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Antifungal activity of *Swertia ciliata* (Family-Gentianaceae), *Acorus calamus* (Family-Araceae) and *Viola serpens* (Family Violaceae) from Pithoragarh, Uttarakhand Himalayas, India

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Abstract
Plant essential oils are potential source of antimicrobials of natural origin. Essential oils and extracts obtained from many plants have recently gained a great popularity and scientific interest. Consumer demand for natural preservatives has increased, whereas the safety aspect of chemical additives has been questioned. The plant oil has been reported to have antibacterial, antifungal, antiviral, antiparasitic and antidermatophytic properties. Thus, this study aimed to investigate the antifungal activity of three essential oils (EOs), against *Picher guilliermondii* and *Candida albicans* strains. Three medicinally important plants *Swertia ciliata*, *Acorus calamus* and *Viola serpens* were evaluated for their antifungal activities. The oil of *Swertia ciliata* had moderate to good activity against the tested *Candida albicans* pathogens and the oil of *Acorus calamus* and *Viola serpens* show good activity against *Pichia guilliermondii* on the basis of zone of inhibition and MIC values. These results support the plant oils can be used to cure mycotic infections and plant oils may have role as pharmaceutical and preservatives.

Keywords: Antimicrobial, antifungal, antiviral and *Swertia ciliate, Viola serpens*

Introduction
Essential oils are volatile aromatic concentrated hydrophobic oily liquids which are obtained from various plant parts such as flowers, buds, seeds, leaves, twigs, bark, woods, fruits and roots. Essential oils are usually terpenoids responsible for the aroma and flavor associated with herbs, spices and perfumes, also called volatile oils because they easily diffuse into the air. The main constituents of essential oils are mono and sesquiterpenes including carbohydrates, phenols, alcohols, ethers, aldehydes and ketones responsible for the biological activity as well as for their fragrance. Phenolic compounds present in essential oils have also been recognized as antimicrobial bioactive components (Sumonrat *et al*., 2008) [17]. Various plant materials are believed to have antifungal activity and many essential oils have been reported to have antifungal activities with no side effects on humans and animals (Sokmen *et al*., 1999) [16].
Previous *in vitro* and *in vivo* investigations suggested that the essential oils could be used as effective antifungal agents (Adam *et al*., 1998) [1]. The selection of plants for evaluation was based on traditional usage for treatment of infectious diseases (Janssen *et al*., 1986, Panizzi *et al*., 1993 and Crespo *et al*., 1990) [9, 14, 6]. However, there are only limited data available on the antifungal activity of essential oils against human and plant fungal pathogens. Fungal species of the genera Aspergillus, Fusarium and Alternaria have been considered to be major plant pathogens Worldwide (Ghafoor and Khan 1976) [7]. Millions of people throughout the world are affected by superficial fungal infections, which are the most common skin diseases. These infections, which occur in both healthy and immunocompromised persons, are caused mainly by dermatophytes. Increasing social and health implications caused by dermatophytes means there is a constant striving to develop safe and new natural antifungal agents to cure human fungal disorders caused by dermatophytes. Many skin diseases such as, tinea and ringworm caused by dermatophytes are existing in tropical and semitropical areas. In general, these fungi live in the dead and top layer of skin cells of moist areas of the body and cause only a minor irritation. Other types of fungal infections could be more serious. They can penetrate into the cells and cause itching, swelling, blistering and scaling (Ping *et al*., 2007) [15].

Essential oils and plant extracts have long been known and used throughout the world for the treatment of many conditions, including skin conditions, and have less deleterious side effects than corresponding synthetic drugs (Tavares *et al*., 2008) [18].

Essential oils are complex mixers comprising many single compounds. Chemically they are derived from terpenes and their oxygenated compounds. Each of these constituents has been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties. (Burt 2004 and Kordali *et al*., 2005) [4, 13]. The antimicrobial activity of different essential oils is known for many centuries. Large number of essential oils and their constituents were investigated for their antimicrobial properties against different bacteria and fungi strains (Bakkali *et al*., 2008, Kalemba and Kunicka 2003) [3, 11]. There are 2600 plant species of which more than 700 are noted for their uses as medicinal herbs (Ali-Shtayeh *et al*., 1999) [2]. In folk medicine, medicinal herbs and plant products were commonly used for the treatment of infectious disease. Generally, the fungal infections are the most common cause of many skin diseases in developing countries (Ping *et al*., 2007) [15].

Fungus is a eukaryotic organism that digests its food externally and absorbs the nutrient molecules into its cells. Fungal infections remain a significant cause of disease. *Cryptococcus neoformans* is the cause of the most common life-threatening meningitis in HIV-positive patients. Candida is one of the non-albicans strains currently emerging in fungal infections. To overcome these alarming problem researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections. Traditional medicines play important role in health services around the globe. About three quarter of the world's population relies on plants and plant extracts for healthcare.

The increasing resistance to antifungal compounds and the reduced number of available drugs led us to search for the new alternatives among aromatic plants and their essential oils, used for their antifungal properties. The antifungal activity can be attributed to the presence of some components such as carvacrol, α-terpinly acetate, cymene, thymol, pinene, linalool which are already known to exhibit antimicrobial activity (Knobloch *et al*., 1985, Juven *et al*., 1994, Harborne *et al*., 1995 and Cimanga *et al*., 2002) [12, 10, 8, 5].

Keeping in view importance of natural sources especially plants, this study was conducted to evaluate antibacterial potential of three important medicinal plants *Swertia ciliata*, Acorus calamus and *Viola serpens*. For antifungal analysis, tube dilution method was adopted.

Materials and Methods

Plant materials

The plant *V. serpens* was collected in the month of October, 2013 from Shama (Kapkote) 52 km away from Bageshwar, Uttarakhand, India. The plant *S. ciliata* (D.Don ex G.Don) was collected in the month of September, 2013 from Munsiyari (an elevation of 2298 meters) 135 km away from Pithoragarh, Uttarakhand, India. The plant *A. calamus* was collected in the month of September, 2013 from Bishar (Pithoragarh) 10 km away from Pithoragarh Discrict, Uttarakhand, India. All three plants were authenticated by Botanical Survey of India (BSI), Dehradun. A voucher specimen (No.114835) of *V. serpens*, voucher specimen (No.114851) of *S. ciliata* and voucher specimen (No.114851) of *A. calamus* deposited in the Herbarium Section at BSI, Dehradun, India.

Extraction of Essential Oils

The plant materials (4 Kg) were subjected to hydro distillation using a Clevenger-type apparatus for 7 h. The collected essential oils were dried with anhydrous sodium sulphate, filtered and stored at 4-6 ºC in the dark.

Fungal Strain Used For the Test

Microorganisms were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India, as Microbial Type Culture Collection (MTCC). All microbial strains were cultured and maintained in Microbiology Laboratory, Department of Biotechnology, Bhimtal Campus, Kumaun University, Nainital, India. Two fungal strain *Pichia guilliermondii* (MTCC4052) and *Candida albicans* (MTCC227) were used.

1. *Picher guilliermondii* (MTCC4052)
2. *Candida albicans* (MTCC227)

Pichia Guilliermondii

Pichia guilliermondii is a species of yeast of the genus *Pichia* whose asexual or anamorphic form is known as *Candida guilliermondii*. *Candida guilliermondii* colonies are flat, moist, smooth, and cream to yellow in color on Sabouraud dextrose agar. It does not grow on the surface when inoculated into Sabouraud broth.

Candida Guilliermondii

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide. It has been isolated from numerous human infections, mostly of cutaneous origin, if only from immunesuppresed patients. *C. guilliermondii* has also been isolated from normal skin and in sea water, faeces of animals, wasps, buttermilk, leather, fish and beer.

Antifungal Activity

Screening essential oil for antifungal activity was done by the well diffusion method which is normally used as preliminary check for antimicrobial efficiency of essential oil. The organisms were cultured in nutrient broth (bacterial strains) and malt yeast broth (fungal strains) and the tests were carried out on Mueller Hinton agar and Potato Dextrose agar plates respectively. The inoculums of the microbial strains were prepared from 24 h broth cultures, the cultures were adjusted to 10^6 CFU/ml with sterile water. The different concentration range from 5µl/ml to 1000 µl/ml of essential oil was prepared by dissolving them in DMSO. Muller Hinton agar and Potato Dextrose agar *was* poured into petriplates. After solidification, 100µL of test strains were spread on the media plates separately. Care was to taken to ensure proper homogenization. The experiment was performed under strict aseptic conditions. After the inoculation, a well was made in the plates with sterile borer (3 mm). The oil sample (30µL/well) of different concentrations (5µl/ml to 100/ml µl) were introduced into the well and plates were incubated at 37°C for 24 hrs. Microbial growth was determined by measuring the zone of inhibition. Nystatin (30µg/ml) for fungal strain was used as positive control. DMSO was used as negative control. Experiments were carried out in triplicate. Inhibition of bacterial growth in the plates containing test oil was analyzed by its comparison with growth in blank control plates. The MICs were determined as the lowest concentration of oil inhibiting visible growth of each organism on the agar plate.

Statistical Analysis

All the measurements were done in triplicate and the statistical analysis was performed by Microsoft Excel 2007. Concentration providing 50% inhibition (IC_{50}) was calculated from the graph of percentage inhibition against oil concentrations by a linear regression using SPSS software.

Results and Discussion

Antifungal activity of essential oil of *Swertia ciliata*

The essential oil of *Swetia ciliata* was screened for antifungal activity against to standard strains of fungal. The results of *in vitro* test (Table 1 and Fig.1) and showed that the oil had moderate to good activity against the tested *Candida albicans* pathogens on the basis of zone of inhibition and MIC values. To the best of our knowledge, this is the first report on the antifungal activity of the essential oil of *Swertia ciliata* collected from Munsiyari, District-Pithoragarh, India.

Table 1: Antifungal activity of the essential oils of *Swertia ciliata* aerial part.

S. No	Fungal strain	MTCC Code	MIC µL/m	*Swertia ciliata* ZI (mm)	Standard Nystatin ZI (mm)
1	*Pichia guilliermondii*	4052	250	14.21	23.3
2	*Candida albicans*	227	250	16.19	21.3

*MTCC- Microbial Type Culture Collection
*MIC- Minimum inhibitory concentration
*ZI- Zone of inhibition

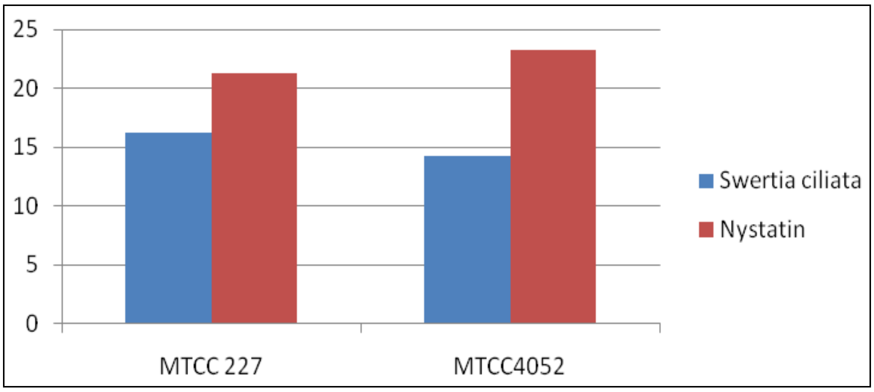

Fig 1: Comparative antifungal activity of *Swertia ciliata* with respect to Nystatin.

Antifungal activity of essential oil of *Acorus calamus*

The essential oil of *Acorus calamus* was screened for antifungal activity against to standard strains of fungal. The results of *in vitro* test (Table.2 and Fig.2) showed that the oil had moderate to good activity against the tested *Pichia guilliermondii* pathogens on the basis of zone of inhibition and MIC values.

Table 2: Antifungal activity of the essential oils of *Acorus calamus* aerial part.

S. No	Fungal strain	MTCC Code	MIC all/m	*Acorus calamus* ZI (mm)	Standard Nystatin ZI (mm)
1	*Pichia guilliermondii*	4052	250	14.12	23.3
2	*Candida albicans*	227	250	11.25	21.3

*MTCC- Microbial Type Culture Collection
*MIC- Minimum inhibitory concentration
*ZI- Zone of inhibition

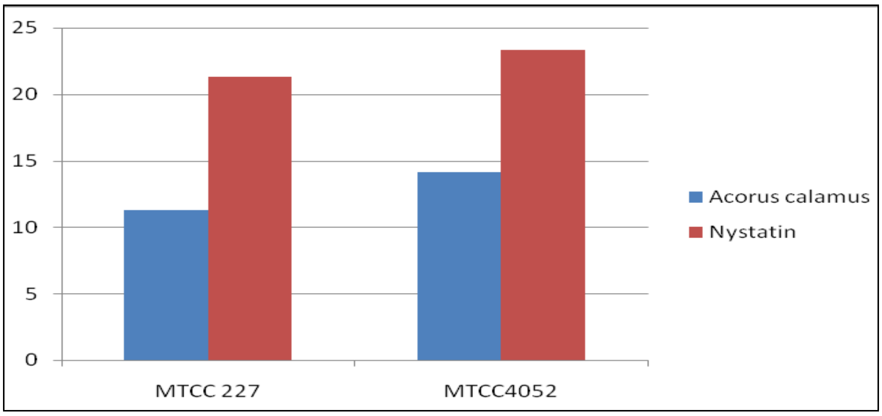

Fig 2: Comparative antifungal activity of *Acorus calamus* with respect to Nystatin

Antifungal activity of essential oil of *Viola serpens*

The essential oil of *Viola serpens* was screened for antifungal activity against to standard strains of fungal. The results of *in vitro* test (Table 3 and Fig.3) showed that the oil had moderate to good activity against the tested *Pichia guilliermondii* pathogens on the basis of zone of inhibition and MIC values.

Table 3: Antifungal activity of the essential oils of *Viola serpens*

S. No	Fungal strain	MTCC Code	MIC µL/m	*Viola serpens* ZI (mm)	Standard Nystatin ZI (mm)
1	*Pichia guilliermondii*	4052	250	17.32	23.3
2	*Candida albicans*	227	250	14.29	21.3

*MTCC- Microbial Type Culture Collection
*MIC- Minimum inhibitory concentration
*ZI- Zone of inhibition

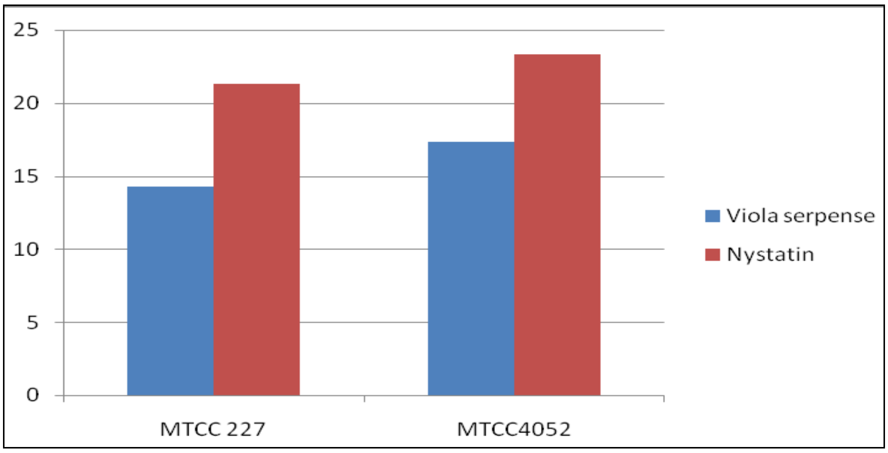

Fig 3: Comparative antifungal activity of *Viola serpense* with respect to Nystatin

Conclusion

Essential oils are naturally occurring phytochemicals which have various applications and have long been known and used throughout the world for treatment of many diseases, and there is at least some evidence that natural products such as essential oil and extracts may tend to have less deleterious side effects than corresponding synthetic drugs. Also, the resurgence of interest in natural control of human infectious fungal pathogens and increasing demand for effective, safe, natural products, that quantitative data on plant oils and extracts are required and could lead to a new antifungal agent, which could support the use of the plant to treat various infective diseases. However, if plant oils are to be used for preservation or medicinal purposes issues of safety and toxicity will need to be addressed. This obviously justifies the use of above mixed oils in traditional medicine to cure mycotic infections in tuberculosis patients.

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