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Original Article

LONG CHAIN FATTY ALCOHOLS FROM *EUPATORIUM ODORATUM* AS ANTI- *CANDIDA* AGENTS

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ABSTRACT

Objective: Novel plant-derived herbal medicines have takenup the pharmacoeconomic hurdle of controlling diseases in a natural way with minimum side effects. In the present study anti-*Candida* screening and phytochemical profiling of a common weed *Eupatorium odoratum* was done to make out this plant as a source to formulate novel naturally derived antifungal formulations.

Methods: Agar well diffusion assay, Bioassay guided fractionation, HPTLC, Bioautography overlay assay, GC-MS, FTIR.

Results: Agar well diffusion assay of hydro-ethanol leaf extracts of *Eupatorium odoratum* showed a dose dependent growth inhibitory effect on *Candida albicans* with an MIC of 25 -50 mg/ml. Bioassay guided fractionation isolated the anti-*Candida* compounds to n-butanol and HPTLC resolved them with ethylacetate: acetonitrile (6:4). Bioautography overlay assay exposed the inhibition zone of bioactive compound & chemical profiling with GC-MS & FT-IR identified long chain fatty alcohols mainly do-decanol & tetra- decanol as growth inhibitory compounds.

Conclusion: The results suggest that *Eupatorium odoratum* leaf extracts could serve as a source of compounds to formulate herbal medicines against *Candida* infections.

Keywords: *Candida albicans, Eupatorium odoratum*, long chain fatty alcohol, HPTLC, GC-MS, FTIR.

INTRODUCTION

Candida albicans is a fungal yeast involved in candidiasis that ranges from none—life-threatening mucocutaneous illnesses to invasive processes. It is estimated that one in every two people will be affected by *Candidiasis* in their lifetime and the infections may lead to morbidity and mortality in immunocompromised patients [1]. The broad range of *Candida* infections requires an equally broad range of diagnostic and therapeutic strategies. However the control of *Candia* infections is proving to be intractable by means of present anti *Candida* agents due to variety of reasons including development of resistance to antimicrobials, expensive nature and undesirable effects on non-target tissues and organisms. Thus there is a constant need for developing a novel, less expensive, specific, anti-*Candida* agent to combat the erosion in the efficiency of antique approaches.

Plants are rich source of organic compounds which contain a broad range of bioactive ingredients that can decrease longevity of *Candida albicans* by intervening the regular biological processes. Several plants have been reported significant for their anti-fungal activity but only a few botanicals have moved from the laboratory to field use, as they are poorly characterized, in most cases active principals are not determined and most of the works are restricted to preliminary screening.

Eupatorium odoratum is an herbaceous perennial plant belongs to the family Asteraceae. This plant is a nuisance weed in agricultural land and commercial plantations and is involved in very few manufacturing processes. Thus considering the vast potentiality of plants as sources for anti-fungal compounds, systemic investigation was undertaken to screen the anti- *Candida* activity of *Eupatorium odoratum*.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Eupatorium odoratum* was collected from local regions of Alappuzha, Kerala. The leaves were washed and dried in oven at a temperature of 60°C. The dried leaves were pulverized to a coarse powder in a mechanical grinder and passed through a sieve.

Solvent Extraction

10gm of powder was suspended each in 100ml of 75% ethanol, Acetone for 72 hrs and distilled water for 24hrs in a shaker. The extract was decanted, filtered with Whitman No. 1 filter paper and concentrated by evaporation.

Fractionation of hydro-ethanol extract

Hydro-ethanol extract was concentrated in a water bath and re-dissolved in distilled water. Bioassay guided Fractionation [2] was done using different polarity based solvents and obtained successively petroleum ether, n-butanol and water fractions.

Evaluation of antimicrobial activity

Preparation of inoculums

Sabouraud's broth medium was inoculated with *Candida albicans* and incubated for 48hrs. Inoculum size was standardized by adjusting the optical density of the fungal suspension to turbidity corresponding to spectrophotometric absorbance 0.5 at 540 nm.

Standard Preparation

Clotrimazole standard was prepared at a final concentration of 100mg/ml in hydro ethanol.

Minimum Inhibitory Concentration (MIC) by agar well diffusion method

The antimicrobial potential of extracts and fractions were evaluated according to their zone of inhibition against *C. albicans* and comparison of their zone with the activity of the standard antifungal agent clotrimazole. Minimum Inhibitory Concentration (MIC) is defined as the least concentration of the extracts that inhibit the growth of organisms [3].

Solvent extracts were diluted using two fold serial dilution method to obtain the concentration ranging from 200 mg/ ml to 1.56mg/ml. Acetone, 75% ethanol, and water were used as controls and clotrimazole, a common anti-Candida agent was used as standard for the assay. 20 ml Sabouraud's agar medium was autoclaved and brought down the temperature around 50°C. 1ml of Candida albicans culture was added to each flask, mixed thoroughly and poured immediately on the plate [4]. Wells were made on the agar plate using a cork borer of 6mm and two fold serial dilutions of the extracts were added to the wells. After 48 hrs of the incubation at 28°C the zone of inhibition was calculated using the formula: Zone of inhibition =Total Diameter

– well diameter (6mm). The MIC values were taken as the lowest concentration range of the extracts that showed >4mm diameter. Each extract was assayed in triplicate.

Phytochemical screening

Ethanol extract and all the fractions were subjected to preliminary phytochemical screening using standard chemical tests [5] to determine the major chemical groups. Observations were made from two independent experiments.

HPTLC analysis of extracts

HPTLC analysis of n butanol fraction was done to resolve the active principals inducing anti-Candida activity. Thin layer chromatographic plates with many ratios of different solvents were analyzed and the optimal solvent system was used further for HPTLC profile to minimize errors in TLC pattern. Merck Silica gel 60 F254 TLC pre-coated plates were spotted with 10µl volume of n-butanol fraction using CAMAG Linomat 5 sample applicator.

The compounds in the fraction were resolved in a CAMAG twin trough chamber saturated with a mobile phase consisting Ethyl acetate: Acetonitrile (6:4, v/v/v) and derivatized by spraying the plate with anisaldehyde regent. After derivatization the plates were scanned with CAMAG Scanner 4 equipped with winCATS Planar Chromatography manager software. The plates were in duplicates. One set was used for bioautography experiment and other for the reference chromatogram. The experiments were repeated trice.

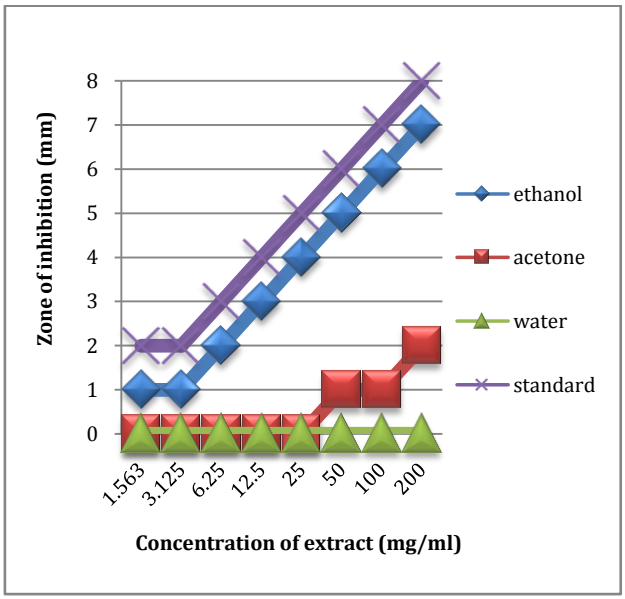

Fig 1: Concentration of extract to zone of inhibition with *c. Albicans*

Bioautography agar-overlay assay with *C. albicans*

HPTLC plates were subjected to bioautography agar-overlay method according to Irena et al [6] with minor modifications. HPTLC plates were dried thoroughly overnight to achieve complete removal of the solvents. The developed TLC plates were placed over Sabouraud's agar (3% agarose) and thinly overlaid with molten Sabourauds agar (2%) inoculated with C. albicans. The plates were incubated in a chamber at 28 °C for 48 h. After incubation, cultures were sprayed with Triphenyl tetrazolium Chloride and further incubated for 30 min at room temperature. Microbial growth inhibition appears as clear zones around active compounds against a pink background.

Identification of bioactive compound

Preparation of sample

Preparative scale thin layer chromatography was chosen for isolation since it was quick and inexpensive. The inhibition zone band was scrapped from the duplicate TLC plates and suspended in 100% ethanol for 24 hrs to dissolve the adsorbed contents. After centrifugation and filtration the sample was collected and subjected to GC-MS analysis and FT-IR analysis.

GC-MS analysis

GC/MS analysis was performed on an Agilent gas chromatograph directly coupled to the mass spectrometer system (JoelAccuTOF GCV). Samples were injected using the split mode (split ratio 1:30), with injector temperature and GC-MS interface temperature both at 280 °C. Column temperature was programmed from 150 °C (2 min), at 8 °C/min to 290 °C (held during 20 min). MS scan range was 50 to 500 a.m.u. Hydrogen was utilized as carrier gas, at the average linear velocity of 40 cm/s.

FTIR spectroscopic analysis

The sample was analyzed on a FT/IR-4100type a spectrometer for the identification of functional groups in bioactive compound.

RESULTS

Antimicrobial activity

Anti-*Candida* assay showed maximum activity in hydro-ethanolic extract of *Eupatorium odoratum* in comparison with water and Acetone extracts, thus the further studies were focused on same extract. Growth of *Candida albicans* was observed over the diffusion zone of all three controls representing the incapability of solvents to inhibit the growth. The activity of hydro-ethanol extract increased linearly with increase in concentration and MIC was identified in a range of 25-50 mg/ml (Figure 1). As compared with clotrimazole, the similar zone of inhibition in hydro-ethanol extract suggests the same as a source of herbal medicine with the standard of commercialized agent available in the market. But the presence of toxic pyrrolizidine alkaloids in the leaves of *Eupatorium odoratum* [7] brought the challenge of using entire plant powder as an antifungal agent and thus the identification of active principals from hydro-ethanol fraction to formulate a medicine from the same.The identification of bioactive compounds involving preliminary qualitative analysis of hydro-alcoholic extract showed the presence of phytochemicals ranging from polar to non-polar moieties(Table 1), which laid the further analysis in a bioassay guided fractionation for polarity based separation. Assessment of different solvent fractions for anti-*Candida* activity showed significant activity in n-butanol fraction which suggested the presence of bioactive compound in the same fraction. Qualitative screening of solvent fractions was done to analyze the unique compounds extracted to n-butanol which instigated further advanced phytochemical analyses (Table 1).

HPTLC analysis of n butanol fraction

Thin layer chromatographic plates developed with many ratios of different solvents were analyzed for anti- *Candida* activity using bioautography overlay method. However the TLC plate developed with Ethyl acetate: Acetonitrile (6: 4) showed the inhibition zone indicating the finest separation of bioactive compound with same solvent system. The developed plate showed a light brown coloured band on derivatization with anisaldehyde-sulphuric acid reagent (Figure 2a) and the HPTLC profile showed a major peak around the Rf value 0.95 (Figure 2b).

Bioautography agar-overlay assay with *C. albicans*

Bioautography is a microbial detection method hyphenated with planar chromatography techniques. It is based mainly on antimicrobial properties of analyzed substances [8].The bioautograms in (Fig. 3) showed zone of inhibition as clear zone on the plate confirming the diffusion of resolved anti-*Candida* principals from the TLC plate to inhibit the growth of *Candida.* The comparison of bioautogram with derivatized TLC plate validated the adsorption of anti-*Candida* compounds inhibiting their growth over the band region.

Table 1: Preliminary phytochemical screening of different extracts of *Eupatorium odoratum*

Phytochemicals	Hydro-ethanol extract	Petroleum ether	n- butanol	water
Phenols	√	×	×	√
Tannins	√	×	×	√
Saponins	√	×	×	√
Essential oil	√	√	√	×
Cardiac glycosides	√	×	×	×
Alkaoids	√	√	×	×

√ - Present
× - Absent

Fig. 2a: HPTLC fingerprint at 550nm after derivatization

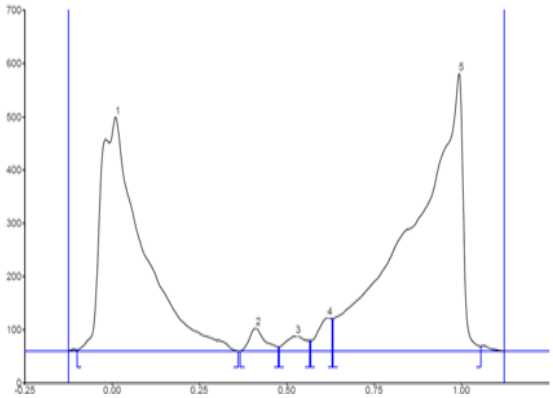

Fig. 2b: TLC plate after derivatization with anisaldehyde reagent

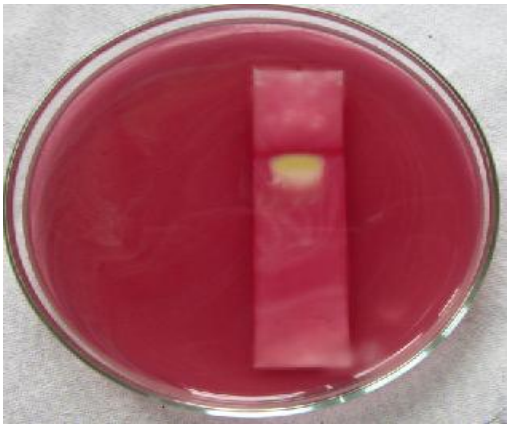

Fig. 3: bioautography overlay assay

Identification of bioactive principles

GC-MS analysis

GC profile showed the presence of two major compounds in the sample with minute impurities & MS analysis identified major compounds as long chain fatty alcohols, do-decanol (retention time:5.5min) and tetra-decanol (retention time : 7.1 min) by comparing their mass spectral fragmentation pattern with those reported in library (Figure 4a & Figure 4b).

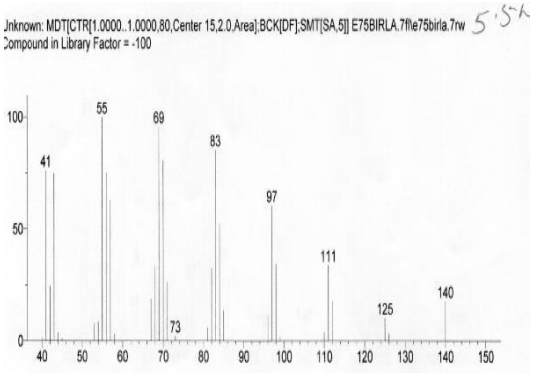

Fig. 4a: GC-MS spectra of do-decanol

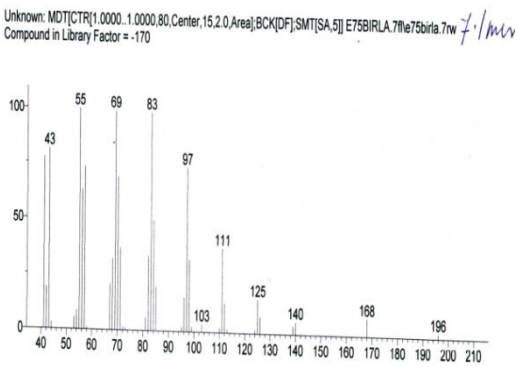

Fig. 4b: GC-MS spectra of tetradecanol

FT-IR

The IR spectrum of the sample showed strong absorption at 3423.03 and 1048.42 indicating the presence hydroxyl group. An intensive absorption in the region of 2976.59 is derived from the aliphatic stretch of CH. While peak at 890 cm-1 indicates the presence of methyl group (Figure 5). Since the band was scrapped from silica plate the bands for silica is also there in the region of 2539.79 and 2413.48. Thus IR spectrum confirms the presence of long chain fatty alcohols with hydroxyl group in its structure.

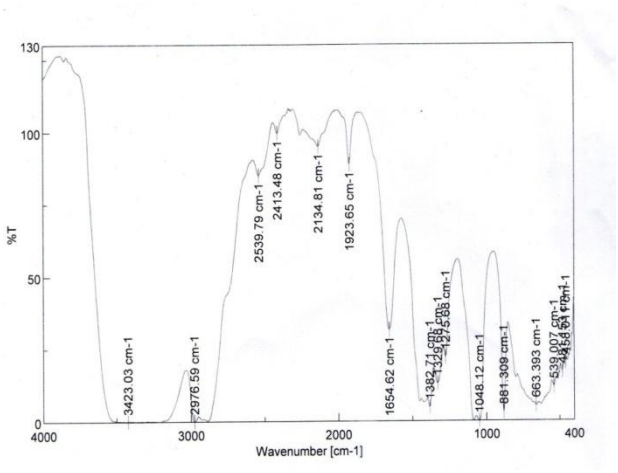

Fig. 5: FT-IR spectrum of sample

DISCUSSION

Ethno botanical studies have become increasingly valuable in the development of green pharmaceuticals hence receiving extraordinary importance and popularity [9]. The *in vitro* assay methods in present study exposed the growth inhibitory activity of *Eupatorium odoratum* against *Candida albicans* & identified the contribution of long chain fattyalcohols (do-decanol and tetra-decanol) in their activity. Previous records are not available for the presence of long chain fatty alcohols in *Eupatorium odoratum*. But prior studies have shown that small molecules such as do-decanol impact the Ras1-Cdc35 pathway in *C. albicans* which affects both hyphae formation and other properties controlled by cAMP regulation [10].

The ability to switch between hyphal and yeast form growth is critical for *C. albicans* virulence [11-13] by promoting invasion and dissemination [14]. Furthermore, many virulence-related genes are coordinately regulated with morphology [15-16]. As well *Eupatorium odoratum* is a weed plant widely distributed in tropical and subtropical regions; leaves of this plant are easily available and involved in very few manufacturing processes. In the view of these all facts the effective isolation of these long chain fatty alcohols from *Eupatorium odoratum* for antifungal herbal preparation may bring up an end to the prevalence of Candidiasis.

CONCLUSION

The findings of the present study demonstrated the potential of long chain fatty alcohols in growth inhibition of *Candida albicans* suggesting leaf extracts of *Eupatorium odoratum* as a source of compounds to formulate novel anti-*Candida herbal* medicines against Candidiasis.

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CONFLICT OF INTEREST STATEMENT

We declare that there are no conflicts of interest.

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