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Studies on Phylloplane Mycoflora of Some Medicinal Plants

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ABSTRACT

The variety and galaxy of fungi and their natural beauty occupy prime place in the biological world. The phylloplane the surface of plant leaves is a complex terrestrial habitat that is characterized by a variety of microorganisms including bacteria, filamentous fungi and yeasts. Phylloplane fungi are the mycota growing on surface of the leaves. Some phylloplane fungi are investigated from the leaves surface of five medicinal plants such as *Ocimum sanctum, Ficus bengalensis, Datura metel, Butea monosperma* and *Stevia rebaudiana.* A total number of 44 fungal species from 28 genera were isolated from surface sterilized leaf segments by dilution plating method. Among them 04 species and 03 genera were isolated from the phylloplane of *Ocimum sanctum*, 17 species and 07 genera from *Butea monosperma*, 03 species and 03 genera from *Datura metel*, 05 species and 05 genera from *Ficus bengalensis* and 15 species and 10 genera were isolated from phylloplane of leaves surfaces.

Keywords: Phylloplane, Terrestrial habitat, Mycota, Investigation, Medicinal plants

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INTRODUCTION

Phylloplane is a natural habitat of various microorganisms including bacteria, fungi, yeasts saprobe and epiphyte etc on the surface of leaf. However, quality and quantity of microorganisms on the leaf surface differ with age of leaf, leaf area, morphology and atmospheric factors such as temperature and humidity. The leaf surface being very rich in nutrients offer a suitable substratum for the colonization of various microorganisms both the parasites and saprophytes. The nonpathogenic fungi that inhabit the phyllosphere depend on nutrients exuded from leaf surface or those deposited from the atmosphere[1]. In addition to nutrient level, growth and abundance of phylloplane fungi are also influence by environmental conditions such as temperature, humidity, light intensity, wind speed, UV radiation and presence of air pollutants[2]. The size, density of hairs and sculpturs of leaf surface seem to be most reliable factors of fungal biodiversity on the studies plant species. Amongst the different microorganisms, two groups of phylloplane fungi i.e. residents and casual are generally present on leaf surface. The effect of certain traces elements on phylloplane microflora, virus multiplication and mutual interaction between virus and have been studied. Phylloplane fungi have been poorly studied as compared to endophytes, saprobes and pathogenic. Medicinal plants have been used up by human being for remedies of different types of diseases because plants contain components of therapeutic value[3]. Different parts of *Taraxacum officinale* are traditionally used for treatment of ailments including gallstone, joint pain, muscle ache, eczema, loss of appetite, intestinal gas. It is also used as skin toner, blood tonic and digestive tonic. Some people use *T. officinale* to treat infection, especially viral infection and cancer. Phytochemicals are natural bioactive compounds found in plants such as vegetables, fruits, flowers, leaves and that work with nutrients and fibers to act defense system which protect against disease. Phytochemicals are divided into two groups, which are primary and secondary constituents, according to their function in plant metabolism. Primary constituents are common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids, phenolic compounds[4] and many more such as flavonoids, saponins, tannins and so on. Following plants are selected for investigation because these plants are available easily anywhere in nature and in abundance .some of them are utilized by human being as a traditional medicine in ancient time. By studying the presence of phytochemical in these plants, uses of these plants can be explained scientifically in respect to their traditional treatment.

- *Ocimum sanctum*
- *Butea monosperma*
- *Datura metel*
- *Ficus bengalensis*
- *Stevia rebaurdiana*

Ficus bengalensis Linn is commonly called Banyan tree. This tree is considered to be sacred in some places. It is used in traditional system of medicine like Ayurvedic, Siddha, Unani, and Homoeopathy. The leaves of *F. bengalensis* are used as ulser protective, leprosy, fever and inflammations (Ayurvedic). The milky juice is aphrodisiac, tonic, vulnerary, maturant also useful in piles, diseases of nose, gonorrhea. As analgesic, antipyretic, anti-alserogenic, inflammatory bowel, antimicrobial, antidiabetic etc. The various chemical constituents present *in F. bengalensis* are Bengalenosides, flavonoids and leucocyanidin glycoside. *Datura metel* is a perennial herbaceous erect shrub. *D. metel* contains tropane alkaloids and are used as sedatives, anti-spasmodic and mydriatic agents[5]. The whole plant, but especially the leaves, has anesthetic, hallucinogenic, antiasthmatic, anti-spasmodic, anti-tussive and bronchodilator effects. Leaves are used as a local application for rheumatic swelling of the joints, lumbago, Scitica, neuralgia, scabies, eczema, allergy and glandular inflammations such as mumps; used externally for earache and smoked to relieve spasmodic asthma. *Butea monosperma* popularily known as Flame of forest, Dhak, palash, or "Bastard teak" which has immense potential and appears to have a broad spectrum activity on several ailments. It is extensively used in Ayurveda, Unani, and Homoeopathy medicine and has become a cynosure of modern medicine. Commonly B. monosperma is used as astringent, aphrodisiac and diuretics[6]. A hot poultice of leaves relieves boils, pimples, skin ulcers, swelling and bleedin piles. The juice of leaves can be used as an enema. A decoction of the leaves treats leucorrhea and diabetes. The leaves are good for eye diseases. *Stevia rebaurdiana* (Bertoni) is a shrub and recently cultivated in India. The main sweet component in S. rebaurdiana is stevioside[7]. The leaf of S. rebaurdiana mainly contains tannins, and alkaloids followed by cardiac glycosides, saponins, sterols, and triterpenes, reducing compounds and anthraquinones. These secondary plant products contribute to its medicinal value as well as exhibiting physiological activity[8]. Tannins have spasmolytic activity in smooth muscle cells[9]. Saponins inhibit Na+ efflux, by the blockage of the entrance of the Na+ out of the cell. This leads to higher Na+ antiporter producing elevated cytosolic Ca+ which strengthens the contractions of heart muscle and thereby reducing congestive heart failure[10]. Though, the stevia leaves contain very low amount of oil, it could not be use for oil extraction , but looking to the fatty acid composition, stevia leaf oil is a rich source of linolenic acid. This high value of linolenic acid may be helpful to maintain ideal fatty acid ratio in human diet.

MATERIALS AND METHODS

Material

The fresh leaves of plants which are taken in this investigation were collected from the nearby area of Kalapipal Mandi distt Shajapur (M.P.) India and brought to the laboratory immediately in separate sterile plastic bags.

Method

Sample collection

Composition of potato dextrose agar medium.

Potato (peeled) - 250 gm

Dextrose - 15 gm

Agar - 18 gm

Distilled water - 1000 ml

Preparation of potato dextrose agar medium

The healthy potato tubers were peeled and weighed for about 250gm and chopped into small pieces with the help of sterile knife. These chopped potatoes were transferred into a conical flask containing about 1000 ml of distilled water. The content was boiled for 20 min. The supernatant were decanted and filter by muslin cloth and filtrate collected. Dextrose (15gm) and agar (18gm) were transferred into the extract and shake well to dissolve the ingredients. The medium was made up to 1 liter by addition of distilled water. The pH of medium adjusted to 5.6 final, the medium was cotton plugged and put into autoclave at 121^0C for 15 min.

Isolation of fungi

Leaf samples of *Ocimum sanctum, Butea monosperma, Datura metel, Ficus bengalensis* and *stevia rebaurdiana* were collected and put into sterile plastic bags and brought to laboratory. A fragment of 1cm of leaf blade was cut from basal part and shaken in flask with 200ml of distilled water. By this we get a suspension of microorganisms and 0.2ml suspension was transferred into patriplates containing PDA medium with streptomycin. The inoculum spread uniformly and kept in a dust free chamber for 5-7 days at room temperature. The fungal colonies were appeared and observed pure cultures were maintained carefully.

Lacto phenol cotton blue mounting

Some part of the mycelium of the representative colonies was picked up with the help of a pair of sterile needles and slides were prepared using Lacto phenol cotton blue (20g-phenol (crystal), 20g lactic acid; 40g glycerine; 20ml water; few drops of cotton blue). For releasing of air bubbles if any present inside the cover slip the slide was heated gently with sprit lamp. The excess stain was removed by using tissue paper and cover glass was sealed by white nail paint.

Identification and photography:

As the dilution plating method yield facultative phylloplane fungi were identified by referring standard mannuals. The manual of soil fungi[11]and dematiaceous Hyphomycetesand more Dematiaceous Hyphomycetes [12]. The photographs were taken using Nikon microscope.

Qualitative phytochemical tests

Phytochemical examinations were carried out for all the extracts as per the standard methods.

1. **Detection of alkaloids:** Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

4. Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

5. Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

6. Detection of phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Libermann Burchard's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols. **7. Detection of phenols**

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

8. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

9. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

10. Detection of proteins and amino acids

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

11. Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes[13-15].

RESULTS

Biodiversity of phylloplane fungi

A total number of 44 fungal species were isolated from surface sterilized segments of five important medicinal plants such as *Ocimum sancum, Ficus bengalensis, Datura metel, Butea monosperma, and Stevia rebaurdiana* by dilution plating method. Among them Aspergillus flavus, Penicilium expansum, Fusarium semitectum, and Fusarium oxysporum were isolated from the phylloplane of Ocimum *sanctum. Fusarium, Aspergillus niger, Chaetomium, Aureobasidium, and Cunninghumella sp.* was isolated from *Ficus bengalensis*. *Collectotricum sp, Pseudosperma sp and Trichoderma viride pers*. isolated from Datura metel. *Alternaria alternata, A. tenuissimia, Aspergillus candida, A. flavus, A.fumigatus, A. niger, A. terreus, Cladosporium cladosporiodes, C. herbarum, Curvularia lunata, Fusarium equiseti, Penicillium citrinum, P.cyclopium, P.purpurogenum, Rhizopus oryzae, Trichoderma koningii* and White sterile form isolated from the phylloplane of Butea monosperma. *Alternaria tenuissima , Aspergillus flavus, A. fumigatus, A.niger, A.ochraceus, Cladosporium cladosporoides, Mucor hiemalis, Penicillium cyclopium, P.notatum, P. perpurogonum, Periconiella sp., Scytalidium lignicola, Sporobolomyces roseus, Trichoderma hamata, and strachybotrys chartarum was isolated from Stevia rebaurdiana Bertoni.* The present investigation done on different medicinal plant sample based on the presence and absence of the medicinally active phytochemical constituents. Alkaloids, Flavonoids and Saponins are present in all samples which are taken in this investigation. Cardiac glycosides are absent in *F. bengalensis* and present in *O. sanctum, D .metel, B. monosperma* and *S. rebaurdiana. Tannins are present in Ocimum sanctum, Ficus bengalensis and Stevia rebaurdiana* but not present in *Datura metel and Datura metel.* Terpenois are present in *Ocimum sanctum, Datura metel* and *Stevia rebaurdiana* but absent in *Ficus bengalensis* and *Butea monosperma.* Steroids are found in *Ficus bengalensis, Datura metel* and *Steviarebaurdiana* but not found in *Ocimum sanctum* and *Butea monosperma.* (Table 2).

Table 1 Fungi isolated from the phylloplane of medicinal plants.

S.No.	Medicinal plants	Fungal species
1.	***Ocimum sanctum***	Aspergillus flavus , Penicilium expansum, Fusarium semitectum, and Fusarium oxysporum
2.	***Ficus bengalensis***	*Fusarium, Aspergillus niger, Chaetomium, Aureobasidium, and Cunninghumella sp*
3.	***Datura metel***	*Collectotricum sp, Pseudosperma sp and Trichoderma viride pers*
4.	***Butea monosperma***	*Alternaria alternata, A. tenuissimia, Aspergillus candida, A. flavus, A.fumigatus, A. niger, A. terreus, Cladosporium cladosporiodes, C. herbarum, Curvularia lunata, Fusarium equiseti, Penicillium citrinum, P.cyclopium, P.purpurogenum, Rhizopus oryzae, Trichoderma koningii* and White sterile form
5.	***Stevia rebaurdiana***	*Alternaria tenuissima , Aspergillus flavus, A. fumigatus, A.niger, A.ochraceus, Cladosporium cladosporoides, Mucor hiemalis, Penicillium cyclopium, P.notatum, P. perpurogonum, Periconiella sp., Scytalidium lignicola, Sporobolomyces roseus, Trichoderma hamata, and strachybotrys chartarum*

Table 2 preliminary phytochemical screening of five medicinal plants

S.No.	*phytochemical constituents*	*Ocimum sanctum*	*Ficus bengalensis*	*Datura metel*	*Butea monosperma*	*Stevia rebaurdiana*
1.	Alkaloids	+	+	+	+	+
2.	Cardiac glycosides	+	-	+	+	+
3.	Flavonoids	+	+	+	+	+
4.	Tannins	+	+	-	-	+
5.	Terpenoids	+	-	+	-	+
6.	Saponins	+	+	+	+	+
7.	Steroids	-	+	+	-	+

+ = Present, **-** = Absent

DISCUSSION AND CONCLUSION

Fungi are extraordinary organisms which are neither plants nor animals, they are also not bacteria. These are one of the important organisms on this planet. They are all show striking diversity in morphology and ecology. There are about 1000000 species of fungi out of them only 400 cause diseases relevant to human, animals and plants. Fungi are some of the world's largest and possibly oldest individuals and are silent killers with deadly poisonous also. Fungi play a significant role in the daily life of the human being besides there utilization in industries, agriculture, medicine, food industries, textiles, bioremediation and many other ways fungal diversity has became an integral part of human welfare[16]. Finally, fungi can be helpful, but they all are important and required in life. Fungi are one of the earth's big recycler without them we could not live. In the present investigation, totally 44 species of fungi were isolated from the phylloplane of medicinal plants by dilution plating technique. They were mention above Table 1. In this investigation fungi show great diversity with these medicinal plants. the diversity of fungi may depends on the methods used in collecting and handling leaf samples, size of leaf fragments and methods used in culture. The frequency of fungal species also differed significantly between the wet and dry season. The number and frequency of colonization were low in dry season but greater in wet season. The greater rainfall in the wet season could promote the dispersal of fungal spores [16]. The number of fungal colonies and the percentage cover of phylloplane fungi can be estimated by direct observation using light microscopy[17]. The leaf washing method cannot provide a quantitative description of the species richness of the phylloplane fungi as leaf washing only indicate the amount of propagules, but not the fungal mass. Thus the leaf washing method is suitable for qualitative studies only [18].

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