the protocol was approved by the institutional animal ethical committee (HKE/COP/IAEC/66/2013) for care and use.

Analysis of non-harmful effects

To determine the non-harmful effect of the effective compound, male Wistar rats (250 g) were used and maintained under the same condition as described above. Wistar rats were randomly divided into five groups and each group containing five rats. According to concentration of applied compound investigated. The compound considered as non-harmful if all the five animals in a group survive after 48 h application. About 0.5 ml of prepared stock solution of the effective compound was diluted in ethanol (0.01, 0.1, 0.2, 0.3, and 0.4%, vol/vol) and injected intraperitoneally. About 0.1% concentration was non-harmful to the animals and selected to use for further investigation.

Wound healing activity

Excision wound was used to evaluate the wound healing activity of compound D1. The rats were inflicted excision wound as described [41,42]. The groups were divided as; 1st group-untreated animals served as a control, 2nd group-animals treated with standard, 3rd group-treated with compound against T. rubrum, 4th group-treated with compound against T. tonsurans, and 5th group treated with compound against *C. albicans*. 4 cm^2 areas were cleaned and depilated on the back of each animal. The inoculum was applied on the back of the animals immediately after depilation and left for 3 days. The establishment of active infection was confirmed on day 4 by isolation of the pathogens from skin scales cultured and by visual examination on 8–10 days. The treatment was initiated on animals with active infections, from the 20th day post-inoculation and continued till complete recovery from infection was achieved. Treatment started, and ointment contained 0.1% (vol/vol) of compound, separately mixed in petroleum jelly, applied once daily on infected area and scored visually for inflammation and scaling. The commercial fungicide ketoconazole used as a control. Clinical assessment of inoculated skin area was performed using a modified lesion score from 0 to 4 as indicated. The recorded score 0 indicates no visible lesion; score 1 few slightly erythematous lesions; score 2 well-defined vesicles; score 3 large areas of marked redness, scaling, blade patches; and score 4 mycotic foci well developed with ulceration. The lesion was scored from 1, 3, 10, 20, 30, and 40 days. The presence of the pathogens was confirmed by cultivation of skin scales from infected loci on SDA plates each day.

RESULTS AND DISCUSSION

Agar well diffusion assay

D. indica seed hexane extract was first subjected to agar well diffusion assay to identify the presence of potential antifungal compounds. The

Table 1: MIC of *D. indica* seed extract

S. No.	Botanical name and part used	Family	Zone of inhibition							
			Test organism	40 mg/ml	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.62 mg/ml
1.	*Derris indica* (Seeds extract)	Fabaceae	*C. albicans*	18.83±0.28	16.83±0.28	13.5±0.5	12.83±0.28	11.16±0.28	10.83±0.28	12.0±0.0
			T. rubrum	16.83±0.28	14.16±0.28	13.83±0.28	12.0±0.0	10.66±0.28	9.66±0.28	7.16±0.28
2.	Positive control		Ketaconazole	24.0±0.0						
3.	Negative control		DMF	NA						

D. indica: *Derris indica*, *T. rubrum*: *Trichophyton rubrum*, MIC: Minimum inhibitory concentration, DMF: Dimethyl formamide

inhibitory activity on tested fungi was quantified by the diameter of zones of inhibition. MIC extract at different concentration showed antifungal activity (0.62–40 mg/ml) against pathogenic fungi *C. albicans* and *T. rubrum* with varying MIC values and summarized (Table 1). Among all the concentrations maximum zones of inhibition showed 18.83±0.28 (*C. albicans*) and 16.83±0.28 (*T. rubrum*) at 40 mg/ml and found to be more effective concentration. Several researchers reported that alkaloids, phenols, triterpenoids, glycosides, and tannins have high potential that could be developed as antimicrobial compounds [43,44]. The result observed in the present study suggests that the hexane extract inhibit the growth of microbes by the presence of these phytochemicals which are responsible to inhibit the fungal metabolism. A varied level of broad-spectrum antimicrobial activity was observed by authors from various parts of *D. indica* on fractionation with different solvents and suggested the use of fatty oil of *D. indica* plant as antimicrobial drugs against pathogenic bacteria and fungi [45-48].

NA-no activity, DMF-Dimethyl formamide

Fractionation and identification

Phytochemical screening tests were conducted to determine the presence of various secondary metabolites present in *D. indica* hexane seeds extract. Phytochemical screening results revealed the presence of steroids, glycosides, flavonoids, alkaloids, and saponins in the extracts [37,38].

Plant was collected based on their ethnopharmacological significance and used as traditional medicine by herbal practitioners and local people. Detailed information was gathered by several interviews [49]. In our previous study, phytochemical screening tests were conducted to determine the presence of various secondary metabolites present in *D. indica* seeds hexane extract and results were compared with reported literature data found to be similar with chloroform extract [50,51]. The results of physicochemical characterizations of hexane extract were found to be soluble in hexane, petroleum ether, chloroform, ethyl acetate, methanol, DMF and DMSO whereas insoluble in water. The extract was brown color with a boiling point (210°C) and the content of hexane extract was weighed (49%). This value was more or less similar to the previously reported literatures which confirmed that seeds are a rich source of lipids [52,53]. The iodine value of the oil indicates that the amount of saturated fatty acid, and iodine value (119.02 mg of KOH/g), acid value (2.0 mg KOH/g), and saponification value (181.9 mg KOH/g), respectively. It is evident from the results of phytochemical studies that seeds are rich source of flavonoids, alkaloids, glycosides, and steroids of pharmacological importance. Thus, an attempt was made here to isolate some of these phytochemical compounds from the seeds of *D. indica* by CC and purified with the help of TLC.

In the present investigation isolated compound D1 was obtained as pale yellow rod shaped crystals (15.5 µm length). There are many reports of isolation of compounds from *D. indica* seed oil in crystal form such as Pongamol [30], Pongapin (II) [54], and Pongol [55]. Further, purity of the fraction was checked by TLC using solvent systems [Hexane-petroleum ether (6:4, v/v)], hRf value (58.28), appeared as a single discrete spot and melting point recorded as 148–150°C. Further, pure fraction was subjected for detailed spectroscopic studies. The UV spectrum of compound D1 showed $UV^{\lambda}{}_{max}$ nm wavelength 665.0 and absorbance at 0.600 ranges indicates the presence of the chromophoric group with an extended conjugation (Fig. 1). The FTIR spectrum reveals the presence

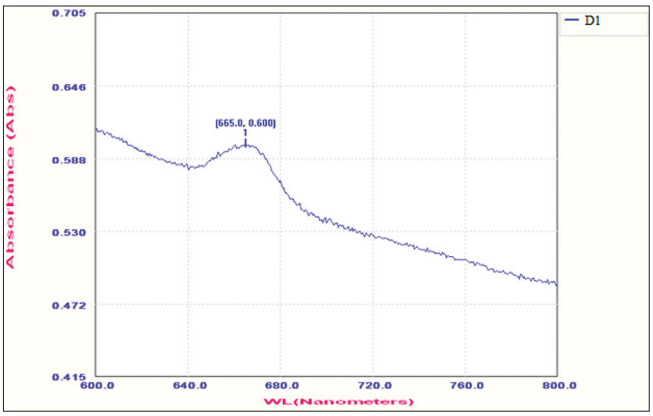

Fig. 1: Ultraviolet spectrum of isolated compound from D*erris indica* seed oil

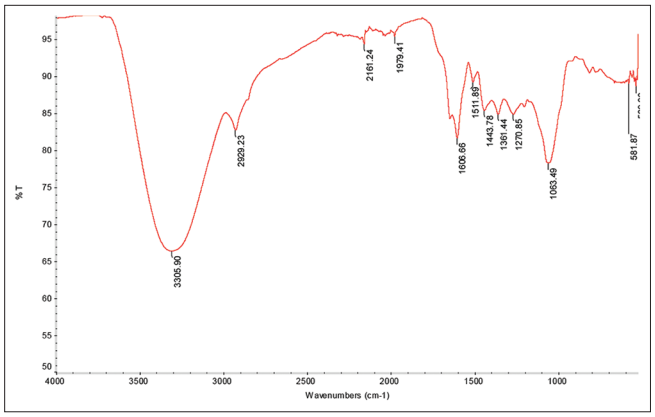

Fig. 2: Fourier-transform infrared spectrum of isolated compound from *Derris indica* seed oil

of Hydroxyl group (-OH) at 3305 cm^{-1} broad main absorption band and the additional moderate to intense band 1063^{cm-1}, 1270^{cm-1}, 1361 $^{cm-1}$, 1443 $^{cm-1}$, and 1511 $^{cm-1}$ is present in between the ranges 600 $^{cm-1}$ and 1600 $^{cm-1}$. Band 2929 cm^{-1} is due to stretching (C-H) and 1606 cm^{-1} is for C=N group stretching (Fig. 2). Its molecular formula was assumed to be $C_{43}H_{36}N_2O_6$ and molecular weight is 676.77. The mass spectrum indicated the molecular ion peak at m/z 676 and base ion peak at m/z 258 (Figs. 3 and 4).

The ^1H NMR spectrum of D1 showed signal at δ_H 8.21 (1H, d, J = 4Hz), and δ 8.15(dd), δ 7.77 (d), multiplet between δ 7.58–7.54 and 7.19 (dd) characteristics to aromatic protons. Signals exhibited at δ_H 3.95(S) correspond to one methoxy proton and δ 1.72 (s) to hydroxyl protons suggested that compound D1 is alkaloid derivatives. Spectroscopic data support the proposed structure D1 (Fig. 5). The structure of isolated compound was established on the basis of spectroscopic studies. This is the first report that crystalline alkaloid had been found as naturally occurring compound from *D. indica* seed oil. The physicochemical properties and spectroscopic

data indicated that compound D1 is an alkaloid and characterized as, 3,5-bis(4,4''-dimethoxy-[1,1':2',1''-terphenyl]-4'-yl) -4H-pyrazole-4,4-diol. According to reports, alkaloids are secondary metabolites originally defined as pharmacologically active compounds, primarily composed of nitrogen [56,57]. Alkaloids are predominant in plant families such as *Fabaceae, Asteraceae*, *Apocynaceae*, and *Boraginaceae* [60].

Table 2: *In vitro* susceptibility assay from compound D1 against *Candida* species

S. No.	Organism	Strains of MTCC	K	D1	C
1.	*C. albicans*	183	23.83±0.28	12.16±0.28	-
		1637	19.66±0.28	16.0±0.0	-
		3017	24.0±0.0	14.83±0.28	-
2.	*C. glabrata*	3814	23.66±0.28	16.83±0.28	-
		3014	20.0±0.0	16.66±0.57	-
		3981	11.33±1.15	-	-
3.	*C. haemulonii*	1966	13.83±0.28	8.16±0.28	-
		2766	16.66±0.28	8.0±0.0	-
		8303	16.66±0.28	8.33±0.57	-
4.	*C. tropicalis*	230	24.0±0.0	20.0±0.0	-
		1406	20.0±0.0	12.33±0.57	-
		2795	16.83±0.28	6.33±0.57	-

C. albicans: *Candida albicans*, *C. glabrata*: *Candida glabrata*, *C. haemulonii*: *Candida haemulonii*, *C. tropicalis*: *Candida tropicalis*

Table 3: *In vivo* studies of isolated compound D1 against pathogenic fungi on male Wistar rats

No. of treated days	Group I control	Group II standard	Group III *T. rubrum*	Group IV *T. tonsurans*	Group V *C. albicans*
1st day	-	-	-	-	-
3rd day	1	1	1	1	1
5th day	2	1	2	3	2
10th day	3	0	2	2	2
20th day	4	0	1	1	1
30th day	3	0	1	0	0
40th day	3	0	0	0	0
45th day	3	0	0	0	0

T. rubrum: *Trichophyton rubrum*, *T. tonsurans*: *Trichophyton tonsurans*, *C. albicans*: *Candida albicans*

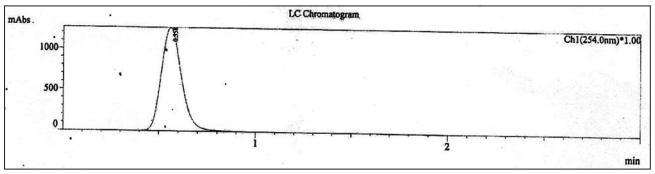

Fig. 3: Liquid chromatographic spectrum of isolated compound from *Derris indica* seed oil

There are reports on the presence of numerous phytocompounds isolated from *D. indica* seeds, such as furanoflavones [15,54,55,59], Furanodiketon [60], Karanjachromene [30], isopongachromene [60], isoponga flavone [61], O-methyl pongaglabol [62], $3,3^1,4^1,7$ - tetramethoxyflavone [60], fatty acids [63], Pongamoside D [7], Glabrachalcone, Karangin, Glabrachalcone [63], and alkaloids [65]. After reviewing literatures, this is the first time report of occurrence of crystalline alkaloid from *D. indica* seed oil and has previously not been reported to the best of our knowledge.

Anticandidal activity of isolated compound D1

The isolated compound D1 was tested against *C. albicans*, *C. glabrata,* and *C. tropicalis* is summarized in Table 2. The purpose of present study was to check the isolated compound D1 (5 mg/ml) against 12 *Candida* strains obtained from (MTCC) Institute of Microbial Technology, Chandigarh, India, and to evaluate their sensitivity or resistance to currently used antifungal agents. A strong anticandidal activity was observed against *C. tropicalis* MTCC 230 (20.0±0.0), *C. glabrata* MTCC 3814 (16.83±0.28) and MTCC 3014 (16.66±0.57), and *C. albicans* MTCC 1637 (16.0±0.0). Furthermore, D1 also showed moderate activity against *C. tropicalis* MTCC 1406 (12.33±0.57) and *C. albicans* MTCC 3017 (14.83±0.28). Interestingly *C. glabrata* MTCC 3981 was found to be resistance against compound and found to be very less effective against Ketoconazole (11.33±1.15) (Fig. 6). *C. tropicalis* MTCC 230 (20.0±0.0) inhibited 80% while compared with standard (24.0±0.0), followed by another strain of *C. tropicalis* MTCC 1406 (12.33±0.57), showed less activity compared with strain MTCC 230 (20.0±0.0). Ketoconazole (1mg/ml) was used as a positive control (Fig. 6). This observation was found to be similar with reported literatures from essential oils of different plants [66-68] and confirmed while comparing with works reported from lemongrass oil inhibits 80% and found to be most active against human dermatophytic strains and was confirmed by the antifungal activity against *C. albicans* and also from reports by traditional users of lemongrass against ring worm infections [87,88]. Several studies have revealed that alkaloids, saponins, tannins, flavonoids, and phenolic compounds possess antimicrobial activities [89]. According to some reports, alkaloids, a class of natural products is complex heterocyclic nitrogenous compounds commonly found to possess antimicrobial properties [90]. Our work proves to be quite interesting due to the presence of alkaloids, flavonoids, and saponins, important classes of bioactive phytochemicals in *D. indica* seed oil and compound D1. Further, it provides scientific validation for usage of the plant extracts in folk medicine in our region [49,50].

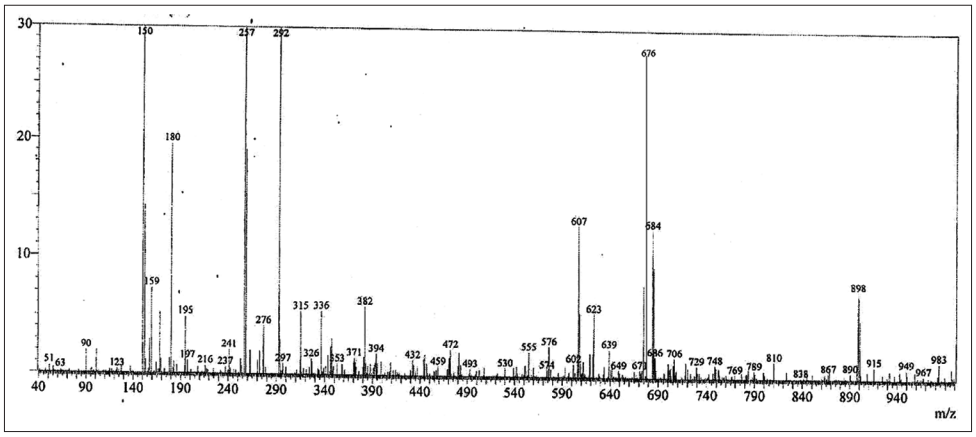

Fig. 4: Mass spectrum of isolated compound from *Derris indica* seed oil

The genus Candida includes several species concerned in human pathology such as C. albicans, C. tropicalis, Candida parapsilosis, C. glabrata, Candida krusei, Candida lusitaniae, Candida kefyr, Candida guilliermondii, and Candida dubliniensis [94]. Among this, C. albicans is extremely known to be the most common cause of mucosal yeast infection [92,93]. People of all ages are affected by skin infections; the causative agents may be yeast cell C. albicans, mold, and dermatophytes [94]. Importance of fungal infections, the difficulties in their treatment and the increase in resistance to antifungal agents have increased research on therapeutic substitutes. Further in vivo studies were undertaken to elucidate the exact mechanism of action by which compound exerts their antifungal effect

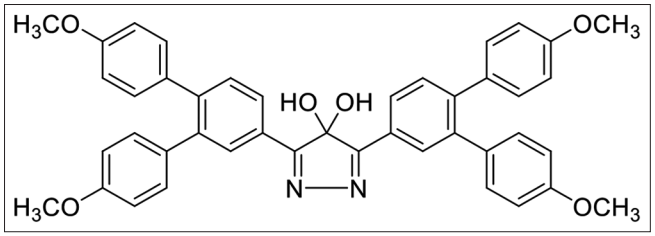

Fig. 5: Structure of 3,5-bis(4,4''-dimethoxy-[1,1':2',1''-terphenyl]-4'-yl)-4H-pyrazole-4,4-diol

K- Ketoconazole, C- Control, D1- *D. indica* isolated compound

Excision wound healing from isolated compound D1

The antifungal and therapeutic potential of the isolated compound D1 was evaluated *in vivo* by using an excision wound healing model on male Wistar rats. No harmful activity was observed for 0.1% extracts in toxicological test; hence, this concentration was used for further investigation. For *in vivo* experiment fungal pathogens such as

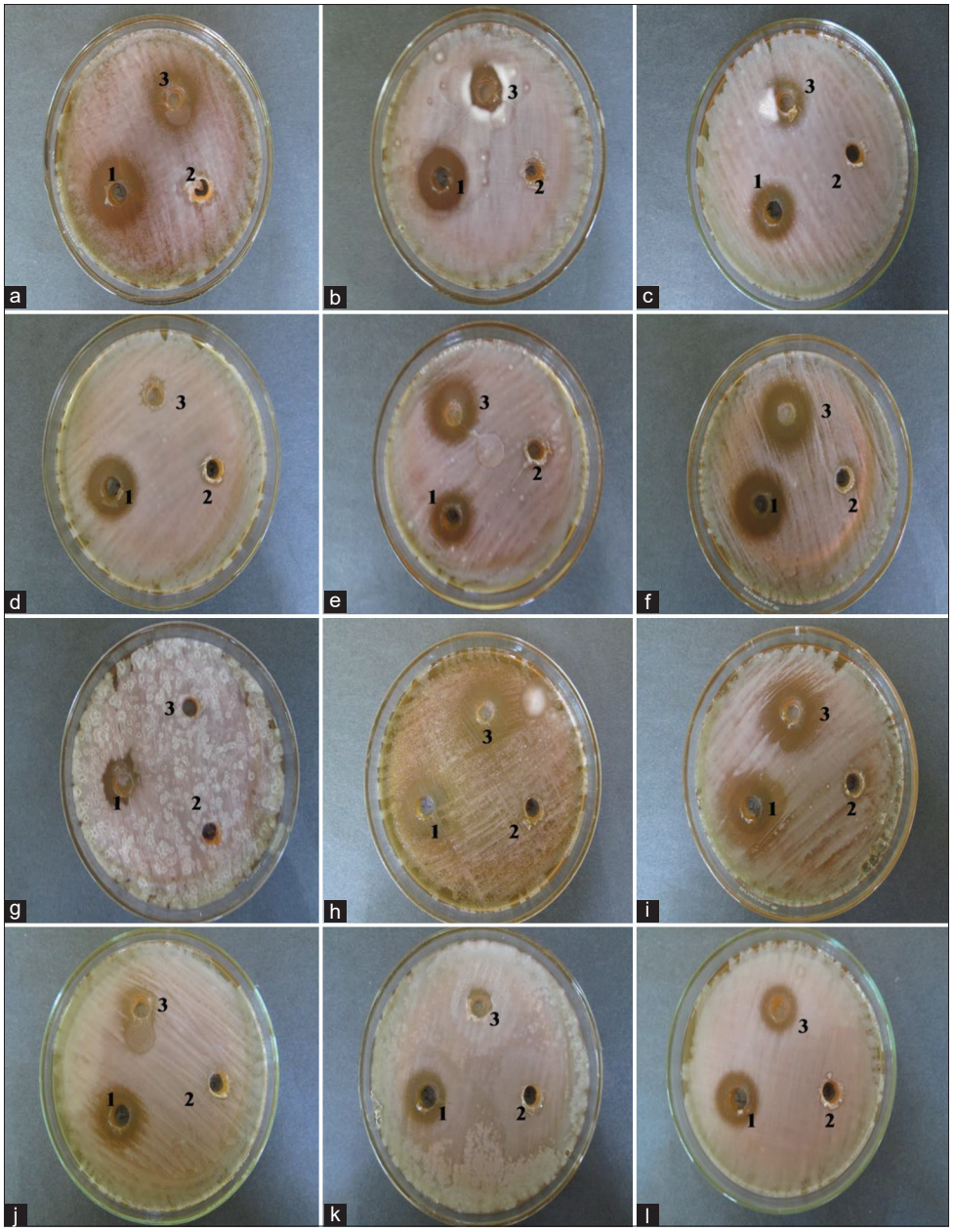

Fig. 6: *In vitro* susceptibility assay of isolated pure compounds D1 against *Candida* species. 1. (+ve control 1mg/ml), 2. (-ve control) and 3. (Isolated compounds D1 5 mg/ml). *Candida albicans*. a (MTCC 3017), b (MTCC 1637) and c (MTCC 183). *Candida tropicalis*.d (MTCC 2795), e (MTCC 1406) and f (MTCC 230). *Candida gaigantae*. g (MTCC 3981), h (MTCC 3841) and i (MTCC 3019). *Candida haemulonii*. j (MTCC 8303), k (MTCC 1966), and l (MTCC 2766)

T. rubrum, T. tonsurans and *C. albicans* were used. These organisms are mainly responsible for skin diseases common in rodents and also in human beings.

The results of *in vivo* study are summarized in Table 3. The treatment was started from the 5th day of the experiment. During the study, it was found that animals induced with fungi *C. albicans* treated with isolated compound was completely cured after 30 days. *T. rubrum* induced fungi takes 40 days to cure wounds completely. The dermatomycete *T. tonsurans* which showed similar results while compare with the rats induced by *T. rubrum*. Compound tested for this experiment observed no visual symptoms at the end of the treatment and showed therapeutic activity. Compound showed antifungal activity against *C. albicans* and *T. tonsurans* after 30 days and against *T. rubrum* after 40 days of treatment. The results demonstrated significant wound healing activity of the tested compound as compared to the established drug, ketoconazole. The animals treated with ketoconazole showed 100% antifungal activity after 10 days of treatment. After this period, the cultures taken from the infected region were inoculated and found negative results. For untreated rats, symptoms were observed at the same time as in treated animals and were present till the end of the experiment. *In vivo* experiment proved that isolated compound to be most effective against tested pathogens. Alkaloids present in the isolated compound believed to be a responsible constituent to this effect. This assumption was confirmed and reported that sterols, alkaloids, proteins, and carbohydrates show significant wound healing activity [39].

CONCLUSION

The phytochemical constituents and pharmacological activities proved that the isolated compound was found to be effective against human fungal pathogens and hence can be used as a base molecule in the design of new antifungal drugs.

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AUTHORS' CONTRIBUTIONS

Vidyasagar GM designed the experiment and involved in the interpretation of data. Nuzhat Tabassum carried out the experimental work, analysis of data and manuscript preparation. Raghunandan and Shivakumar involved in *in vivo* activity. Authors went through the final manuscript.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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