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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 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 *Candida 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 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, Chennai, 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, Pune, 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, Mumbai, 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 ratio) 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).

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}.

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.

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.

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