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In vitro Antifungal Activity of Plant Extracts against Pathogens of Clinical and Agricultural Importance and Phytochemical Analysis of the Active Compounds

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Abstract This study was carried out with an objective to investigate the antifungal potentials of 14 plants known for medicinal properties against the human pathogen, *Candida albicans* and the tea pathogen, *L. theobromae.* Soxhlet extracts prepared from leaves in various organic solvents were first screened for their antifungal activity by agar diffusion method. While all tested plants showed antifungal activity, evident from distinct inhibition zones on PDA plates, extracts from *Clausena excavata, Ocimum sanctum, Piper betle, Polyalthia longifolia* and *Xanthium strumarium* exhibited higher activities and were selected for phytochemical analysis. *L. theobromae* was found to be more susceptible than *C. albicans.* Bioautography with the test pathogens revealed the presence of antifungal compounds which appeared as clear zones of inhibition against fungus growth on developed TLC plates. *X. strumarium* and *C. excavata* showed two antifungal zones each while each of the other 3 extracts produced single antifungal zone. Application of spray reagents on TLC revealed the chemical nature of all active compounds. Both compounds from *X. strumarium* were sesquiterpenes and those from *C. excavata* were furano-coumarins. Bioactive compounds from *O. sanctum*, *P. longifolia* and *P. betle* were found to be monoterpene, diterpene and phenolic respectively. The results form the basis for further characterization and development of newer fungicidal compounds.

Keywords Antifungal, Bioautography, *Clausena excavata, X. strumarium, P. betle.*

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Introduction

Plant extracts has been traditionally used in India for treating various ailments and forms part of the cultural practice of this country. Scientific reports indicate that plants are an immense reservoir of valuable source of molecules with strong antimicrobial potential and considered as the prime source for the discovery of novel drugs. Different types of compounds produced by plants, particularly the secondary metabolites, may possess antifungal activity and some have successfully been utilized for clinical application (Atanasov

et al. 2015). In addition,many such antifungal compounds are now used in agriculture because synthetic chemical fungicides are unpopular for their residual toxicity, non targeted environmental impacts and direct or indirect effects on animal health system (Saha et al. 2012). Plant products are safer and eco-friendly in comparison to synthetic fungicides, as they have no deleterious effect on environment and on non target organisms in nature.

In recent years, *Candida* infections pose a serious threat to immunocompromised patients, especially those tested HIV positive and those who are receiving immunosuppressive drugs (Sanglard 2016, Campoy and Adrio 2017). Fungal infections lead to 1.5 to 2 million deaths per year, which is higher than either malaria or tuberculosis related deaths (Denning and Bromley 2015). Moreover, increasing resistances towards synthetic antifungal drugs are being reported (Cowen et al. 2014). *Lasiodiplodia theobromae*, a pathogen causing several crop diseases including diplodia disease in tea has also exhibited resistance against the fungicide thiopahanate methyl (Al-Jabri et al. 2017). Since tea is an important contributor to the economy of North East India, it is essential to look for effective alternatives for controlling diseases in tea plants.

The present study reports the antifungal activity of 14 plant extracts against the human pathogen, *Candida albicans* and the tea pathogen, *L.theobromae.* The chemical nature of the antifungal compounds present in the botanical extracts was determined following separation by thin layer chromatography and bioautography.

Materials and Methods

Selection and collection of plant materials

The plant materials were selected on the basis of literature reports of their traditional ethnomedicinal uses and bioactivity as well as availability in sub-Himalayan region of West Bengal. Altogether 14 plant

Table 1. List of plants used in this study.

Name of plants	Local/English name	Family	Traditional application and biological activity
Bidens pilosa Linn.	Spanish needle	Asteraceae	Leaf extract used for treatment of cough, laryngitis, headache, conjunctivitis, rheumatism, infection, digestive and stomach disorder including peptic ulcer
Clausena excavata Burm. f.	Agnijal	Rutaceae	Used in cold, malaria, abdominal pain, snake-bite, preliminary stage of AIDS and dermatopathy
Datura stramonium L.	Datura	Solanaceae	Smoke of leaves is used for asthma, causes sleepiness. Roots are good for tooth-ache
Datura innoxia Mill.	Safed dhatura, Indian-apple	Solanaceae	Leaves are used as repellent and vermicide, used in asthma, wound, malaria and Leishmaniasis. Seed are grind and cooked in mustard oil to cure scabies
Emblica officinialis Gaertn.	Amla	Phyllanthaceae	Fruits used to promote longevity, enhance digestion, strengthen heart, purify blood, stimulate hair growth and enhance intellect. Used against constipation, fever and cough
Eucalyptus globules Labill.	Eucalyptus	Myrtaceae	Leaf essential oil used as antiseptic, against coughs and colds, sore throats and other infections. Used as mouthwash toothpaste. Leaf extracts used in anti-bacterial, antioxidant and anti-inflammation deodorant
Lantana camara L. var aculeate Moldenke	*Raimuniya,* Guye genda	Verbenaceae	Used in bronchitis, stomach problems, rheumatism and to clean teeth
Leonurus sibiricus L.	Guma & Raktadron	Lamiaceae	Anti-inflammatory and anti-diarrhoea. Leaf extract used in hemorrhage, weakness
Measa indica (Roxb.) A. DC.	Ramjani	Primulaceae	Leaves used as an agent for clearing the throat/vocal cord for producing a melodious sound

Table 1. Continued.

Name of plants	Local/English name	Family	Traditional application and biological activity
Ocimum sanctum L.	Tulsi	Lamiaceae	Leaf juice with honey is given for 3-7 days for cough and cold. 1 : 1 ratio of tulsi leaf and neem leaf paste is very effective for diabetes
Polyalthia longifolia var *pendula*	Ashok tree	Annonaceae	Powder of stem bark mixed with curd and sugar and mixture is given orally thrice a day to cure diarrhoea. Stem bark is dried, powdered and given orally in the treatment of gout
Piper betle L.	Paan	Piperaceae	Leaf paste with *Acacia catchu* bark paste massaged on the skin of children in maggots. Herbal dye, antipyretic, antioxidant, anticancer, antiulcer, anti-inflammatory, pain reliever and immunomodulating
Syzygium cumini (L.) Skeels	*Jamun*	Myrtaceae	Hypoglycaemic, diuretics, analgesic, anti-inflammatory, antiplaque, antimicrobial antidiarrhoel, antioxidant and gastroprotective
Xanthium strumarium L.	Chotagokhru	Asteraceae	Dry fruits kept on dried stem of *Calotropis procera* are burnt and the smoke is inhaled

species were studied for their biological activity and phytochemical analysis. Table 1 shows a list of the plants along with their local names, families and traditional uses (Rastogi and Mehrotra 1995, Chatterjee and Pakrashi 1997). Most of the plant materials were coolected from local areas within and outside campus of University of North Bengal. Some of the plants were collected from forest areas of Sukna located in the Terai region of the Eastern Himalayas. Fresh disease free leaves of different plant species were collected. Voucher specimen of each species was deposited in the herbarium of Department of Botany, University of North Bengal.

Fungal pathogens

Two pathogens of clinical and agricultural importance were used in this study. A virulent strain of *Lasiodiplodia theobromae* (ITCC 5446.02) was earlier isolated in the laboratory from young tea plants showing diplodia disease from a nursery in the Darjeeling district of West Bengal and its identity was authenticated by IARI, New Delhi. *Candida albicans* (MTCC 183) which is an opportunistic human pathogen causes candidiasis especially in immunocompromised patients. This strain was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Soxhlet extraction

Fresh leaves of fourteen different plant species (Table 1) were thoroughly washed with distilled water and shade dried at room temperature for 5 to 10 days. The dried leaves (120 g) of each plant were ground to moderately fine powder (1mm) and extracted in a soxhlet apparatus using appropriate solvents (Table 2), for fifteen h at 35—45°C. The extract was concentrated to dryness under vacuum in a rotary evaporator (Eyela, Japan). The residue (15 g) obtained as a gummy solid mass was dissolved in proper solvent at 3 different concentrations (100 mg/ml, 10 mg/ml and 1 mg/ml) and used for further studies.

Agar cup bioassay

The plant extracts were screened for antifungal activity by agar cup diffusion method. Potato dextrose agar (PDA) medium was autoclaved at121°C for 15 min, cooled to 45 °C and 1ml of pure cell suspension or spore suspension (10^6 /ml) or of the test pathogen was mixed with 19 ml of molten medium and poured into sterile petriplates of 9 cm diameter. Agar cups were prepared with sterile cork-borer (4 mm diameter) in the PDA plates after solidification of the medium seeded with spores of the test fungi. Plant extracts (50 µl) were introduced into each well and the plates were incubated for 48-72 h at 28°C. The antifungal activity

Table 2. Screening of antifungal potential of crude soxhlet extracts of leaves from different plant species against *Candida albicans* and *Lasiodiplodia theobromae* by agar diffusion assay.* The inhibition zone includes the diameter of the agar cup (6 mm).

Name of plants	Solvent for extraction	Diameter of inhibition zone (mm) obtained at different concentrations of leaf extracts (mg/ml)* *C. albicans* 100	*C. albicans* 10	*C. albicans* 1	*L. theobromae* 100	*L. theobromae* 10	*L. theobromae* 1
Bidens pilosa	Ethyl acetate	21.3 ± 3.0	15.0 ± 1.7	9.3 ± 1.5	25.0 ± 2.6	16.6 ± 2.1	11.3 ± 2.5
Clausena excavata	Ethyl acetate	24.3 ± 3.5	17.3 ± 2.5	7.6 ± 0.6	34.6 ± 3.2	22.3 ± 3.0	14.3 ± 2.1
Datura stramonium	Benzene	17.0 ± 4.5	10.6 ± 1.2	0	26.3 ± 2.5	15.0 ± 2.6	0
Datura innoxia	Ethanol	18.6 ± 3.8	12.0 ± 2.6	8.3 ± 0.6	28.6 ± 3.2	18.3 ± 2.3	8.6 ± 1.1
Emblica officinalis	Ethyl acetate	13.3 ± 2.1	7.6 ± 0.5	0	17.6 ± 3.8	13.3 ± 2.1	0
Eucalyptus globulus	Ethanol	18.3 ± 1.5	9.3 ± 2.1	0	21.6 ± 3.2	14.6 ± 2.1	9.0 ± 1.0
Lantana camara	Ethanol	11.6 ± 1.1	0	0	15.6 ± 2.5	10.6 ± 1.5	0
Leonurus sibiricus	Ethanol	15.6 ± 2.1	10.6 ± 0.6	0	20.3 ± 2.3	14.0 ± 3.6	7.3 ± 0.6
Maesa indica	Dichloromethane	18.6 ± 2.9	14.3 ± 3.0	8.3 ± 0.6	25.6 ± 2.5	15.6 ± 2.5	10.3 ± 2.1
Ocimum sanctum	Ethyl acetate	22.0 ± 2.6	13.6 ± 2.1	9.6 ± 1.5	26.3 ± 1.5	18.3 ± 4.6	11.6 ± 3.0
Polyalthia longifolia	Ethyl acetate	31.3 ± 2.5	16.3 ± 2.3	11.0 ± 2.6	28.3 ± 2.3	23.3 ± 3.2	15.6 ± 3.0
Piper betle	Dichloromethane	29.6 ± 3.2	17.3 ± 1.5	12.6 ± 1.5	36.3 ± 2.9	24.6 ± 4.0	14.3 ± 1.5
Syzygium cumini	Ethyl acetate	15.3 ± 3.0	9.6 ± 1.5	0	22.3 ± 3.8	13.6 ± 0.6	11.0 ± 1.0
Xanthium strumarium	Benzene	24.6 ± 3.0	18.3 ± 2.1	9.6 ± 1.5	35.3 ± 3.5	24.3 ± 2.5	15.6 ± 3.0

was evaluated by measuring zones of inhibition of fungal growth around the plant extracts. Complete antifungal assay was carried out under strict aseptic conditions. The zones of inhibition were measured in mm and the experiment was carried out in triplicate. The average of 3 replications and standard deviation were computed by MS Excel 2007.

Thin layer chromatography

Thin layer chromatography was used both for detection of antifungal compounds and their phytochemical analysis. Antifungal activities were tested by bioautography following the method of Kumar et al. (2012). Phytochemical analysis was performed using ultraviolet light and different spray reagents (Wagner and Bladt 1996). Precoated silica gel 60 F254 aluminium TLC plate (Merck, India) was activated by heating at 70°C for 45 minutes prior to sample-loading. Each concentrated extract (10 mg/ml) was loaded on the activated TLC plate at 2 different spots (20 µl each) 2 cm apart and developed either in hexane : ethyl acetate : methanol (60 : 40 : l v/v) or in hexane : ethyl acetate (70 : 30 or 80 : 20 v/v) as solvent. The plates were air-dried until the solvent evaporated completely and subsequently cut symmetrically into 2 parts to separate the 2 developed chromatograms. These were used independently for phytochemical analysis and bioautography and the results were compared in order to determine the nature of antifungal compound.

Bioautography

For bioautography with *L. theobromae*, spore suspension (10^6 spores/ml) was prepared from 7 d old culture in Richard's medium and sprayed with an atomizer on dried TLC plates (Kumar et al. 2012). The plates were incubated in a humid chamber at 28°C for 2-5 days. For *C. albicans* developed chromatograms were placed in sterile petri plates. Molten PDA medium was mixed with phenol red (0.02%) and an inoculum of cell suspension of *Candida albicans,* at tolerable temperature. The mixture was poured evenly over developed TLC plates, covered with lid and incubated at 28°C for 24 h. Inhibition zones, which appeared as clear spots on a background of fungal growth, indicated the presence of antifungal compounds. R_f values of the inhibition zones and the zone diameters swere noted.

Phytochemical analysis

For phytochemical analysis, the developed chromatogram was viewed under UV light (254 and 365 nm) and sprayed with vanillin-sulfuric acid, anisaldehyde-sulfuric acid or Folin ciocalteu's reagent. The color of the developed spots, if any, was noted and

Table 3. Antifungal activity of crude soxhlet extracts of leaves from potential plant species against *Candida albicans* and *Lasiodiplodia theobromae* assessed by bioauto graphy technique and phytochemical detection of antifungal compounds.

Plants	No. of zones	R_f	Antifungal activity Zone of inhibition (mm) *C. albicans*	*L. theobromae*	Chemical nature of active compound
C. excavata	2	0.39	18	25	Furano-coumarin
		0.30	15	22	Furano-coumarin
O. sanctum	1	0.85	10	15	Monoterpene
P. betle	1	0.64	30	38	Phenolic
P. longifolia	1	0.73	15	27	Diterpene
X. strumarium	2	0.56	32	38	Sesquiterpene
		0.25	24	32	Sesquiterpene

the R_f was matched with that of the active compound. The result of chemical analysis was determined based on color of the spots (Wagner and Bladt 1996, Harborne 2005).

Results and Discussion

Screening of botanicals for antifungal activity

Agar cup bioassay showed that all 14 plants were active against both the tested fungal pathogens (Table 2). But overall *L. theobromae* was found to be more susceptible than *C. albicans*. *Piper betle* and *Xanthium struaium* was found to be the most active plants as it produced largest inhibition zones of 36.3 mm and 35.3 mm respectively at 100 mg/ml against *L. theobromae.* Other plants showing strong antifungal activity were *Polyalthia longifolia, Clausena excavata* and *Ocimum sanctum*. Besides *Datura innoxia* and *D. stramonium* also showed good antifungal activity and 100mg/ml which however reduced greatly at 1 mg/ml. *Emblica officinalis* and *Lantana camara* were less effective. Reports of antifungal activity of *Maesa indica* and *Leonurus sibiricus* is extremely rare (Yashoda et al. 2014). Considering the overall performances of the plant species, 5 plants, viz. *P. betle, O.sanctum, P.longifolia, C. excavata* and *X. strumarium* were selected for further phytochemical analysis.

Activity monitoring and phytochemical analysis of active compounds

Bioautography of the concentrated crude extracts prepared from *C.excavata, X. strumarium,P. longifolia, P.betle* and *O. sanctum* revealed antifungal activity against *C. albicans* and *L. theobromae* (Table 3). The occurrence of antifungal components was evident by the presence of clear zones of inhibition on TLC plates. *X. strumarium* and *C. excavata* showed 2 antifungal zones each while the other 3 plant extracts produced single antifungal zone each. The largest inhibition zone of 38 mm was produced by *X.strumarium* (R_f 0.56) and *P. betle* (R_f 0.64) extracts against *L.theobromae. X. strumarium* showed 2 antifungal compounds both of which produced green fluorescence under UV_{254}. The compounds produced reddish brown spots when sprayed with anisaldehyde-sulfuric acid and brown spots with vanillin-sulfuric acid which indicated presence of sesquiterpene derivatives. *X. strumarium* has been reported to contain an antimicrobial sesquiterpine lactone named xanthatin (Saha et al.2012). Another sesquiterpine lactone, deacetyl xanthumin, has been reported but this also structurally resembles xanthatin (Kim et al. 2002). Occurrence of a second antifungal compound in our extract indicates that this plant contains yet undetected bioactive compounds which warrants further study.

The single antifungal compound of *P. betel* produced green fluorescence under UV_{254} and deep blue and brown spots when sprayed with Folin ciocaltu's and vanillin-sulfuric acid respectively indicating phenolic compound. Row and Ho (2009) studied the chemical compositions of the crude oil by GC/MS analysis and identified 36 compounds including eugenol (36.2%) and chavibetol acetate (16.9%) both of which are antifungal phenolic compounds or their derivatives (Kumar et al. 2010b). In this study, only one large antifungal zone was observed possibly because the compounds failed to separate under the current experimental conditions.

P. longifolia (27 mm) and *C. excavata* (25 mm) also exhibited large inhibition zones against *L. theobromae*, however, zone diameters produced by *O. sanctum* (15 mm) were much lower than other

extracts. The two antifungal compounds from *C. excavata* showed intense quenching under UV_{254} but fluoresced with a bright blue color under UV_{365}. Both produced blackish blue spot upon spraying with anisaldehyde-sulfuric acid indicating presence of furano-coumarins. Literature review has revealed that root of *C. excavata* have diverse group of coumarins with broad range of biological activities (Wang et al. 2008). However, till date, very few studies have been done concerning the antifungal activity of phytochemicals of *C. excavata* leaves. We earlier reported a new compound excavarin A (Kumar et al. 2012) from dichloromethane extract. Characterization of this second compound with antifungal activity in the ethyl acetate extract is in progress.

The antifungal compound from *P.longifolia* appeared as a single blue spot on TLC plates upon spraying with anisaldehyde reagent and heating at 110°C which indicated the presence of diterpene. The occurrence of clerodane and halimane diterpenes in *P. longifolia* has been reported by several authors (Katkar et al. 2010, Bhattacharya et al. 2015). The antifungal compound from *O. sanctum* produced brown spots when sprayed with vanillin sulfuric acid indicating monoterpene derivative. Essential oils from *O.sanctum* leaves have been found to posses antifungal properties (Kumar et al. 2010 a). Eugenol, which is also a monoterpene has been reported to be the major compound (43%) in essential oil extract by GC-MS analysis (Devendran and Balasubramanian 2011). Thus it is most probable that the antifungal compound detected in this study is eugenol, but further studies are necessary to ascertain this.

Conclusion

The studies showed that extracts from several plants, especially, *X. strumarium* and *P. betle* has strong antifungal activity against *L. theobromae* and *C. albicans.* The phytochemical analysis coupled with bioautography revealed occurrences of several types of antifungal compounds which may form basis for further characterization and development of newer fungicidal compounds.

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