RESEARCH ARTICLE

Antifungal Activity of *Iris ensata* against *Trichosporon asahii* Causing Invasive Trichosporonosis

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ABSTRACT- Trichosporon sp. is widely distributed in nature and can predominantly be found in the environmental substrates, such as soil, birds, vegetables, water, and decomposing wood. These fungi can colonize skin and, less frequently, respiratory and gastrointestinal tracts of humans. Currently, the genus Trichosporon sp. is considered one of the most important emerging causes of invasive infection in immune-compromised patients, with *Trichosporon asahii* being the most frequently reported species. In this study, *T. asahii* was procured from MTCC and its sensitivity was checked against different solvents (Methanol, Ethanol, Acetone, and Chloroform) of *Iris ensata*, a medicinal plant. Zone of inhibition and MIC were evaluated using agar well diffusion assay and tube dilution broth assay. Ketoconazole and Nystatin B were used as positive controls. Phytochemical screening was done to determine the phytochemicals present in the plant. The methanol extract was found to be most effective compared to other solvent extracts and positive controls. Phytochemicals play a major role in this anti-Trichosporon activity.

Key-words- Medicinal plant, Phytochemicals test, Trichosporon, Yeast infection

INTRODUCTION

Trichosporon species are soil inhabitants and can be part of the normal flora of the human skin and gastrointestinal tract [1,2]. Invasive infection due to Trichosporon species is rare. However, during the past 2 decades Trichosporon species have emerged as important opportunistic pathogens in immune-compromised individuals [3-8].

Because the traditional classification and nomenclature for Trichosporon species were complicated, a new nomenclature based on molecular techniques has been proposed [1,9-12]. The previously named *T. beigelii*, the main pathogen that causes Trichosporon diseases, refers to 6 species in the new nomenclature (*T. asahii, T. cutaneum, T. inkin, T. asteroides, T. mucoides*, and *T. ovoides*) [1,4]. Another clinically important species, *T. capitatum*, had been referred to as *Blastoschizomyces capitatus* [13]. *T. japonicum*, first isolated from the air in Japan, was recently reported to cause infection in humans [9].

The basidiomycetous yeast, Trichosporon Behrend, are a medically important genus that includes the causative agents of white piedra in immunocompetent hosts and disseminated infections in immunocompromised hosts. Disseminated infection due to Trichosporon species is one of the emerging mycoses in neutropenic patients, particularly when they are treated for haematological malignancy with cytotoxic and immunosuppressive therapy [14,15]. Until recently, most of Trichosporon isolates originating from clinical material were designated as *T. beigelii* or *T. cutaneum*. *T. asahii* and *T. mucoides* are involved in deep seated infections whereas *T. asteroides*, *T. ovoides* and *T. cutaneum* are responsible for white piedra or other superficial infections. The sixth species, *T. inkin*, has been reported in superficial as well as disseminated infections [16]. Recently Moylett *et al.*, 2003 [17] have added *T. pullulans* to the list of emerging pathogenic species of Trichosporon. They reported two cases of *T. pullulans* infection in patients with chronic granulomatous disease and reviewed five additional cases from the literature. It is believed that most cases of trichosporonosis attributed previously to *T. beigelii* and *T. cutaneum* were probably caused by *T. asahii* which is now emerging as an important life threatening opportunistic systemic pathogen, especially in granulocytopenic and immunocompromised hosts [4]. Besides, it has been implicated as a cause of hypersensitivity pneumonitis in Japan [18]. Trichosporonosis is usually an insidious disease and its diagnosis is likely to be missed, particularly in developing countries, because of a general lack of awareness and lack

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of acquaintance with the salient diagnostic features of the etiologic agent. In an earlier study of yeasts and yeast like fungi associated with foodstuffs undertaken in this laboratory in 1978, Misra [19] isolated *T. cutaneum* and *T. pullulans* from milk, meat, butter and coarsely ground gram seeds. He also isolated *T. capitatum* currently classified as *Geotrichum capitatum* from sputum of a patient with bronchopulmonary disorders. In a related experimental study on the pathogenicity of some of this yeast like fungi for cortisone treated mice, Khan *et al*. [20] reported macroscopic and microscopic lesions in their brain, heart, kidney, liver, lung and spleen due to *T. cutaneum* with a mortality of 38%. However, barring a few sporadic case reports, there is no information on the prevalence of disseminated trichosporonosis in India [16, 21-24].

MATERIALS AND METHODS

Collection, identification and extraction methods- Fresh leaves of the *I. ensata* plant was collected from the various regions of Dehradun city, India. The taxonomic identity of the plants was confirmed by Department of Botany of Forest Research Institute, Dehradun, India in the duration of 2010. Leaves were washed under running tap water, air dried and then homogenized to a fine powder and stored in air tight bottles. The air-dried and powdered plant material (100 g) was extracted with 200 ml of each solvent (Methanol, ethanol, acetone and chloroform), kept on a rotary shaker for 24 hrs. Thereafter it was filtered and centrifuged at 5000xg for 15 min. The supernatant was collected and evaporated to dryness to give the crude dried extract.

Fungal cultures- The test fungal species *T. asahii* was procured from MTCC, Chandigarh (MTCC No. 6179). It was maintained on selective media Yeast Malt Agar slants and plates at an optimum temperature of 25^0C and experiments were carried out on Sabouraud Dextrose Agar media.

Antifungal assays

Agar well diffusion assay- Preliminary analysis of antifungal activity was conducted using Agar Well Diffusion Assay [25]. The fungal inoculum was prepared in saline solution and incubated for 1 hour. Molten Sabouraud Dextrose Agar (SDA) was added with 1 ml of fungal inoculum into pre-sterilized petri plates. After solidification, wells of 6 mm diameter were punctured in the culture medium using sterile cork borer. A fixed volume (100 µl) of respective crude extract prepared in 5% Dimethyl Sulphoxide (DMSO) was loaded in the well using sterilized micropipettes. Plates were incubated for 2 days at 25^0C and zone of inhibition (in mm) of different extracts was determined after 48 hrs. Sterile 5% aqueous DMSO was used as negative control while Ketoconazole and Nystatin B were used as the positive control. All experiments were carried out in triplicates.

Tube dilution broth assay- MIC of the *I. ensata* plant extract against the test fungi i.e. *T. asahii* was determined using the Broth Dilution Method [26]. Various concentrations (600–1.55 mg/ml) of the extracts were prepared by dissolving extracts in 5 % DMSO. One ml of the plant extract (100 mg/ml) was added to 1 ml of Sabouraud Dextrose broth in test tubes and subsequent concentrations were prepared by using serial dilution technique. 100 µl fungal culture prepared in saline water was inoculated into each test tube and mixed thoroughly on a vortex mixer. The test tubes were then incubated at 25°C for 2 days. The MIC values were determined microscopically after 48 hrs of incubation in comparison with the growth and sterility controls. Ketoconazole and Nystatin B were used as positive controls. All the experiments were performed in triplicate.

Phytochemical screening of the plant extracts- The Phytochemical components of the medicinal plant was screened using the following methods [27,28].

Saponins- 25 g each of the powdered samples were boiled in 25 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion.

Steroids- Two ml of acetic anhydride was added to 0.5 g ethanol extract of each sample with the addition of 2 ml H_2SO_4. A color change from violet to blue or green indicates the presence of steroids.

Flavonoids- A portion of the powdered plant samples were separately heated with 10 ml of ethyl acetate in a water bath for 3 min. The mixtures were filtered and 4 ml of each filtrate was shaken with 1 ml of dilute ammonia solution. A yellow color observation indicates the presence of flavonoids.

Tannins- 0.5 g of each powdered samples were boiled in 20 ml of water in a test tube and then filtered. Few drops of 0.1 % ferric chloride were added and observed for brownish green or blue-black color.

Total Phenol- Total 2 g each of the samples was defatted with 1 ml of diethyl ether using a soxhlet apparatus for 2 hrs. The fat free samples were boiled with 50 ml of ether for the extraction of the phenolic components for 15 minutes. 5 ml of the extracts were pipetted into 50 ml flask and then 10 ml distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The *Iris ensata* were made up to mark and left to react for 30 minutes for color development.

Alkaloid- Five g of each sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 48 hrs. After filtration, the extracts were concentrated on a water bath to ¼ of the original volume.

Concentrated ammonium hydroxide was added in drops to the extract until the precipitation was collected, washed with dilute ammonium hydroxide and then filtered. The residue obtained is the alkaloid and was dried and weighed.

Terpenoids- To 0.5 g of extract was added 2 ml of chloroform. 3 ml of concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. A reddish-brown coloration of the interface indicated the presence of terpenoids.

Statistical Analysis- The inhibitory zones of plant extracts were expressed as the Mean±Standard deviation at $P<0.05$.

RESULTS AND DISCUSSION

Table 1 clearly shows that methanol extract of the plant was more effective as compared to other solvents as it inhibited the growth of *T. asahii* with maximum zone of inhibition (35 mm) and with minimum concentration i.e. MIC at 6.2 mg/ml. The solvents exhibited the overall effectiveness in the order Methanol > Ethanol > Acetone > Chloroform. Whereas positive controls Ketoconazole and Nystatin B showed inhibition zones of 25 mm and 22 mm respectively and MIC was found to be 50 mg/ml and 200 mg/ml.

Table 1: Antifungal activity of four extracts of *Iris ensata* Thunb. against *Trichosporon asahii*

Measurement of antifungal activity	**Solvent extracts (100mg/ml)**				**Positive controls**	
Zone of inhibition (mm)	35±0.5	20±0.1	16±0.5	18±0.5	25±0.0	22±0.0
Minimum Inhibitory concentration (mg/ml)	6.2±0.5	100±0.6	200±0.5	100±0.5	50±0.1	200±0.1

M- Methanol; E- Ethanol; Ch- Chloroform; A- Acetone; Ke- Ketoconazole; Ny- Nystatin

Phytochemical screening of the plant extracts revealed the presence of certain phyto-constituents like alkaloids, steroids, flavonoids, phenols, glycosides, tannins etc (Table 2).

Table 2: Phytochemical screening of the extracts of *I. ensata* Linn

I. ensata	**Phenols**	**Terpenoids**	**Alkaloids**	**Flavonoids**	**Steroids**	**Tannins**	**Saponins**
Methanol	+	-	+	+	-	+	+
Ethanol	+	-	+	+	-	+	+
Chloroform	-	+	-	-	+	-	-
Acetone	-	-	+	+	+	+	-

To our knowledge, this study appeared to be the first one to work against *T. asahii* with the help of extracts of *I. ensata*. Infections with Trichosporon species have been recognized with increasing frequency over the last two decades [29]. They can be found as commensally in the human gut or skin flora and in the environment. These non-Candida yeasts have a broad spectrum of clinical manifestations from self-limiting cutaneous infections to life-threatening invasive disease in the immune-compromised host [30,31]. There is no clear antifungal agent of choice for the treatment of trichosporonosis and *in vitro* sensitivity assays have not been standardized. There were data to support the use of triazoles (fluconazole, voriconazole) and some authors had recommended combination therapy with Amphotericin B. [31].

In this study, the plant extract of *I. ensata* in different solvents had been used and found effective against *T. asahii*. Methanol extract of the plant was found to be most effective with a zone of inhibition 35 mm and MIC at 6.2 mg/ml followed by ethanol with zone of inhibition 20 mm and MIC at 100 mg/ml. Methanol extract was found to be more effective compared to positive control Ketoconazole and Nystatin. Earlier reports also revealed the antimicrobial activity of *I. ensata* [32,33].

CONCLUSIONS

As discussed above, *Iris ensata* plant extract can be used to treat Invasive Trichosporonosis in patients suffering from it. The study also suggests the use of plants in treating Trichosporon infections in India as no reports of medicinal plants against Trichosnosporosis has been published yet. *I. ensata* is a medicinal plant previously

used to treat various infections and now from this study, we can conclude that it can also be used to treat Trichosporonosis.

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