Examining the anti-candidal activity of 10 selected Indian herbs and investigating the effect of *Lawsonia inermis* extract on germ tube formation, protease, phospholipase, and aspartate dehydrogenase enzyme activity in *Candida albicans*

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**ABSTRACT**

**Objective:** The objective of the study is to identify potential anti-candidal agents from natural resources and elucidate the effect of *Lawsonia inermis* extract on major virulent factors of *Candida albicans*.

**Materials and Methods:** Plants, the most abundant and readily available resource of diverse bioactives, were chosen for the anti-candidal screening study. Ten different plants that were proven to have antimicrobial activity but not explored much for anti-candidal activity were chosen for this study. Ethyl acetate extract of these plant leaves were tested for the anti-candidal activity. Extracts with good anti-candidal activity were further screened for its effect in *C. albicans* germ tube formation and enzyme (protease, phospholipase, and aspartate dehydrogenase) activity.

**Results:** Among 10 plants screened, *L. inermis* extract showed complete inhibition of *C. albicans*. On further evaluation, this extract completely inhibited *C. albicans* germ tube formation in serum until the end of incubation period (3 h). This extract also exhibited dose-dependent inhibitory activity against two major virulent enzymes of *C. albicans*, proteases (27–33%) and phospholipases (44.5%). In addition to it, this extract completely inhibited both the isoforms of constitutive candidal enzyme aspartate dehydrogenase, thereby affecting amino acid biosynthesis.

**Conclusion:** Thus, this study confirms the anti-candidal potential of *L. inermis* and hence can be considered further for development of anti-candidal drug.

**KEY WORDS:** Anti-candidal activity, *Candida albicans*, enzyme inhibition, germ tube inhibition, *Lawsonia inermis*

**Introduction**

Among the opportunistic fungal infection, candidiasis especially, invasive candidiasis form the most common cause of fungal infections leading to high rates of morbidity and mortality, globally. Candidiasis accounts for 8–10% of the total systemic nosocomial infection. Oral candidiasis is the most common opportunistic infection observed in HIV-infected patients. Invasive candidiasis remains the prominent cause of mortality since 1997 with 0.4 deaths per 100,000 populations. Causative agent *Candida* spp. occurs as a commensal in vagina, skin, oral cavity, and gastrointestinal tract. Dimorphism of *Candida* and its ability to relapse to pathogenic form by swiftly sensing and adapting to the changing environment (especially
in immunocompromised condition) is one of the unique characteristics of this opportunistic fungal pathogen. A cascade of reactions initiated, during this adaptation phase, which leads to the expression of hyphal specific genes and also genes encoding vital virulence factors such as adhesins, proteases, phospholipases, and methyltransferases. These enzymes play a vital role in survival and virulence of Candida spp.\(^5\) High resistance of drug to currently available drugs such as azoles, polyenes, and echinocandins were observed in Candida spp. and its degree of resistance varies from species to species. Increase in drug resistance, emergence of new species, and toxicity of excising drugs are major factors that entail the need for development of new drugs to combat candidiasis.

Natural resources are rich in diverse bioactive compounds. Therapeutic applications of such phytochemicals are in practice for thousands of years in Indian traditional treatment systems. Abundant availability of conspicuous structural variations, potent bioactivity, and comparatively less or no toxicity of phytochemicals are major reasons for increased preference for these compounds of natural origin over synthetic compounds for drug development. About 50% of the approved drugs in the market are from natural origin or its derivative. Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycins), anticancer drugs (e.g., taxol, doxorubicin), central nervous system-active agents, cytoprotective drugs, immunomodulators, and chemotherapeutic agents are few among those large number of approved drug products of natural origin.\(^7\) With the increase in development of drugs from natural resources, simultaneous increase in interest for screening antimicrobial or anti-infective components from plants were observed.\(^8\) The present investigation is one such effort to screen potential anti-candidal lead from Indian medicinal plants well known for its antimicrobial properties. Indian medicinal plants remain the most promising sources for identification and development of new lead as among the 17,000 species of higher plants found in India, 7500 were well known for its medicinal uses. With this rich pool of natural resources, India remains the hub for herbal drug development.\(^9\) Considering all these, in this study, potential anti-candidal properties were screened from 10 different Indian plants.

In the present investigation, anti-candidal activity of leaf extracts of Tridax procumbence, Adhatoda vasica, Azadirachta indica, Ocimum sanctum, Lawsonia inermis, Acacia cavena, Pongamia pinnata, Eucalyptus spp., Solanum trilobatum, and Acalypha indica were evaluated. These 10 plants were well known for its antimicrobial and medicinal properties.\(^8\) Though these plants were known for its antimicrobial property, its specific effect in control of Candida spp. has not much explored, except for few preliminary anti-candidal screening. Different types of solvent and aqueous extracts of these plants were tested elsewhere for anti-candidal activity. Many of the aqueous extracts failed to exhibit anti-candidal activity. Solvent used for such extraction of anti-candidal bioactives from these plants include alcohol (methanol, ethanol), chloroform, benzene, ether, and hexane. Among these, alcoholic extracts of certain plants exhibited some promise,\(^10-12\) still other than these preliminary screening, they were not tested further for its specific effect on Candida spp. To the best of our knowledge, there is no extensive study on the effect of ethyl acetate extract of these plants on Candida spp. Hence, ethyl acetate extracts of these plants were chosen for this study.

**Materials and Methods**

**Candida Strains (Standard Strain)**

Candida albicans NCIM 3074 strains obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India, was used as standards test organism.

**Plant Extract Preparation**

Leaves from selected plants were collected individually, air dried in shade and ground to fine powder. Ethyl acetate was used for the extraction of phytochemicals. Powdered leaf was soaked with equal volume of ethyl acetate for 3 h and then ground well-using mortar and pestle with required amount of ethyl acetate (about twice the volume of powered leaf) and then subjected to filtration using Whatman No. 1 filter paper. Filtered extracts were air dried. Weighed quantity of air-dried plant extracts were dissolved in measured quantity of dimethyl sulfoxide (DMSO) and used for further studies.

**Disc Agar Cylinder Method**

Screening of all the plant extracts was done using disc agar cylinder method. Briefly, C. albicans lane was prepared using Sabouraud dextrose agar (SDA), and agar cylinders of it were made using sterile borer. Sterile disc containing plant extracts (500 µg/ml) were placed on fresh sterile SDA plates, and cylinders containing culture on the top were placed over the disc with respective controls and incubated for 48–72 h and observed for the growth around the disc.

**Germ Tube Inhibition Assay**

Germ tube inhibitory activity of plant extracts were screened using the method described by Kretschmar et al.\(^13\) with minor modification. Dried plant extract, which showed anti-candidal activity in disk agar cylinder method, was taken at 500 µg/ml concentration in a test tube and resuspended in 10 µl of DMSO. To this, 990 µl of bovine serum was added, and loop full of mid-log phase (10^5–10^7 cells/ml) C. albicans culture was inoculated in the serum and incubated for 3 h at 37°C. Germ tube formation was observed under the microscope, after incubation. Control tubes were maintained similarly without extract treatment and observed for comparison of germ tube formation.

**Secreted Aspartyi Proteinase Plate Assay**

Stationary phase culture of C. albicans was used for SAP inhibition assay using the method described by Kumar et al. (2006). To Sabouraud Dextrose (SD) broth inoculated with 100 µl of mid-log phase C. albicans, L. inermis extracts were added in the concentration of 0.5 µg/ml and 0.75 µg/ml, in respective test tubes. Total volume was made up to one ml using SD broth. The test tubes were incubated at 37°C for 6 h with 140 rpm. After incubation, pellets collected and washed thrice with phosphate buffer saline (pH 7.2). Washed pellet was resuspended in 100 µl of SD broth. Resuspended pellet was loaded onto the sterile disc placed over SAP induction medium (23.4% Yeast Carbon Base, 2% yeast extract and 4% BSA, pH 5.0). The plates were incubated at
37°C for 3–5 days. After incubation, the plates were flooded with 20% TCA (10 min), stained with 1.25% amido black dissolved in methanol acidified with 10% acetic acid (10 min) and destained with 15% acetic acid (10 min) and observed for clear zone around the disc. Zone of proteolysis (Pz) ratio was measured by comparing Pz of control and treated groups.

Protease Activity

Protease inhibitory activity of L. inermis extract in C. albicans was evaluated using zymogram method described by Schmidt et al. with minor modification. Pellet of mid-log phase culture of C. albicans NCIM 3074 was washed twice with normal saline and centrifuged to collect the pellets. 100 µl of C. albicans cell suspension was inoculated in protease induction medium containing 1% glucose, 3% yeast carbon base, and 1% bovine serum albumin (BSA). A volume of 500 µg mL − 1 ethyl acetate extract was added to respective test tubes immediately after the inoculation of culture. The mixture was incubated for 24–48 h at 37°C in a shaker at 150 rpm. After incubation, cells pelleted and lysed using ultrasonicator. Cell lysate after protein estimation using Lowry et al. were used for zymographic studies to check protease activity. Zymogram was performed with 1% casein incorporated in the acrylamide gel to detect the protease inhibition activity. The protease inhibitory activity was indicated by the absence of cleavage of casein (no band formation), and it can be compared to complete cleavage of casein by proteases (white band formation) in control groups.

Phospholipase Inhibitory Activity

Screening of phospholipase activity of C. albicans treated with L. inermis extract (300 µg/ml) was done using the protocol of Leidich et al. Briefly, assay mixture containing 1 ml of sample (cell lysate) and 1 ml of 60% perchloric acid was heated in a sand bath at 200°C until the solution turns colorless. Subsequently, 1 ml of 60% perchloric acid was added and the final volume was made to 9.1 ml using distilled water. To this, 0.5 ml of molybdate II reagent and 0.4 ml ANSA reagent mixture was added. The tubes were shaken well and heated in a boiling water bath for 8 min. The blue color developed was read using spectrophotometer at 680 nm. Percentage change in phospholipase activity was calculated comparing the absorbance of control and treated groups.

Aspartate Dehydrogenase Activity

Assay done using method described by Gennady. Mid-log phase SD broth culture of C. albicans treated with L. inermis extracts at the concentration of 0.5 mg/ml were used for this study. Untreated culture and DMSO-treated cultures were used as control and DMSO control samples, respectively. After incubation period, the cells were pelleted out, washed, and resuspended in ice-cold extraction buffer (62.5 mM tris-Cl pH 6.8). Total soluble proteins were extracted by sonication and used for the enzyme activity.

Native gel electrophoresis was carried out at 15 ± 1°C using tris-glycine as running buffer (pH 8.3) under standard non-denaturing and non-reducing native conditions. Electrophoresed samples were subjected to staining using staining solution containing 0.015 M sodium phosphate buffer pH 7.0–50 ml, L-aspartic acid (sodium salt) - 50 mg, 1.25% MTT 1 ml, 1% PMS 0.5 ml, and 1% NAD 2 ml. Gel was incubated in staining solution in the dark at 37°C until dark blue bands appear. Stained gel was fixed using 25% ethanol and observed for bands showing aspartate dehydrogenase activity.

Results

Screening of Anti-candidal Activity of Plant Extracts

Ethyl acetate extracts of all plant were screened for anti-candidal activity against C. albicans using disk agar cylinder method. Among the 10 plant extracts screened, extracts from five plants, L. inermis (BDUE0110), A. indica, S. trilobatum, A. indica, and A. vasica showed considerable anti-candidal activity in preliminary screening, done using disk agar cylinder method [Figure 1]. Among these, L. inermis (BDUE0110) and A. indica extracts showed complete inhibition of candidal growth over the disk. Surprisingly, extracts of other plants, with well-known antimicrobial activity including Eucalyptus spp. and O. sanctum did not show promising anti-candidal activity.

Germ Tube Inhibitory Activity

Germ tube inhibitory activity of all the plant extracts was tested against C. albicans at 500 µg/ml concentration. Six plant extracts L. inermis, Eucalyptus spp., A. indica, S. trilobatum, A. indica, and P. pinnata showed germ tube inhibitory activity against C. albicans at the tested concentration. Of the plant extracts tested, L. inermis showed complete inhibition of germ tube formation till end of incubation period (3 h) [Figure 2 and Table 1]. Other plant extracts showed germ tube inhibitory activity on C. albicans in the first hour of incubation and at the end of incubation period (third hour), germ tube induction was observed.

![Figure 1: Screening of anti-candidal activity of plant extracts in Candida albicans](image)

Table 1:

Consolidated anti-candidal activity screening results of plant extracts

| Plant            | Disc agar cylinder method | Germ tube inhibition assay |
|------------------|---------------------------|----------------------------|
| Eucalyptus spp.  | −                         | +                          |
| Solanum trilobatum | +                         | ++                         |
| Azadirachta indica | +                         | +                          |
| Adhatoda vasica  | −                         | −                          |
| Acacia cavena    | −                         | −                          |
| Ocimum sanctum   | −                         | −                          |
| Pongamia pinnata | −                         | +                          |
| Tridox procumbence | −                        | −                          |
| Lawsonia inermis (BDUE0110) | ++     | ++                        |
| Acalypha indica  | +                         | +                          |

+++ = Complete inhibition, ++ = Inhibition, += No inhibition
Secreted Aspartyl Protease Plate Assay

Effect of *L. inermis* extract on *C. albicans* protease enzyme activity was tested at two different concentrations 500 µg/ml and 750 µg/ml. SAP assay showed dose-dependent protease inhibitory activity in *C. albicans* on *L. inermis* extract treatment. When compared to control, extract treatment resulted in 27% and 33% protease inhibition in 500 µg/ml and 750 µg/ml concentration treatment of extracts, respectively [Table 2].

Protease Inhibitory Activity Screening

As in Figure 3, Zymogram results shows degradation of casein (thick white band formation) in control samples and intact casein (less intense band formation) in treated samples. This indicates protease inhibitory activity of *L. inermis* extract.

Phospholipase Inhibitory Activity

Spectroscopic analysis of change in phospholipase activity of *C. albicans* upon treatment of *L. inermis* extract has been tabulated in Table 3. Henna extract treatment shows phospholipase inhibitory activity. Percentage inhibition of phospholipase activity in *C. albicans* treated with henna extract when compared to untreated control group was found to be 44.5% at 300 µg/ml treatment.

Aspartate Semialdehyde Dehydrogenase Inhibitory Activity

*L. inermis* extract was found to completely inhibit aspartate semialdehyde dehydrogenase (ASADH) activity in *C. albicans*. Untreated control and DMSO control *C. albicans* group showed two distinct bands at Rm 0.422 and 0.672, respectively. Among these, band with Rm 0.672 was found to be prominent band. Surprisingly *L. inermis* (500 µg/ml) treatment completely inhibited ASADH expression in *C. albicans* [Figure 4] as evident by absence of both the isoforms (bands) in treatment group.

Discussion

Candidiasis is most abundant and well characterized dimorphic fungal infection affecting immunocompromised patients. Differential drug susceptibility, emergence of new drug-resistant species, and differential genotypic and phenotypic expression of *Candida* spp. in immunocompromised conditions such as diabetes, cancer, and AIDS exaggerate the treatment challenge. This subsequently potentiates the need for development of new anti-candidal drugs. Probably, as a consequence of such challenges, there is increase in screening of antimicrobial or anti-infective components from natural resources, especially from plants. With similar interest, in this study, effort has been made to screen and identify anti-candidal potential(s) from 10 different plants. These plants were already proven to have antimicrobial properties, but there is not any convincing evidence or detailed study on its anti-candidal properties. Such studies done using these plants used water, alcohol (ethanol, methanol), and organic solvents such as chloroform and hexane as medium to extract phytochemicals for anti-candidal screening. Evaluation of ethyl acetate as extraction medium for extraction of antifungal potential from these plants was very sparse. Ethyl acetate is a Class III solvent, which is comparatively safe and preferred more, over methanol, ethanol, chloroform, and hexane for use in pharmaceutical industry for drug development. Since this solvent is not explored for screening anti-candidal activity, in this study, ethyl acetate was chosen as the solvent of choice for the extraction of potential anti-candidal leads from selected plants.

Disk Agar Cylinder Diffusion Method

Disk agar cylinder method was used to effectively screen antimicrobial potential of both hydrophobic and hydrophilic compounds. This method is suitable for screening antimicrobial activities of hydrophobic compounds which cannot diffuse in the media. When loaded onto the disk, these compounds will remain entrapped on the disk surface. Seed inoculums, containing agar cylinder, when placed on the disk will not grow over the disk, provided if these entrapped hydrophobic compounds possess antimicrobial properties. Among the 10 plant extracts tested, *L. inermis* and *A. indica* extracts showed complete inhibition of growth indicating the probable presence of potential anti-candidal compounds in these extracts. This warrants further phytochemical profiling studies to confirm the presence of specific anti-candidal agents.

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**Table 2:**

| Organism        | Zone of inhibition |
|-----------------|--------------------|
|                 | 500 µg/mL (percentage of inhibition) | 750 µg/mL (percentage of inhibition) |
| *Candida albicans* | 6.0 mm (27)         | 6.5 mm (33)               |

**Table 3:**

| Organism        | Henna extract treatment (300 µg/mL) (%) |
|-----------------|----------------------------------------|
| *Candida albicans* | 44.45                                  |
This is one of the most rapid, simple and effective way to screen yeast mold shift inhibitor is germ tube inhibitory study. In the present study, we identified that L. inermis extract completely inhibits germ tube formation in C. albicans until the end of incubation period. While other plant extracts Eucalyptus spp., S. trilobatum, A. indica, F. pinnata, and A. indica showed partial or temporary germ tube inhibitory activity but could not exert complete inhibition. To the best of our knowledge, this is the first study to report germ tube inhibitory activity of L. inermis extract. Since this extract can completely inhibit germ tube formation, this can be further investigated for its potential to inhibit hyphal transition and hence can be used in prevention of invasive infections.

As discussed in Table 1, among the 10 plants screened, only L. inermis showed complete inhibition of germ tube formation and candidal growth. Hence, further studies were done using this plant extract.

**Protease Inhibitory Activity**

Extracellular protease produced by Candida spp. is well proven to be one of its most important virulence factors required for its pathogenesis. L. inermis extract was found to have protease inhibitory activity in this study. To the best of our knowledge, this is the first report on protease inhibitory effect of L. inermis extract on C. albicans. Protease production is associated with a number of other putative virulence attributes of C. albicans including hyphal formation, adhesion, and phenotypic switching. Since this extract is found to inhibit protease, an important virulence factor for candidal pathogenesis, this can be a potential source for development of anti-candidal lead. Further to this preliminary investigation, more studies on the exact mechanism and extent of protease inhibition of L. inermis extract need to be evaluated.

**Germ Tube Inhibitory Activity**

Phospholipase inhibitory activity of L. inermis extract was evaluated for overall phospholipase inhibitory activity. Spectrophotometric analysis confirms phospholipase inhibitory activity of L. inermis. To the best of our literature search, this is the first report on phospholipase inhibitory activity of L. inermis on C. albicans. Further to this preliminary investigation, more studies on the exact mechanism and extent of phospholipase inhibition of L. inermis extract need to be evaluated.

**Aspartate Semialdehyde Dehydrogenase Inhibitory Activity**

L. inermis extract treatment completely inhibits the expression of ASADH. Such ASADH inhibitory activity of L. inermis has not been reported elsewhere. ASADH is an oxidoreductase enzyme is found in microorganisms and plants where it catalyzes the reductive dephosphorylation of beta-aspartyl phosphate to L-aspartate-β-semialdehyde in the aspartate biosynthetic pathway. Absence of aspartate pathway in humans and other eukaryotic organisms makes ASADH an attractive target for the development of new fungicidal compounds. Inhibition of this enzyme leads to inhibition of (methionine, threonine, and isoleucine) amino acids synthesis and defect in cell wall biosynthesis which ultimately leads to mortality. Thus, L. inermis extract with ASADH inhibitory activity can be a potential source for development of anti-candidal drug.

**Conclusion**

In this study, 10 plants which have demonstrated antimicrobial property were screened for anti-candidal activity. Ethyl acetate, a polar aprotic solvent useful in extraction of organic salts, was used for extraction of anti-candidal leads from these plants. Among which, L. inermis showed very effective anti-candidal activity. This extract is found to have multiple sites of action, including germ tube inhibition, protease, phospholipases, and aspartate dehydrogenase inhibitory activity. We conclude that ethyl acetate extract of L. inermis can be a potential lead for effective anti-candidal drug development.
Conflicts of Interest

There are no conflicts of interest.

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