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Antifungal and HPLC analysis of the crude extracts of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus*

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The antifungal activity of methanolic crude extract of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* were investigated against *Alternaria solani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris* sp. and *Helminthosporium* sp. at different concentrations (1000, 2000, 3000, 4000 and 5000 µg/ml). At 5000 µg/ml crude extract of *Tinospora cordifolia* is found to be highly effective against *Helminthosporium* sp. followed by *Acorus calamus* against *Alternaria solani*. On the other hand at 5000 ug/ml, *Celestrus paniculatus* showed better activity against *Alternaria solani* and *Helminthosporium* followed by *Acorus calamus* against *Alternaria solani* at 4000 ug/ml. At 5000 ug/ml, all the three crude extracts showed least activity against fungus *Curvularia lunata* and *Fusarium* sp. except *Acorus calamus* that showed better activity against *Curvularia lunata*. The increase in the production of phenolics in the extract can be correlated with the induction of resistance in treated plants against phytopathogenic fungi. HPLC analysis of the crude extract of medicinal plants showed six different phenolic acids (Benzoic acid, Cinnamic acid, Caffeic acid, Ferulic acid, Gallic acid and Tannic acid) present in varying amount. The results of the study provide scientific basis for the use of the plant extract in the future development as antioxidant, antibacterial, antifungal and anti-inflammatory agent.

Key words: Antifungal activity, *Acorus calamus*, *Tinospora cordifolia*, *Celestrus paniculatus*, HPLC, phenolic acid

Introduction

Plants have evolved a number of inducible defence mechanisms against pathogen attack. Some of the responses are constitutive and pathogen non-specific, but the majority of them are induced after recognition of the pathogen.

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Recognition results in the activation of a variety of defence responses, including rapid localized cell death (Hammond and Jones, 1996), synthesis of pathogenesis-related (PR) proteins and induction of systemic acquired resistance (Selitrennikoff, 2001). Systemic acquired resistance is characterized by the activation of a broad spectrum of host defence responses, locally at the site of the initial pathogen attack and systemically in distal tissues, providing resistance against widely diverse organisms such as fungi, bacteria and viruses (Durrant and Dong, 2004). *Acorus calamus* Linn. (Family Araceae) commonly known as "sweet flag" or Waan-Nam, is a well known medicinal plant. The rhizomes are considered to possess anti-spasmodic, carminative and antihelmintic properties and also used for treatment of epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and tumors. It is listed as an insecticide, an antifungal agent, an antibacterial agent and a fish toxin (Anonymous, 2000). *Tinospora cordifolia* Miers, commonly known as 'Guduchi' (family Menispermaceae) is a plant prescribed in Ayurveda, the Indian traditional system of Medicine as a 'Rasayana' or general tonic (Thatte and Dahanukar, 1986). Dry barks of *T. cordifolia* has anti-spasmodic, anti-pyretic (Ikram *et al*., 1987), anti-allergic (Nayampalli *et al*, 1986), anti-inflammatory (Pendse *et al*., 1977) and anti-leprotic (Asthana *et al*., 2001) properties. Guduchi is a promising drug entity which should enter the world market by evidence-based research for therapeutics (Jagetia and Rao**,** 2006)**.** Celastrus paniculatus or Jyotishmati is a herbal plant belonging to the Celastraceae family. It is also called Black-Oil tree or Climbing Staff tree. It is also considered to be analgesic, aphrodisiac, diaphoretic, emetic, emmenagogue, stimulant and tonic (Duke and Ayensu, 1985).

The objective of this research was to auntheticate the antifungal sensitivity and HPLC analysis of methanolic extracts of phenolic acid present in *Acorus* calamus, *Tinospora cordifolia* and *Celestrus paniculatus* to lengthen the queue of antimicrobial herbs.

Materials and methods

Collection and extraction of medicinal plant material

The raw material of medicinal plants such as *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* were collected from different regions of India. Voucher specimens deposited at Institute of Bioengineering and Biological Sciences, Varanasi, India for future reference. The dried powdered of plant materials (roots and aerial parts) were extracted separately with methanol: sterile water (1:1) using soxhlet apparatus for 48 hrs. The solvent was distilled off at lower temperature under reduced pressure in rotory flash

evaporator and concentrated on water bath to get the crude extract which is stored in dessicator for future use.

Antifungal activity

Three different medicinal crude extract which showed *in vitro* antifungal activity against some plant pathogens such as *Alternaria solani*, *Helminthosporium* sp., *Bipolaris* sp., *Curvularia lunata* and *Fusarium* sp., were used in the present experiment. Test fungi were isolated on potato dextrose agar (PDA) (peeled potato 250 g, dextrose 20 g, agar 15 g, distilled water 1 L) medium from their respective hosts collected from experimental farm of Banaras Hindu University, Varanasi, India. The cultures were further purified by single spore isolation technique and maintained at 25±2 °C on PDA slants 7-10 days old culture were used in the experiment.

Stock solution (5000 µg/ml) of the crude extract was prepared by dissolving 5 ml of the culture in 1 ml of distilled water. Required concentrations (1000, 2000, 3000, 4000 and 5000 µg/ml) were prepared from each stock solution by diluting with distilled water. One drop (40 µl) from each concentration was placed on grease-free glass slides. Fungal spores (200-300) were picked up from 7-10 days old culture with sterilized inoculation needle and mixed in solution of the fraction of different concentrations separately. The slides were placed in moist chambers made by placing two sterile filter papers each on the lid and base of the petriplates. The slides with spores were then incubated at 25±2°C for 24 hr. Germination was observed after staining with cotton blue prepared in lactophenol under binocular microscope (Nikon, Japan Type 102). Spores mixed in sterile distilled water only served as control. All the experiments were conducted in triplicate.

Sample preparation of phenolic compounds

The phenolic acids were extracted as per the method of Singh *et al.* (2002). Three crude extracts of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* were collected from different places of India. One gram of each extract was macerated and suspended in 5 ml methanol-water (80:20; v/v). The collected samples were subjected to ultrasonication (Branson Sonifier, Danbury, CT, USA) for 15 min at 4°C followed by centrifugation at 12,500 x g for 15 min. The clear supernatant was subjected to charcoal treatment. The residue was re-extracted twice with the same extracting solution and the supernatant was pooled prior to evaporation under vacuum (Buchi Rotavapor Re Type, Labco, India; Ambala Cantt. India). Dried extract were resuspended in 1.0 ml high-performance liquid chromatography (HPLC)-grade methanol by

vortexing and filtered through ultra membrane filter (pore size 0.45 µm: Millipore) before HPLC analysis.

HPLC analysis

Quantitative analysis of the sample was performed according to the method of Singh *et al*. (2002). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 µl. The peak area was calculated with a Winchrom integrator. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6 mm i.d., particle size 5 µm, Luna 5µ C-18(2); phenomenex, Torrance, CA, USA) at 25°C. Running conditions included: injection volume, 5µl; mobile phase, methanol: 0.4% acetic acid (80: 20 v/v); flow rate, 1 ml/min; and detection at 290 mm. Samples were filtered through an ultra membrane filter (pore size 0.45 µm; E-Merck, Darmstadt, Germany) prior to injection in the sample loop. Cinnamic acid, Caffeic acid, Ferulic acid, Gallic acid and Tannic acid were used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (R_t) of individual standards and further confirmed by co-injection with isolated standards. The amount of each phenolic acid is expressed as micrograms per gram of fresh weight unless otherwise stated.

Results and discussion

Comparative analysis of antifungal activity

Crude extract of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* were tested against phtopathogenic fungi such as *Alternaria solani*, *Helminthosporium* sp., *Bipolaris* sp., *Curvularia lunata* and *Fusarium* sp. at concentrations of 1000, 2000, 3000, 4000 and 5000 µg/ml. The effects of the different concentrations of crude extracts on five different phytopathogenic fungi are presented in Fig. 1.

The methanolic extract, on the other hand, inhibited growth of the test fungi to varying degrees. A considerable reduction in the sporulation was also recorded. In most of the cases concentrations at 1000, 2000 and 3000 µg/ml brought minimal inhibition against test fungi. The methanolic extract tested at 5000 µg/ml against a number of pathogenic fungi was found effective at higher concentrations. Among the three extract tested, the extract of *Tinispora cordifolia* was found to be most effective and evinced excellent inhibitory

activity against *Helminthosporium* sp. (93.48%) followed by *Acorus calamus* against *Alternaria solani* (89.28%) at the concentration of 5000 µg/ml. At 5000 µg/ml, *Celestrus paniculatus* showed almost similar activity against *Alternaria solani* (79.31%) and *Helminthosporium* sp. (79.49%) followed by *Acorus calamus against Alternaria solani* (79.17%) at 4000 µg/ml. At 5000 µg/ml, all the three crude extracts showed least activity against fungus *Curvularia lunata* and *Fusarium* sp. except *Acorus calamus* that showed antifungal activity of 78.57% against *Curvularia lunata*. It is revealed from the above statement, that higher concentration of the methanolic extract impart maximal antifungal activity.

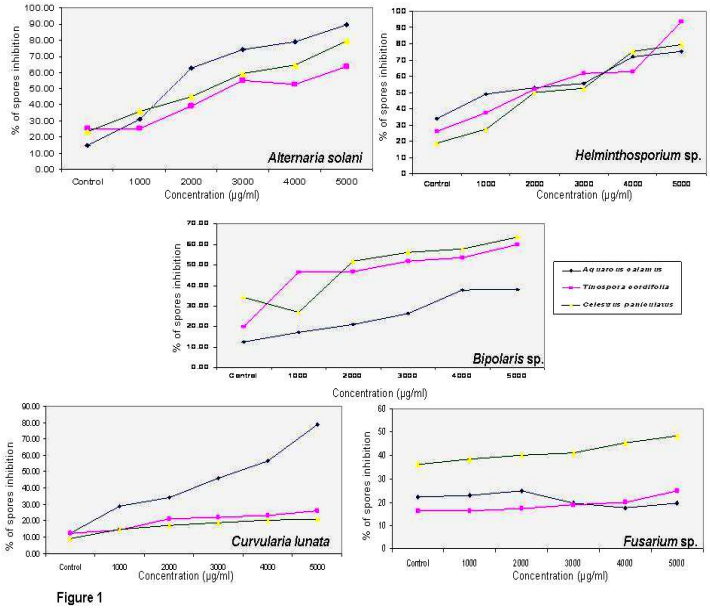

Fig. 1. Antifungal activity of *Acorus calamus, Tinospora cordifolia* and *Celestrus paniculatus* against different phytopathogenic fungi.

Methanol extract of *A. calamus* containing Asarone as a major component showed high antifungal activity against *M. gypseum, T. rubrum* and *P. marneffei* and had moderate activity against *C. albicans* and *C. neoformans* (Phongpaichit *et al*., 2005). Azaron or 1, 2, 4-trimethoxy-5-(1-propenyl) Benzene, isolated form the rhizome extract of *Acorus calamus* showed strong antifungal activity against three phytopathogenic fungi viz., *Macrophomina phaseolina, C. lunata* and *Alternaria alternata* at 400 µg ml^{-1} (Begum *et al*., 2004). A constitutively expressed protein was purified from leaves of *Acorus calamus* exhibited antifungal activity against phytopathogens such as *M. phaseolina, Fusarium moniliforme* and *Trichosporium*

vesiculosum (Ghosh, 2006). *T. cordifolia* exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria (Srinivasan *et al*., 2001). Pre-treatment with *T. cordifolia* was to impart protection against mortality induced by intra-abdominal sepsis following coecal ligation in rats (Singh *et al*., 2003). *Celastrus paniculatus* exhibited anti-fungal activity against six species of fungi (*Trichophyton mentagrophytes*, *T. rubrum*, *T. soudanense*, *Candida albicans*, *Torulopsis glabrata* and *C. krusei* (Vonshak *et al*., 2003). The aqueous extract of *Celastrus paniculatus* seed has cognitive-enhancing properties and an antioxidant effect (Kumar and Gupta, 2002). *T. cordifolia* was tested for their antifungal potential against eight important species of *Aspergillus* such as *A. candidus*, *A. columnaris*, *A. flavipes*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus,* and *A. tamari* (Satish *et al*., 2007).

HPLC analysis

According to Bauer and Tittel (1996) and Springfield *et al*. (2005), they reported HPLC fingerprinting is the best way for chemical characterization, and therefore this study also established HPLC fingerprint for the active phenolic acids that can act as antioxidant, antifungal, antibacterial and anti-inflammatory. The diverse pharmacological activities have been accredited to phenolic acids for instance, gallic acid is reported to be anti-inflammatory (Kroes *et al*., 1992) and antibacterial (Ravn *et al*., 1989), caffeic acid with anti inflammatory.(Fernandez *et al*., 1998) and antibacterial, antifungal (Ravn *et al*., 1989), Ferulic acid with anti-inflammatory (Fernandez *et al*., 1998) and antifungal (Fernandez *et al*., 1998), cinnamic acid with antifungal (Fernandez *et al*., 1998) and antihelmintic (Tawata *et al*., 1996), salicylic acid with antipyretic and anti-inflammatory (Simon and Kerry, 2000), externally used as antiseptic, antifungal and for various skin conditions (Tawata *et al*., 1996). Recent researches indicate that the polyphenols, being secondary metabolites, are present in rich amount in several plants. Many of them possess antioxidant, anti-inflammatory and several others therapeutic properties. Recent researches indicate that phytophenols, being chief secondary metabolites, are present in rich amount in several plants. Many of them posses antioxidant, antiinflammatory and several other therapeutic properties. (Table 1). The HPLC fingerprints (Fig. 2a, 2b and 2c.) of the crude extracts of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* showed six types of the Phenolic acids i.e. Benzoic acid, Cinnamic acid, Caffeic acid, Ferulic acid, Gallic acid and Tannic acid that are present in varying amount (Table 1). Although a primary objective of carrying out HPLC may be to standardize dosage, more information may be obtained during the course of a run, if appropriate detection hardware and software are used.

Table 1. Amount of phenolic acid in the crude extract of *Acorus calamus*, *Tinospora cordiifolia* and *Celestrus paniculatus*.

Crude Extract	Phenolic acid (µg/g dry wt)					
	Benzoic acid	**Caffeic acid**	**Cinnamic acid**	**Ferulic acid**	**Gallic acid**	**Tannic acid**
Acorus calamus	ND	5.90	ND	ND	1432.00	ND
Tinospora cordifolia	ND	85.40	10.46	45.70	ND	5852.00
Celestrus paniculatus	3629.70	ND	ND	ND	393.40	3652.20

The HPLC 'fingerprint' (Figs. 2a., 2b. and 2c.) of the methanolic extract of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* show major peaks at the retention times (min.) of 6.92, 5.73, 3.70, 3.40, 3.10 and 2.58 at a wavelength of 290 nm. Out of the three extracts, *Tinospora cordifolia* showed maximum amount of tannic acid (5,852.10 µg/g) followed by *Celestrus paniculatus* (3,652.20 µg/g). Out of the six different Phenolic acids, Ferulic acid and Cinnamic acid showed amount content of 45.70 µg/g and 10.46 µg/g which are detected only in *Tinospora cordifolia*. *Tinospora cordifolia* also showed maximum amount of Caffeic acid (85.40 µg/g) followed by *Acorus calamus* (5.90 µg/g). *Celestrus paniculata* revealed Benzoic acid (3,629.70 µg/g) in large amount. HPLC analysis of the samples revealed wide-variability in their Phenolic acid content (Fig. 2a). As per my knowledge this is the first report of Phenolic acids viz. Benzoic acid, Cinnamic acid, Caffeic acid, Ferulic acid, Gallic acid and Tannic acid in medicinal crude extracts from *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus*.

In the leaf oils of *A. calamus*, phenolic compounds [(Z)-asarone (15.7–25.5%) and (Z)-methyl isoeugenol (2.0–4.9%)] analysed using GC and GC-MS showed strong inhibitory effect against *Mycobacterium* sp., *Bacillus subtilis*, *Fusarium avenacium* and *Rhizomucor pusillus* (Radusiene *et al*., 2006). Guruchi contains tinosporine, tinosporide, tinosporaside, cordifolide, cordifol, heptacosanol, clerodane furano diterpene, diterpenoid furanolactone tinosporidine, columbin and b-sitosterol. Guduchi has been reported to treat throat cancer in humans (Thatte *et al*., 1992). Azaron or 1, 2, 4-trimethoxy-5-(1-propenyl) Benzene, isolated form the rhizome extract of *Acorus calamus* exhibited antifungal activity against three phytopathogenic fungi viz., *Macrophomina phaseolina* (Maubl) Ash by, *Curvularia lunata* Wakker Boedijn and *Alternaria alternata* (Fr.) Kedissler (Begum *et al.*, 2004). Alkaloids like berberine, palmatine, tembetarine, choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine have been isolated from the non-polar fraction of extracts of stem and roots of *T. cordifolia* (Jagetia and Rao, 2006). A high-performance liquid chromatographic

method was used for the estimation of berberine in the stem of *Tinospora cordifolia* and *Tinospora sinensis* and the concentration of berberine was determined using a C-18 reverse phase column with a mobile phase of acetonitrile:water (10:90 v/v) at a flow rate of 0.6 ml/min and with UV detection at 266 nm. This observation becomes important in the context of the use of *T. sinensis* in place of the genuine drug *T. cordifolia* (Srinivasan *et al*., 2008). A new sesquiterpene polyol ester characterized as 1α,6β,8β-triacetoxy-9β-benzoyloxydihydro-β-agarofuran, along with the three known compounds: 1α,6β,8α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran, angulatueoid C, and 1α,6β,8β,14-tetraacetoxy-9α-benzoyloxydihydro-β-agaro furan , was isolated from methanolic seed extract of *Celastrus paniculatus* and these compounds showed a relaxant effect on the isolated rat ileum (Borbone *et al*., 2007).

The results of the antifungal activity and chemical profiling of the various crude extracts were in agreement with the uses of the extract of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatus* in traditional medicine. The rhizome and aerial parts of the plants appeared to be a potential source of broad spectrum antibiotics.

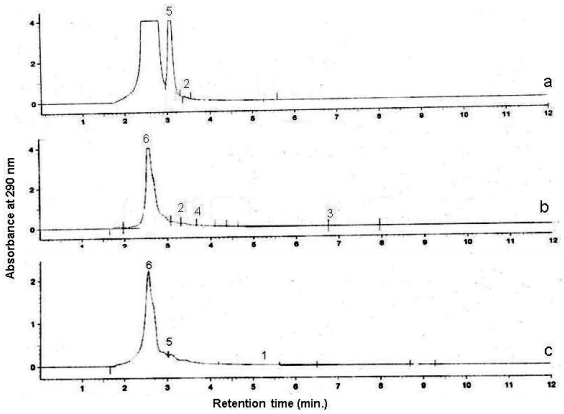

Fig. 2. HPLC analysis of methanolic extracts of a. *Acorus calamus*, *b. Tinospora cordifolia and c. Celestrus paniculatus*, Peak Nos. 1= Benzoic acid, 2=Caffeic acid, 3= Cinnamic acid, 4= Ferulic acid, 5= Gallic acid, 6= tannic acid.

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