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Full Length Article

Diphyllin: An effective anticandidal agent isolated from *Cleistanthus collinus* leaf extract

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ABSTRACT

In this study, diphyllin [9-(1,3-benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho[2,3-*c*]furan-1(3*H*)-one] was isolated from *Cleistanthus collinus* leaf extract. The isolated compound and leaf extract were evaluated for their *in vitro* anticandidal activity against Candida strains such as *Candida albicans*, *C. tropicalis*, and *C. glabrata*. Diphyllin was found to possess higher anticandidal activity against various *Candida* species with the Minimal Fungicidal Concentration (MFC) of 85–145 μg and inhibition zone of 9.5 ± 0.5–13.5 ± 0.5 mm at 200 μg concentration against the yeast pathogens studied. Thus, diphyllin was twice more active than miconazole against *C. glabrata*.

1. Introduction

In recent years, candidiasis is a major fungal infection caused by *Candida* species in humans and veterinary animals. Among them, 90% of nosocomial candidemia cases were due to *C. albicans* as causative agent associated with other candidal species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, and *C. krusei* in the subcontinent [1–3]. In recent years, although a number of synthetic and natural derivative antifungal drugs developed in pharmacological industries were effective in controlling *Candida* infections, the toxicity, high cost, side effects, and development of drug-resistant strains due to frequent use of the drugs have led to several problems in candidiasis management [4–6]. Henceforth, a plant-derived novel agent with low toxicity and side effects has been examined to overcome and enhance the efficiency of treatment of fungal infections [7,8].

Anticandidal activities of plant extracts, oils, toxicants, metals, synthetic drugs, and natural products have been reported by many researchers and the frequency of discovery of new antifungal agents from plant sources emphasizes the increasing interest in the broad spectrum of activity against *Candida* species [9,10]. Bioactive compounds are extracted from aromatic, toxic, and medicinal plants in pure or crude forms were considered recently as effective agents in controlling bacterial and fungal pathogens. Review of literature and the examination of botanicals against *Candida* species were significantly increased in the last decade [11,12]. The population of the Indian subcontinent has been traditionally using many plants as medicine for treatment of several microbial infections.

Cleistanthus collinus (Euphorbiaceae) is distributed in Asian countries with many potential pharmacological properties [13–16]. In this work, anticandidal activity of *C. collinus* leaf extract and its fraction against *C. albicans*, *C. tropicalis*, and *C. glabrata* have been examined. To the best of our knowledge, no study has been investigated the inhibitory effects of *C. collinus* extract and its fraction against different *Candida* species till date.

2. Materials and methods

2.1. Preparation of extracts

C. collinus samples were collected from Viralimalai, Tamil Nadu, India, in August 2011. The plant leaves were carefully separated and washed with running tap water and subsequently with distilled water to remove pollutants. The samples were shade-dried and minced to precede extraction. About 2 kg dried plant material

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was subjected to crude extract preparation using Soxhlet apparatus (Sigma Soxhlet Mantle, Tamil Nadu, India). Distilled water and ethyl acetate (Merck, Darmstadt, Germany) were used as solvents. Crude extracts were concentrated under reduced vacuum and stored for further analysis.

2.2. Isolation and characterization of diphyllin

About 87 g of ethyl acetate extract was obtained and exactly 7 g was mixed with activated silica and filled at the top of the column. It was then subjected to first elution with 50 ml toluene. Thereafter, toluene was mixed with ethyl acetate in different ratios (9:1–1:9, and 0:10). Eighty-one fractions of 5 ml were collected in test tubes. These fractions were concentrated by evaporation and subjected to thin-layer chromatography (TLC). After TLC, comparable fractions (14–21) were obtained as a single compound. The isolated compound was further subjected to column chromatography and TLC for verifying the purity of the compound. This isolated compound was named as compound TE (toluene/ethyl acetate fractions). Fractionated compound TE was characterized using TLC, ultraviolet–visible (UV–Vis) spectral analysis, Fourier transform infrared (FTIR) spectral, nuclear magnetic resonance spectroscopy, mass spectrometry, and elemental analyses.

2.3. Anticandidal activity

C. albicans (NCIM 3471), *C. tropicalis* (NCIM 3118), and *C. glabrata* (NCIM 3236) were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Maharashtra, India, and used for anticandidal analysis. A primarily anticandidal test was carried out for aqueous, ethyl acetate extracts, and fractioned compound by well-diffusion method as described by Magaldi et al. [17]. Yeast was inocula prepared from 18-h-old mother cultures. Yeast inocula were spread on petri dishes containing Sabouraud dextrose agar and wells were made using a sterile cork borer. Extracts and fractioned compound were dissolved in sterile 4% dimethyl sulfoxide (DMSO). Thereafter, 100, 200, 400, and 800 μg extracts and fractions were loaded on the wells. Standard antifungal agent miconazole 50 μg and 4% DMSO were used as positive and negative controls. The plates were incubated at 37 °C for 24–48 h and the zone of inhibition (ZOI) was measured.

2.4. Minimal fungicidal concentration

Minimal fungicidal concentration of the fractioned compound was evaluated by the broth macro dilution method, to determine the minimum inhibitory concentration (MIC) of the fractioned compound that inhibited visible growth of test pathogens. Mid exponential culture (10 μl) was seeded with the fractioned compound at concentrations of 2–200 μg in 1 ml total volume of Sabouraud dextrose broth incubated at 37 °C for 24 h with mild agitation at 100 rpm. After the incubation, the culture pellets were obtained by centrifugation (REMI, Maharashtra, India), resuspended in 100 μl sterile broth, and the total suspension swabbed onto the Sabouraud dextrose agar plates and allowed to incubate for a further 24–48 h at 37 °C [18].

3. Results

3.1. Characterization of isolated compound TE

3.1.1. Physical properties of compound TE

About 179 mg dry weight of the residue was obtained from identified fractions. Fractionated compound was crystal in nature and green in color, soluble in all organic solvents. The R_f value of this compound was 0.37 in toluene/ethyl acetate (4:1) in mobile phase.

3.1.2. Ultraviolet–visible spectral analysis

The UV–vis spectra exhibited an absorption bond at 278 nm, which can be assigned to π–π* transition of carboxyl and aromatic groups. This gives an idea about the structured compound containing hetero atom having nonbonding electrons (Fig. 1).

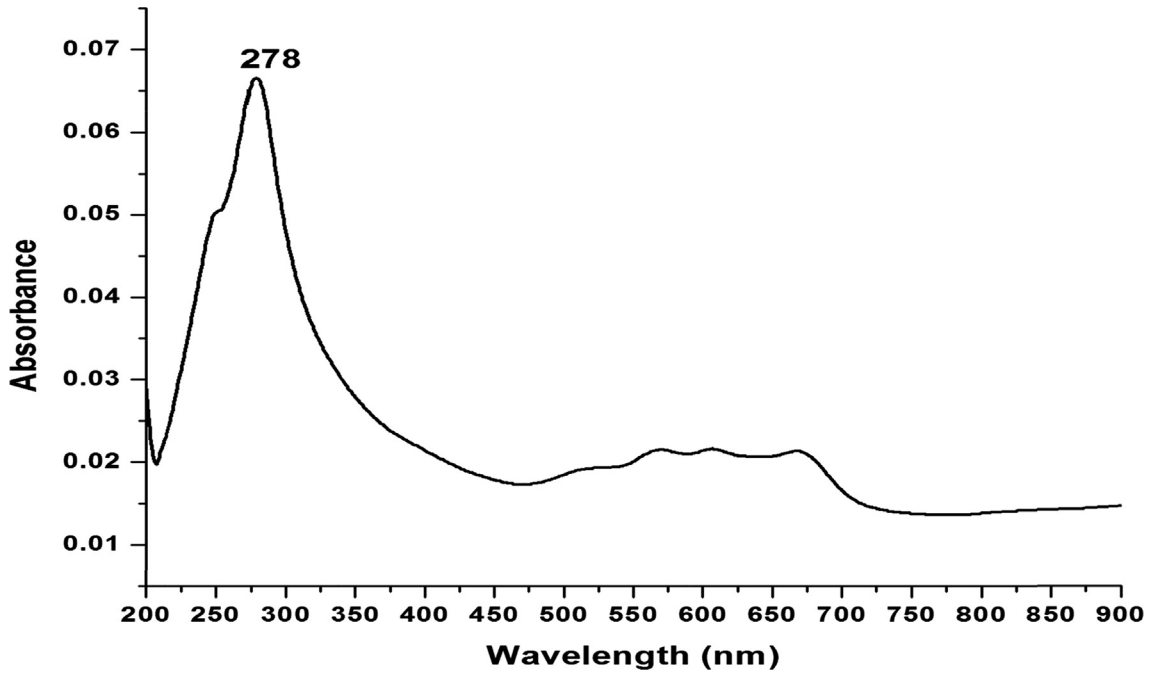

Fig. 1. UV–Visible spectra of fractioned compound TE (Diphyllin).

3.1.3. FTIR spectral analysis of compound TE

Infrared spectrum of TE recorded in KBr medium (4000–450 cm^{-1}) showed a number of bands (Fig. 2). The tentative assignments of various stretching and bending frequencies for fractioned compound TE are listed in Table 1. A broad band observed at 3437 cm^{-1} can be assigned to the OH stretching vibration. The medium bands at 3037 and 3031 cm^{-1} are attributed to aromatic C=H stretching vibration. The ketonic bond observed due to stretching vibration, that is C = O, appears at 1764 cm^{-1}. In in-plane bending, bands appear in the region 1378 cm^{-1}. The median band observed at 1086 cm^{-1} can be attributed to C—O—C bending vibration. The presence of absorption bands in the region 846–727 cm^{-1} is due to out-of-plane bending vibrations of C—H bands at 727 cm^{-1}. The aromatic substituted vibration appears as a strong absorption band at 626 cm^{-1}.

Table 1
FT-IR spectrum of fractioned compound TE.

Absorption (cm^{-1})	Assignment
3437	OH(b)
3037–3031	C—H aromatic
2931	C—H aliphatic
1764	C=O
1378	In plan bending bass of aromatic ring
1056	C—O—C
846–727	Out of plan bending of aromatic
727	Substitutes aromatic ring

3.1.4. ^{1}H NMR spectral studies of compound TE

The proton magnetic resonance spectra of the fractioned compound TE was recorded (Table 2) in $CDCl_3$ solvent (Fig. 3) and the resonance signals were given in. The integration of the spectra indicates the number of proton to be 16. Resonance signal at δ 3.98 ppm is due to O—CH_3 protons. The aromatic proton appears as multiples in the range of δ 6.83–6.56 ppm and the substitute benzene ring appears in the range of δ 6.40–6.37 ppm. The OH proton appears at δ 7.76 ppm signal. The signal due to methoxy proton appears, that is O—CH_2—O, at δ 5.995.95 ppm and the —CH_2—O proton appears at δ 5.27 ppm. Thus, the ^{1}H NMR spectra reveal the presence of aromatic, methoxy, O—CH_2—O, and —CH_2—O groups in the compound. The intensity ratio obtained for signals correlates well with the total number of protons under chemically equivalent and magnetically active nuclei.

Table 2
^{1}H NMR spectra of fractioned compound TE.

Resonance signals	Assignment
3.98	O—CH_3
6.83–6.56	Aromatic proton
6.40–6.37	Substitutes benzene ring
7.76	OH
5.99–5.95	O—CH_2—O
5.27	—CH_2—O

3.1.5. ^{13}C NMR spectral studies of compound TE

The spectra of the fractioned compound TE were recorded in the $CDCl_3$ solvent, as shown in Fig. 4, and the data are presented in Table 3. A spectrum shows absorption of carboxyl carbon at 169.55 and 169.44 ppm. The chemical shift of aromatic carbons appear at 146.71, 129.8–118.35 ppm. The substitute's aromatic carbon can be distinguished from other carbons by its decreased peak height. Its lacks a proton and hence suffers from longer relaxation time with a diminished nuclear Overhauser effect. The peak at 149.52 ppm may be assigned to the substitute's carbon in the ring. The peaks at 151.09 and 149.52 are due to aromatic carbon with O atom. The sharp signal at 149.71 is due to aromatic *ortho*-carbon bond with OH group. Peaks at 134.76 and 129.82 ppm are due to aromatic ring attached with another aromatic ring as a single O bond carbon. The peaks at 11.08–100.59 are due to aromatic carbons. The peak at 69.02 ppm is due to Ar—C—O carbon. The chemical shifts of ^{13}C atoms of the fractioned compound have been assigned relative to the assignments available for individuals of the compound. The ^{13}C NMR signals of the compound and various assignments to different carbon atoms are in good agreement with the ^{1}H NMR.

3.1.6. Mass spectrum analysis of compound TE

The mass spectrum of the fractionated compound was obtained on element ionization mode. The molecular mass was observed at 378 *m*/*z*, which is close to the expected value of 380 *m*/*z* (Fig. 5). The mass spectral fragment studies show that the molecular ions peak at *m*/*z* 378, that is M^{-2} peak ($C_{21}H_{16}O_7$), which confirms the molecular mass of the compound. The peak at *m*/*z* 366, 345, 319,

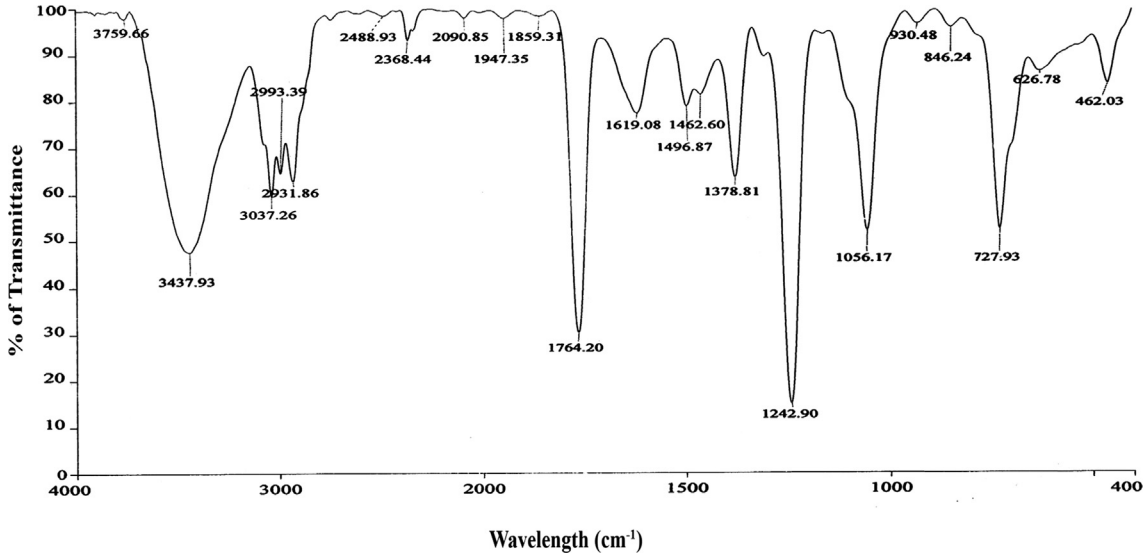

Fig. 2. FT-IR spectrum of fractioned compound TE (Diphyllin).

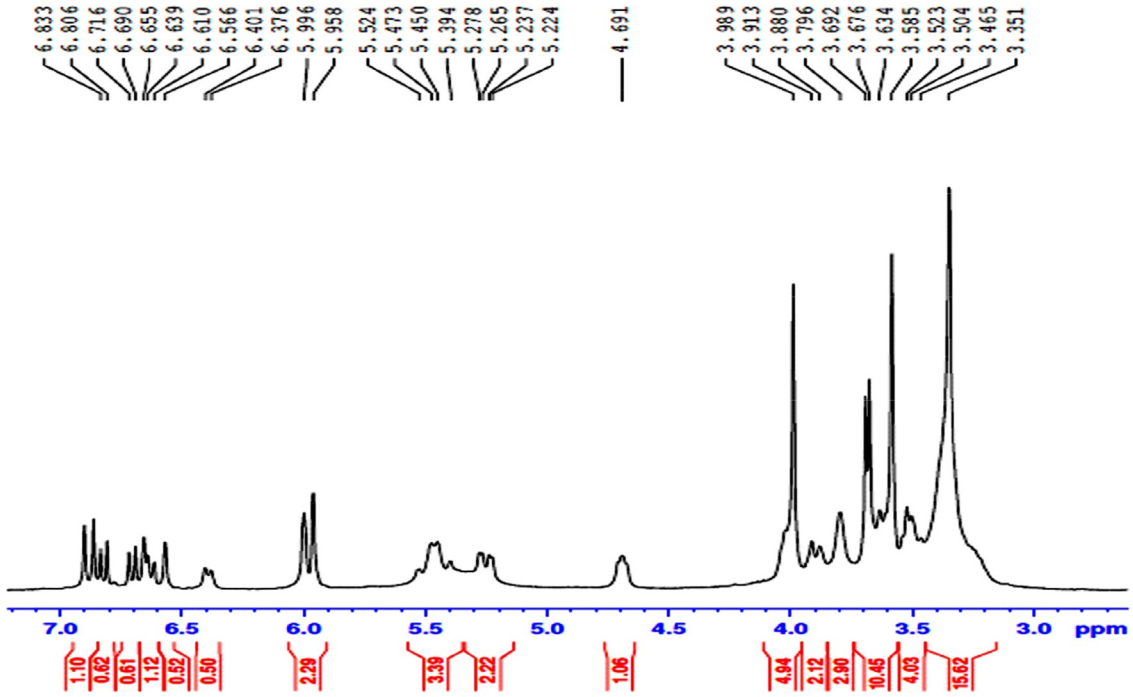

Fig. 3. ^{1}H NMR spectra of fractioned compound TE (Diphyllin).

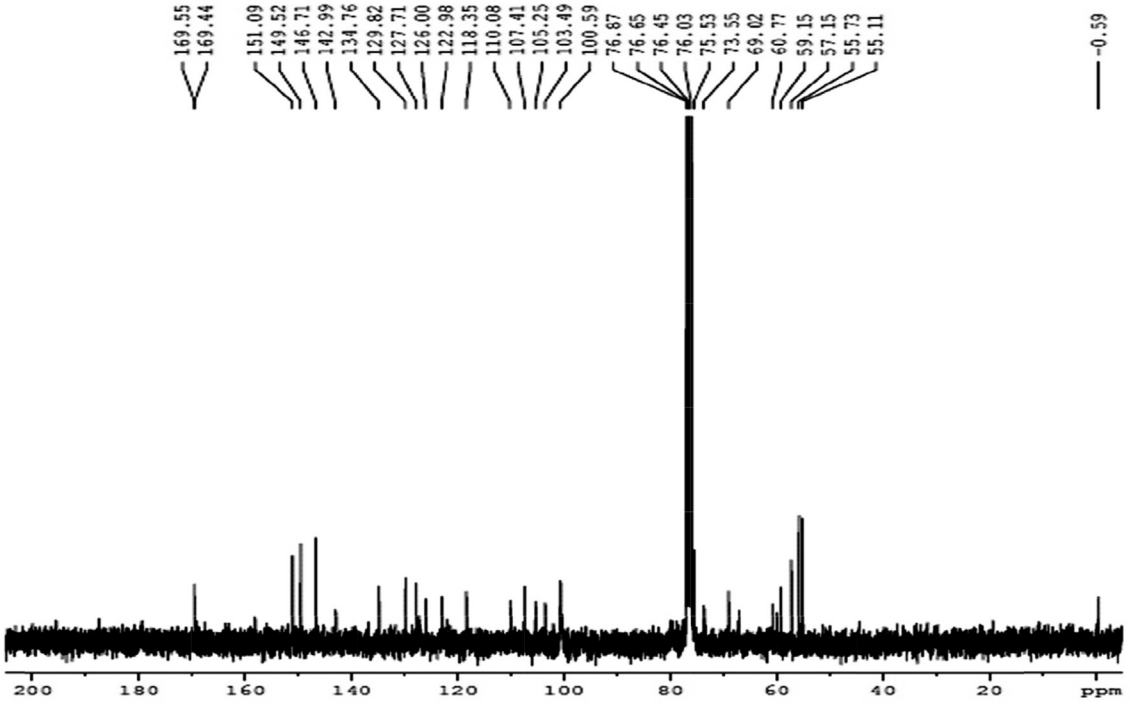

Fig. 4. ^{13}C NMR spectra of fractioned compound TE (Diphyllin).

and 285 are due to $C_{21}H_{15}O_6^+$, $C_{20}H_{14}O_5^+$, $C_{19}H_{11}O_5^+$, and $C_{18}H_8O_4^+$, respectively.

3.1.7. Elemental analysis of compound TE

The compound TE was analyzed for carbon, hydrogen, and nitrogen. The results of elemental analyses are given below

	C%	H%	O%
Calculated	66.31	4.24	29.45
Observed	66.29	4.24	29.27

Table 3
^{13}C NMR spectra of fractioned compound TE.

Resonance signals	Assignment
169.55 & 169.47	C=O
146.71 & 129.82, 129.82–118.35	Aromatic carbon
118.85	Substitutes aromatic carbon
149.52	Ar—O—CH_2
55.73, 55.11	O—CH_3
151.09 & 149.52	Aromatic carbon bonded with O—CH_3
142.99	Ar—OH
134.76 & 129.82	Ar—Ar carbon
60.77 & 59.15	Ar—CH_2—O
69.02	Ar—C—O

The above data indicate that the molecular formula of the fractioned compound TE is $C_{21}H_{16}O_7$ and the molecular weight of the compound is 380.

3.1.8. 2D structure elucidation and name of the compound TE

On the basis of the spectral studies, the fractioned compound TE was successfully drawn in the ChemDraw® Standard 14.0 software and the name was identified as 9-(1,3-benzodioxol-5-yl)-4-hydroxy-6,7dimethoxynaphtho[2,3-*c*]furan-1(3*H*)-one (diphyllin) (Fig. 6). The name and synonyms of the diphyllin and the source of the plant and properties of the compound are given in Table 4.

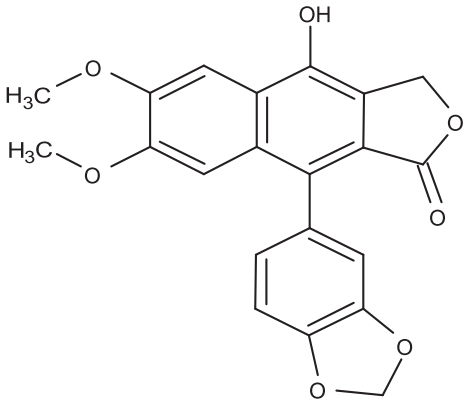

Fig. 6. Structure of Diphyllin (9-(1,3-benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxy-naphtho[2,3-*c*]furan-1(3*H*)-one).

3.2. Anticandidal activity

The anticandidal activity was determined against *C. albicans*, *C. tropicalis*, and *C. glabrata* from the aqueous and ethyl acetate solvent-based *C. collinus* leaf extracts. The ethyl acetate extract only showed activity against *C. albicans* at 800 μg and other pathogens were resistant (Table 5). Further, the ethyl acetate extract was fractionated with toluene/ethyl acetate (3:2) solvents to get one single compound. Thereafter, the fraction, identified as a compound diphyllin, was tested against selected yeast pathogens (Fig. 7). All selected *Candida* species were found to be highly sensitive to the fractionated compound at 200 μg. *C. glabrata* and *C. albicans* were found to be highly susceptible (11–13.5 ± 0.5 mm ZOI at 200 μg) to the isolated botanicals. The MFC values of the compound were observed at 85–145 μg/ml (Table 5) against all tested *Candida* species. For the standard antifungal drug miconazole used in the test, zones of inhibition in the range of 17.5 ± 0.5–20.5 ± 0.5 mm were observed against the tested pathogens.

4. Discussion

In recent years, the number of researches focused on drug development from plant sources to treat infectious has notably increased. This plant material has been used in various biological studies [19,20]. Among all the properties, antifungal activity has received the most attention. *Candida* species are normal flora, harmless yeast-like fungi in healthy humans, but they can cause infections in skin and mucosal membranes under immune-compromised situations [21]. In this study, we evaluated the anticandidal activity of *C. collinus* leaf extracts and its fractions.

Hot aqueous extract of *C. collinus* did not show any activity against different species at least concentration but its displayed moderate anticandidal activity against only *C. albicans* at 800 μg. But earlier it was reported that cold aqueous extract if *C. collinus* exhibited good anticandidal activity (>11 mm as maximum ZOI)

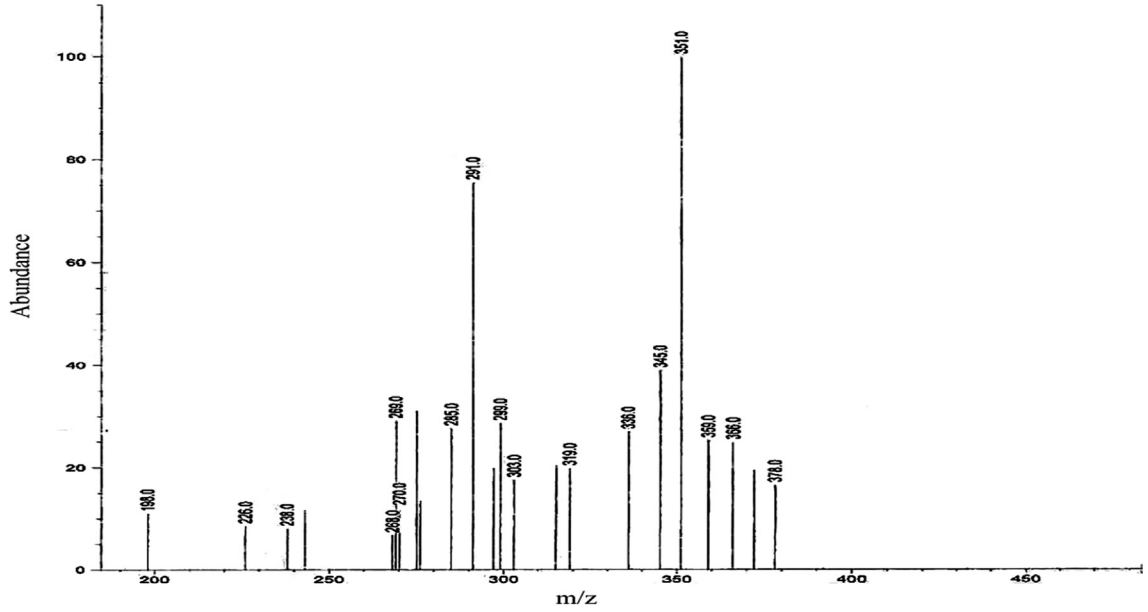

Fig. 5. The mass spectrum of fractioned compound TE (Diphyllin).

Table 4
Isolated compound name and synonyms, compound source and bio activity.

S. no	Name and synonyms	Compound source	Bio activity
1	4-Hydroxy-6,7-dimethoxy-9-[3,4-(methylenedioxy) phenyl]-naphtho[2,3-*c*]furan-1(3H)-one	Lignan from roots of ***Diphylleia grayi***, leaves of ***Cleistanthus collinus***, ***Justicia procumbens*** and ***Haplophyllum hispanicum*** **Zerenex Molecular** [ZBioX-0173]	**Cytotoxin; Zerenex Molecular** [ZBioX-0173]
2	9-(1,3-Benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho[2,3-*c*]furan-1(3H) on *[German] [ACD/IUPAC Name]*		
3	9-(1,3-Benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho[2,3-*c*]furan-1(3H)-one *[ACD/IUPAC Name]*		
4	9-(1,3-Benzodioxol-5-yl)-4-hydroxy-6,7-diméthoxynaphto[2,3-*c*]furan-1(3H)-one *[French] [ACD/IUPAC Name]*		
5	Naphtho(2,3-c)furan-1(3H)-one, 4-hydroxy-6,7-dimethoxy-9-(3,4-(methylenedioxy)phenyl)-		
6	Naphtho(2,3-c)furan-1(3H)-one, 9-(1,3-benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxy-		
7	Naphtho[2,3-*c*]furan-1(3H)-one, 9-(1,3-benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxy- *[ACD/Index Name]* 22055-22-7 *[RN]*		
8	9-(13-Benzodioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho(2,3-c)furan-1(3H)-one		
9	9-(Benzo[d][1,3]dioxol-5-yl)-4-hydroxy-6,7-dimethoxynaphtho[2,3-*c*]furan-1(3H)-one		
10	9-Benzo[1,3]dioxol-5-yl-4-hydroxy-6,7-dimethoxy-3H-naphtho[2,3-*c*]furan-1-one		
11	Diphyllin		

Source of information: http://www.chemspider.com/Chemical-Structure.90798.html?rid=86c435ce-27e1-4d77-9f1d-1d1410b5091b.

Table 5
Anticandidal activity of *C. collinus* extracts and Diphyllin.

Yeast pathogens		*Candida albicans*		*Candida tropicalis*		*Candida glabrata*	
Samples	Concentrations (μg)	ZI*	MFC** (μg/mL)	ZI*	MFC** (μg/mL)	ZI*	MFC** (μg/mL)
Aqueous extracts	100	–	–	–	–	–	–
	200	–		–		–	
	400	–		–		–	
	800	M		–		–	
Ethyl acetate extract	100	–	–	–	–	–	–
	200	–		–		–	
	400	M		–		–	
	800	9.5 ± 0.5		M		–	
Diphyllin	100	M	≥85	M	≥110	M	≥145
	200	11 ± 0		9.5 ± 0.5		13.5 ± 0.5	
	400	13.25 ± 0.25		11.25 ± 0.25		14.5 ± 0.5	
	800	15 ± 0.5		12.5 ± 0.5		17.5 ± 0.5	
4% DMSO		–	–	–	–	–	–
Micoconazole (50 μg) (Positive control)		19.5 ± 0.5		17.5 ± 0.5		22.5 ± 0.5	

* Results are expressed as mean ± standard deviation of values from triplicate experiments.
** Average (MFC) minimal fungal inhibition concentration; M – Moderate activity; DMSO – Dimethyl sulphoxide

and exhibited MICs 600 μg/ml and MFC 750 μg/ml against *C. albicans* [13]. Significant ZOI was observed in ethyl acetate extract at 800 μg compared to the aqueous extract. From this study, it was found that some important phytocompounds might be present in ethyl acetate extract. Earlier, we investigated the preliminary phytochemicals, and aqueous and ethyl acetate extracts were qualitatively screened using gas chromatography–mass spectrometry (GC–MS). Fifteen major phytocompounds were found to be present in ethyl acetate extract, major compounds among them being silane, trimethyl[5-methyl-2-(1-methyl ethyl)phenoxy]-anthracene (7.06%). Tannins, terpenoids, flavonoids, saponins, glycosides, steroids, and alkaloids were also found in ethyl acetate extract. Tannins, terpenoids, glycosides, flavonoids, and saponins were observed in the aqueous extract [22]. The major compounds were observed in the ethyl acetate extract other than aqueous extract by TLC and GC–MS analyses [23,24]. The isolated phytocompound diphyllin showed higher level of inhibition against all tested *Candida* species at 200 μg. Before that many researcher reported good anticandidal activity of medicinal plants. They reported only the anticandidal activity of crude extracts and did not find any promising fractionated compounds [25,26].

Diphyllin is a major glycoside compound present in *C. collinus* plant. Anjaneyulu et al. [27] reported a new diphyllin diglycoside from *C. collinus* heartwood. From the methanolic extract, the $CHCl_3$

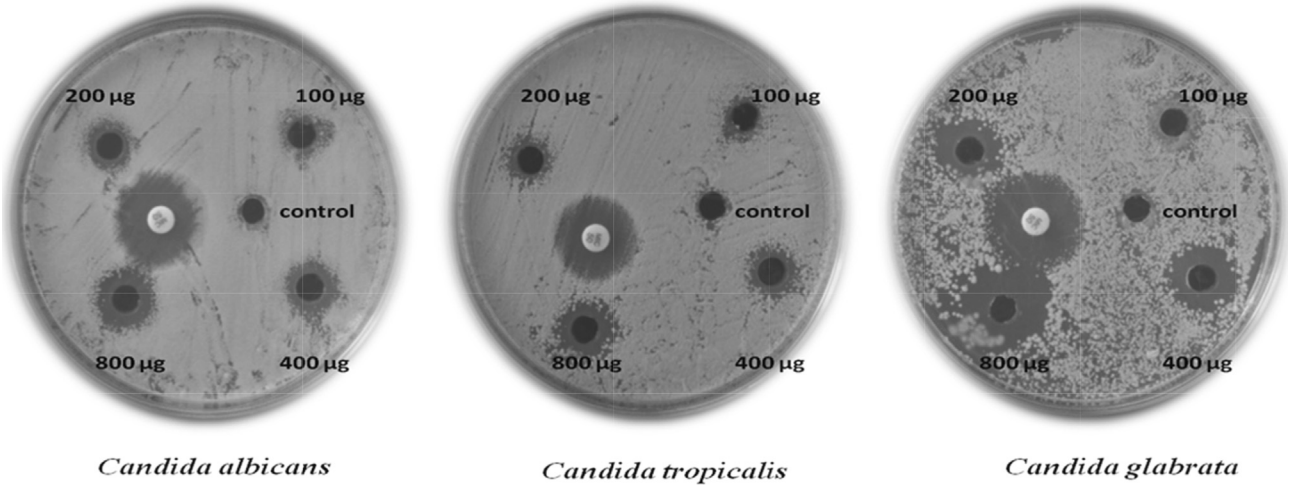

Fig. 7. Anticandidal activity of Diphyllin.

soluble fraction was treated with benzene and the benzene-insoluble residues were crystallized ($CHCl_3$–MeOH) as colorless plates. As the isolated fraction of methanol extract showed Molisch's test as positive, the glycoside was hydrolyzed and the aglycone was identified as diphyllin through spectral analyses. Two glycosides of diphyllin containing 2,3- and 3,4-di-*O*-methyl xyloses were identified for this plant. Similarly, 4-*O*-(3″-*O*-methy1-β-D-glucopyranosyl)diphyllin and Cleistanthoside-A were successfully isolated and identified in *C. collinus* fruits [28].

Investigations of extracts from *C. collinus* plant leaves revealed a complex group of compounds [29,30]. The toxic active principles of *C. collinus* in the leaves are arylnaphthalene lignin lactones—diphyllin and its glycoside derivatives Cleistanthin A and B, and Collinusin [31,32]. Diphyllin and Cleistanthin A and B were commonly known as "Oduvin" in the past. Also, the lignans Cleistanone, Cleistanthin C, and Cleistanthin D are present in *C. collinus*. The toxicity of the *C. collinus* leaves have been primarily due to Cleistanthin A and B [33]. Diphyllin was isolated free and also as 3,4-di-*O*-methylxylopyranoside from *C. collinus* leaves and as its fi-D-glucopyranoside from its bark [34]. The fruits of *C. collinus* have been shown to contain sitosterol and lupeol [35].

Aligiannis et al. [36] proposed a classification on MIC of plant material extracts (strong inhibitors, MIC up to 500 µg/ml); moderate inhibitors (MIC between 600 and 1500 µg/ml); and weak inhibitors (MIC above 1600 µg/ml). On the basis of our MIC results, fractionated compound of *C. collinus* extract showed strong inhibition (85–145 µg/ml) against *C. albicans* followed by *C. tropicalis* and *C. glabrata*. *Candida* species is responsible for the majority of yeast infections in humans and veterinary animals at immune-compromised situations. Among them, in 90% of cases, *C. albicans* is the most causative agent associated with disease to serious fungal infection. Moreover, *C. albicans* form biofilm with *C. tropicalis*, *C. glabrata*, and other *Candida* species, which has also been associated with disease [37,38]. Previously, the toxicity property of fractioned compound was studied against mouse 3 T3–L1 preadipocytes cell proliferation. The ethyl acetate fraction (diphyllin) showed 23–59% anti-proliferative activity (concentration necessary to inhibit cell growth at 50% is ~180 µg/ml) [39]. From our study, we found an assured isolated compound with anticandidal activity against *Candida* species with less toxicity.

In conclusion, it can be said that the results of this study indicated that diphyllin, the fractionated compound of *C. collinus* ethyl acetate extract, exhibited strong inhibition against *C. albicans*, *C. tropicalis*, and *C. glabrata*. Also, to the best of our knowledge, this is the first detailed study of *C. collinus* extract and its fractioned compound against *Candida* species. Further studies on the mode of action of diphyllin are required to understand its anticandidal effects.

Conflict of interest

The authors declared that no conflict of interest.

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