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INFLUENCE OF FUNGAL DISEASES ON PHYTOCHEMICAL COMPOSITION OF SELECTED MEDICINAL PLANTS WITH SPECIAL REFERENCE TO SECONDARY METABOLITES

G. E. Mallikarjuna Swamy
P. Sujanapal
R. Jayaraj

Kerala Forest Research Institute
(An Institution of Kerala State Council for Science, Technology and Environment)
Peechi – 680 653 Thrissur, Kerala

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BRIEF DETAILS OF THE PROJECT PROPOSAL

Project No.	:	KFRI RP 650/2012
Title of Project	:	**Influence of Fungal Diseases on Phytochemical Composition of Selected Medicinal Plants with Special Reference to Secondary Metabolites**
Objectives	:	1. To undertake a survey of diseases due to fungal pathogens on selected species of medicinal plants, document the seasonal occurrence of diseases, isolation and characterization of fungal pathogens. 2. To determine the qualitative and quantitative changes in quality of phytochemical constituents due to the disease. 3. Metabolite profiling of selected medicinal plants and fungal pathogens for important secondary metabolites including alkaloids and phenols.
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ABSTRACT

Medicinal plants have been one of the valuable sources of medicines since the time of human civilization. Diverse biotic and abiotic factors affect plants. Plant pathogens causes the majority of plant diseases compared to abiotic factors. Fungal disease survey of ten selected medicinal plants - *Centella asiatica, Cyclea peltata, Desmodium gangeticum, Hemidesmus indicus, Ichnocarpus frutescens, Pseudothria viscida, Rauvolfia serpentina, Rubia cardifolia, Solanum violaceum,* and *Strobilanthus ciliates* has been carried out in eight locations of northern Kerala part of Westerns Ghats which included Kakkayam, Kuruva, Muthanga, Nadukani, Sulthan Bathery, Thamarassery, Thirunelli and Tholpetty during three different seasons. A total 185 fungal cultures were isolated and 43 fungal species belong to 10 genera were found to be associated with the surfaces of diseased samples of selected medicinal species. The dominant fungal genera were *Alternaria*, *Colletotrichum, Cladosporium*, *Curvularia, Fusarium, Myrothecium, Pestalotia, Pestalotiopsis, Phoma* and *Phomopsis.* The disease symptoms were manifested mainly as leaf spots and blight. Among the dominating species, *Colletotrichum gloeosporioides* was found associated with disease samples of four medicinal plants *Centella asiatica, Desmodium gangeticum, Pseudorthria visida* and *Solanum violaceum*. *Phomopsis* sp. was found associated with leaf infection in *Hemidesmus indicus* and *Ichnocarpus frutescens*. In case of, *Alternaria alternata* and *Pestalotiopsis versicolor* were found associated with leaf spot and blight disease on *Rauvolfia serpentina* and *Cyclea peltata,* respectively. *Rubia cordifolia* and *Solanum violacenum* were found affected by foliar disease caused by *Fusarium oxysporum* and *Fusarium* sp., respectively. Pathogenicity tests have been conducted for dominant fungal species and all dominant fungal species produced symptoms of disease similar to plants growing in nature.

The disease incidence of pathogenic fungi in each of the study area was determined based on the number of infected plants out of the total number of plants in an area. Disease incidence was determined in medicinal plants species - *C. asiatica, C. peltata, D. gangeticum, H. indicus, I. frutescens, P. viscida, R. serpentina, R. cordifolia, S. violaceum* and *S. ciliates.* The disease incidence in selected medicinal plants was high during the rainy season followed by winter and summer seasons.

The preliminary screening of secondary metabolites suggested the presence of alkaloids, phenols and flavonoids in most of the medicinal plants. This further indicated that the secondary metabolites could be estimated quantitatively by standard analytical methods. The quantitative estimation of alkaloid in leaves indicated an increase in both partially infected and totally diseased samples as compared to the healthy leaf samples of all the plant species tested. But, for of *S. violaceum* and *S. ciliatus* the alkaloid content decreased in infected leaves. However, the content of flavonoids and phenols differed in infected and healthy samples in different plant species.

1. Introduction

Medicinal plants are the cornerstones of both human and veterinary medical system worldwide. In developing countries, where traditional medical system prevails, a majority of people still depend on medicinal plants to meet their daily health needs. Industrialized nations also use medicinal plants, as many pharmaceuticals are based on or derived from plant compounds. Across the globe, many cosmetics and house hold products contain plants of therapeutic and medicinal value. A growing problem, which is compounded by global population growth and increased demand for raw materials, is over exploitation of these important resources.

A few plants are cultivated for medicinal purpose, the remaining are not cultivated and less exploited. Such uncultivated plants are an integral part of the forest ecosystem and any damage caused to them might alter the population status in the ecosystem. Considerable attention has been paid to the study of diseases of agriculture and plantation crops. However, diseases of plants growing in the forests have not received much attention. Since, medicinal plants growing in the forests are also prone to such diseases. So, there is a scope for detailed investigation in view of their importance as herbal drugs. The bio active molecules of plant origin are promising chemical substances which are used in the therapy of wide range of diseases including Cancer and hepatitis. Microorganisms produce toxins and other secondary metabolites, and alter the metabolic pathway of the host plant. Nutritional as well as medicinal properties of plants and their products are expected to change drastically due to infection. Such infected plants may not be suitable for medicinal use and might even pose danger to humans and livestock, if consumed.

Today, scientific research reveals that not only chemical from the plant has effect against a particular disease, but that antioxidant property of the plant extracts also gives beneficial effect to human health. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. Increased fungal infections, toxicity of some antifungal agents and their interactions with other drugs, and development of resistance of some species of fungi have led many studies to search for new antifungal agents.

At present, a few developing countries have the resources or institutional capability to advice on policy and regulatory mechanisms to provide the level of research required to guarantee the production of medicinal plants to sustain local pharmaceutical industries and provide for healthcare needs. The subject tends to fall into two government ministries that normally don’t deal directly with each other:

agriculture and health. They would have to coordinate programs if medicinal plants were to be cultivated.

At present, the farming of medicinal plants is small, scattered, and largely informal given the increasing global population and consequent rise in demand for medicinal plants. To overcome this situation one strategy is to regard medicinally important species as under-utilized crops. An increasing number of developing countries are already showing interest in farming medicinal plants – trees, shrubs, lianas and herbs, annuals as well as perennials.

An increase in commercialization of medicinal plants will provide opportunities to the local communities to enhance their livelihoods. But in order to realize this potential, there is need to ensure involvement of all stakeholders in the production-to-consumption and marketing continuum and ensure that the resources, especially market information, are made available to all the players.

Forests have been and are subject to a variety of anthropogenic activities including, commercial logging and mining and natural calamities like, forest fires, diseases and pests. These have exerted tremendous pressure on biological diversity, including the diversity of medicinal plants. The process of loss of forest covers in the Western Ghats thus been contained, pressure leading to erosion of forest biomass and local extinctions of species continue (Pascal 1988; Gadgil and Chandran 1989; Nadakarni *et al*., 1989). But diseases are responsible for major productivity loss of the forest and are more destructive in terms of volume loss than forest fires. The diseases of plants are found not only to cause depletion of biodiversity or their population, but also, cause immense economic loss to the country.

Local/trible collectors indiscriminately harvest most medicinal plants and their parts from wild. This might result in the harvesting of diseased plant parts along with healthy ones and herbal drugs prepared from such admixture might pose great danger to human and animal life and certain vital herbal formulations prepared out of the infected materials might cause entirely different side-effects. Very limited knowledge is available on the production of toxins by pathogens in crude plant drugs and the effect of probable use of diseased medicinal plant materials in the preparation of the herbal drugs. Therefore, in order to ensure the quality of medicinal plant products, it is necessary to study diseases of medicinal plants and their effect on the phytochemical constitution of these plants. Study on the occurrence of diseases and their seasonality in such plants help to understand the role of disease as a limiting factor.

In this regard, an attempt has been made to study fungal diseases of medicinal plants species in Northern Kerala Parts of Western Ghats and also the effect of pathogens on secondary metabolites of diseased in comparison to healthy plants.

2. Objectives

The present study was undertaken with the following objectives

1. To undertake a survey of disease due to fungal pathogens on selected species of medicinal plants, document the seasonal occurrence of diseases, isolation and characterization of fungal pathogens.
2. To determine the qualitative and quantitative changes in quality of phytochemical constituents due to the disease.
3. Metabolite profiling of selected medicinal plants and fungal pathogens for important secondary metabolites including alkaloids and phenols.

3. Materials and Methods

a. Survey of medicinal plant species in northern Kerala part of Western Ghats for diseases caused by fungi

Disease survey was carried out in 08 locations - Kakkeyam, Kuruwa, Muthanga, Nadukani, Sulthan Batheri, Thamarassery, Thirunelly and Tholpetty during three seasons. A total of twenty four study sites were established and in each forest, there were atleast three study sites. Each individual study site consisted of three replicates and in each replicate three quadrates (10x10m) were randomly established. The study was conducted by frame quadrate method (Sutherland, 1996). The area is an abode for a variety of plant species with medicinal values. Selected medicinal plants include herbs, shrubs and climbers and their medicinal importance of the species in the area are detailed in Table 1.

Table 1. Selected Medicinal plants growing in Northern Kerala Parts of Western Ghats and their medicinal uses

Sl. No.	Plant species, (Malayalam common name), and family	Parts used	Medicinal Uses
1	*Centella asiatica* (Muthil, Kudangal) Apiaceae	Whole plant	Is a mild adaptogen, mildly antibacterial, antiviral, anti-inflammatory, antiulcerogenic, anxiolytic, nervine and vulnerary, and can act as a cerebral tonic, a circulatory stimulant and diuretic.
2	*Cyclea peltata* (Kattuvalli) Menispermaceae	Root and stem	It has bitter, digestant, antipyretic and astringent properties and used in the disease like fever, diarrhea, pruritus, dermatoses, worms, asthma, tumors, heart disease and wounds.

3	*Desmodium gangeticum* (Orila) Fabaceae	Whole plant	It is used in fevers, edema, kidney disorders and post-delivery complications. Roots have febrifuge, expectorant, diuretic properties. It is anti-dysenteric, anti-diarrhoeal and galactogogue.
4	*Hemidesmus indicus* (Nannari) Apocynaceae	Roots	Root is a tonic, demulcent, diaphoretic, diuretic and blood purifier. It is employed in nutritional disorders, syphilis, and chronic rheumatism and gravel other urinary and skin affections.
5	*Ichnocarpus frutescens* (Palvalli) Apocynaceae	Whole plant	It is used as a tribal medicine in atrophy, bleeding gums, convulsion, cough, delirium, dysentery, glossitis, heamaturia, measles, night blindness, relieves pain due to insect bite, splenomegaly and tuberculosis.
6	*Pseudarthria viscida* (Moovila) Fabaceae	Whole plant	It is used for asthma and nervous dysfunction. Also used in the treatment of insect bites and used against inflammation and vomiting.
7	*Rauvolfia serpentina* (Sarpagandhi) Apocynaceae	Roots and leaves	Root is bitter, acrid, heating, sharp, pungent and anthelminthic. It is also used for the treatment of various central nervous system disorders associated with psychosis, schizophrenia, insanity, insomnia and epilepsy.
8	*Rubia cordifolia* (Manjetti) Rubiaceae	Root	The root is sweetish, followed by acrid and bitter taste. Roots are credited with tonic, astringent, antidysenteric, antiseptic, de-obstruent properties.
9	*Solanum violaceum* (Cheruchunda) Solanaceae	Roots and fruits	Root paste is applied for poison. Seeds are used for the preparation of oil used in the case of cough and bronchial disease.
10	*Strobilanthus ciliatus* (Karimkurinji) Amaryllidaceae	Whole plant	The Roots are bitter, sweet, thermogenic, emollient, diuretic, febrifuge, diaphoretic, depurative, anti-inflammatory, expectorant and tonic. Leaves used in the treatment of jaundice, dropsy, rheumatism, anasarca and disease of urinogenital tract.

Source: Anon. (1986) and Kirtikar and Basu (1995),

b. Collection and symptomatology of diseased medicinal plants and characterization of fungal pathogens

Once the identification of plant species was confirmed, plants - *Centella asiatica, Cyclea peltata, Desmodium gangeticum, Hemidesmus indicus, Ichnocarpus frutescens, Pseudorthria viscida, Rauvolfia serpentina, Rubia cordifolia, Solanum violaceum,* and *Strobilanthus ciliates* were screened for fungal diseases. Infected leaves samples were collected in sterile moistened polypropylene covers separately and brought to the laboratory in all the three seasons for further studies. The symptomatology was studied and the data on colour, shape and size of the disease symptoms were recorded. Infected plant materials were observed by the naked eye, hand-held lenses and stereoscopic binocular microscope for the presence of fungal propagules. The fungal species were identified based on their characteristics of colony, hyphae, fruiting bodies, spore colour, shape and size using identification manuals (Arx, 1981; Barnett and Hunter, 1972; Boerema *et al*., 1993; Booth, 1971; Cole and Kendrick, 1981 (Vol-I & II); Domesch, and Gams, 1972 and 1980; Ellis and Ellis, 2001; Ellis, 1971 and 1976; Ramarao and Manoharachary; 1990; Sivanesan, 1983; Subramanian, 1983; Sutton, 1980).

For isolation of fungal pathogens, portions of infected leaves were first washed in running tap water for 10-15 min. followed by distilled water. They were surface disinfected with 1.5 to 2% sodium hypochlorite (NaOCl) solution for two minutes, then washed in sterile distilled water and incubated on PDA medium (Dhingra and Sinclair, 1993). Petridishes were incubated under alternating light and darkness cycle of 12/12 h at 23±2ºC for about 5-7 days. Infected and uninfected plant materials incubated on PDA were observed under stereobinocular microscope and fungal colonies were identified based on the characteristics of colonies, mycelia, fruiting bodies and spores. Identity of the fungal species was confirmed by comparing the illustrations described in identification manuals as described earlier.

c. Pathogenicity test

Pathogenicity of fungal isolates was tested using seedlings of the respective selected host plants raised in the glasshouse. Pathogenicity of all the fungi isolated to their respective host plants to establish the pathogenic status, only selected dominant fungi (by quantitative method) were screened. Pathogenicity of the fungal isolates to the respective hosts was tested by using 4-6 month-old seedlings and spraying conidial suspension ($2x10^4$, $4x10^4$ and $6x10^4$spores ml^{-1}) of the respective fungus. Three

seedlings of the respective host plants were inoculated and were incubated in humidity chamber. Disease symptoms developed in the host plants were recorded and fungus was re-isolated from the diseased host tissues and pathogenicity of the respective fungal species confirmed.

Preparation of spore suspension and mycelia discs for inoculation: The young hyphal tips of the 3-5 day-old-colony culture of the fungus produced on or around incubated diseased plant material on PDA were transferred aseptically into sterilized glass petriplates containing PDA amended with antibiotic streptomycin (25 mg L^{-1}). Plates were incubated as described previously. The candidate fungal species was then prepared for pathogenicity.

The spore suspension of candidate fungal species was prepared by flooding the sporulated five- to seven-day-old culture with sterile distilled water and filtered through three layers of cheese-cloth to remove mycelial fragments and the spore density was fixed to $2x10^4$, $4x10^4$ and $6x10^4$spores ml^{-1} by using a haemocytometre. The spore suspension was used within 2h of preparation.

d. Seasonal occurrence of fungal diseases in medicinal plant species

The incidence of fungal diseases on different plant species was determined in all the forest regions during summer, rainy and winter seasons for the year 2013, 2014 and 2015. During each season, visits were paid, at least once in a season, to assess diseases in all the three study sites of each region.

The disease incidence in a particular medicinal plant due to a specific pathogen was determined seasonally based on the number of infected plants out of the total number of the plants of each species, in each forest region.

Per cent disease incidence was calculated using formula:

$$\textbf{Disease incidence (\%)} = \frac{\text{Number of plants of a species diseased in an area}}{\text{Total number of plants of candidate species observed in an area}} \times 100$$

e. Assay of secondary metabolites

The apparently healthy and diseased samples of selected medicinal plants were first subjected for preliminary screening for qualitative analysis and later following the confirmation of the presence of secondary metabolites, quantitative analysis were done.

f. Preparation of plant extracts

The leaves were washed in clean water and dried at room temperature. The dried plant material was milled to coarse powder using grinder and stored in dark at room temperature in air tight container. Powders of the sample were first subjected to successive extraction starting from non-polar to polar solvents by using petroleum ether, chloroform, ethanol (95% v/v) and distilled water using Soxhlet extractor. The extracts were concentrated *in vacuo* and dried to obtain the respective crude phytochemical extract. These extracts were first subjected to preliminary screening for the presence of alkaloids, flavonoids, and phenols.

g. Qualitative assay for secondary metabolites (Fiegel, 1960 and Gibbs, 1974)

Test for Alkaloids

1. **Mayer's reagent test**
   When 2 ml of Mayer's reagent and 1 ml of dil. HCl was added to plant extract, yellow precipitate was produced indicating the presence of alkaloid.
2. **Wagner's reagent test**
   Production of white precipitate by the plant extract when added with 2 ml of Wagner's reagent and 1 ml of dil. HCl indicate the presence of alkaloids.
3. **Dragondroff's reagent test**
   In this test, addition of 2 ml of Dragondroff's reagent and 1 ml of dil. HCl to the plant extracts produced orange precipitation indicating the presence of alkaloids.

Test for flavonoids

1. **Flavonoid test**
   In this test, the plant extract containing a few magnesium turnings when added with conc. H_2SO_4 through sides of the test tube produced magenta color indicating the presence of flavonoid, scarlet color indicating flavones or deep cherry color indicating flavonoid.
2. **Ferric chloride test**
   When neutral ferric chloride solution was added to the extract, blackish green colour was produced indicating the presence of flavonoid.

3. **Lead acetate test**

   In this test, 10% (v/v) lead acetate solution with plant extract produced a yellow precipitation indicating the presence of flavonoids.

Test for Phenol

1. **Phenol test**

   This test was done by adding 0.5 ml of ferric chloride solution to the plant extract. The presence of phenol was indicated by the formation of the intensive colour in the solution.
2. **Ellagic acid test -** This test yielded muddy yellow, olive brown, niger brown or deep chocolate colour upon reaction of plant extract with a few drops of 5% mixture containing glacial acetic acid and 5% (w/v) sodium nitrate solution.
3. **Hot water test -** In this test, leaf and stem pieces, when partially dipped in hot water, produced intense colour at the junction.

Quantitative analysis of Secondary Metabolites

Estimation of Alkaloids by Ikan (1969) method

A 500 mg of samples was extracted with methanol, and the methanol extract was condensed. To this mixture, 20 ml dil. acetic acid (1:5) was added and shaken well in a separating funnel. The acetic acid layer was collected added with 25 ml N-hexane and chloroform. This was shaken again in a separating funnel for 3 times and pH was adjusted to 8 using sodium hydroxide solution and shaken for 30 min. In a separating funnel, the chloroform layer was collected washed with water, and pH was adjusted to 11 to 12 by adding ammonium hydroxide and the chloroform layer was collected and filtered by using dry filter paper. The filtrate was transferred to a pre- weighed beaker and dried under reduced pressure at 60°C for 6 h. The amount of alkaloid was calculated using the formula-

$$\textbf{Total Alkaloid (mg g}^{-1}\textbf{)} = \frac{\text{The weight of alkaloid residue (X)}}{\text{Weight of sample (Y)}} \text{ X } 100$$

Where,
X= Weight of the residue, Y = Weight of the empty evaporating dish, Z = Weight of the empty dish + alkaloid residue, Total (X) = Z-Y

Estimation of flavonol Swain- Hills (1959) method

500mg of plant material was used to extract repeatedly with alcohol. The extract was centrifuged at 2000 rpm for 20 minutes. Supernatant was collected. Thus collected supernatant was evaporated to dryness and the residue was dissolved in 5 ml of distilled water. 0.2 ml of extract was taken in a test tube and the final volume was made to 2 ml with distilled water and to this 4 ml of Vanillin reagent was added rapidly. Extract after 15 minutes, the absorbance was recorded at 500nm against blank.

From the standard graph of phloroglucinol amount of flavonol present in the plant material was calculated.

Estimation of phenols by Folin Denis reagent (Folin and Denin, 1939) method

In this method, 500 mg of sample suspended in 5 ml of 80 % ethanol (v/v) and shaken. The homogenized solution was centrifuged at 1000 rpm for 20 minutes and the supernatant was collected. The residue was extracted 5 to 7 times and all the supernatant residue were combined and evaporated to dryness. The residue obtained was dissolved in 5 ml of distilled water and estimated for phenol.

Into a 50 ml of Nessler's reagent tube, 0.5 ml of the supernatant was taken and added with 2 ml of Folin Denis reagent and 10 ml of sodium carbonate (20% v/v). The final volume was made up to 50 ml by adding distilled water and the absorbance was read at 660 nm. A standard graph was prepared using different dilutions of tannic acid and the amount of phenol (mg $g^{-1)}$ was calculated.

Statistical analyses

Experiments were conducted in a factorial design. The data of repeated trails in 2013, 2014 and 2015 were tested for homogeneity of results. The data were subjected to analysis of variance (ANOVA). Once 'F' values are significant, means were separated by fisher's least significant difference test (LSD, $P_{0.05}$ & $P_{0.01}$) (Gomez and Gomez, 1983) or by Duncan's multiple range test (DMRT, P = 0.05) (SPSS 16).

4. Results and Discussion

Upon incubation on PDA, fungal colonies developed on the surface and around the incubated diseased samples and on the surface of medium. The fungal species were identified based on the colony morphology and spore characteristics. Most often, the presence of fungal fruiting bodies on the affected parts indicated the association of the specific fungus with the disease. Details of the infected plants and fungi associated with disease are given in Table 2. A total of 185 fungal cultures were isolated and 43 fungal species belong to 10 genera were found to be associated with the surfaces of incubated diseased samples of selected medicinal species. The most commonly occurring fungal genera were *Alternaria*, *Colletotrichum, Cladosporium*, *Curvularia, Fusarium, Myrothecium, Pestalotia, Pestalotiopsis, Phoma* and *Phomopsis.*

The disease symptoms were manifested mainly as leaf spots and blight. These leaf spots appeared initially as lesions which enlarged and coalesced later during favorable conditions leading to blight.

When incubated, the diseased samples were found associated with more than one fungal species. When more than one species is prevalent, the dominant fungus was assumed to be the possible causal organism. The dominant fungal species produced large number of fruiting bodies and innumerable number of spores on the surface of disease samples upon incubation.

While identifying the causal organism of foliar diseases of plant species, certain authors have recorded many species of fungi (more than two) that were associated with the diseased symptom and upon testing their pathogenicity, more often, single species of fungus has been found to be the causal organism of the particular disease (Barber, *et*. *al*., 2003; Mendes and Muchovej, 1991 and Sharma and Florence, 1996).

Table 2. Disease symptomatology and fungi associated with diseased parts of medicinal species growing in the Northern Kerala parts of Western Ghats

SI No.	Botanical Name Disease symptoms and description	Pathogen associated	Northern Kerala parts of Western Ghats
1	***Centella asiatica*:** Leaf spot: Circular to irregular, dark brown spots surrounded by chlorotic pale margin; measures 4x5 and 4x6 mm.	*Alternaria alternata* *Cladosporium* sp. *Colletotrichum gloeosporioides* * *Curvularia lunata* *Fusarium* sp. *Phoma* sp.	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thamarassery Thirunelli Tholpetty

2	***Cyclea peltata*:** Leaf spot and blight: Circular to irregular dark brown lesions surrounded by yellow necrotic margin, spots coalesce; result in blight, and severity leads to defoliation; measures 4x30 and 4x33 mm.	*C. gloeosporioides* *Cladosporium* sp. *Curvularia lunata* *Fusarium* sp. *Pestalotiopsis versicolor**	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thamarassery Thirunelli Tholpetty
3	***Desmodium gangeticum*:** Leaf spot: Circular reddish brown spots later becomes irregular dark brown surrounded by dark necrotic margin; measures 4x3 and 5x4 mm.	*C. gloeosporioides** *Fusarium* sp. *Myrothecium roridum* *Pestalotiopsis* sp.	Kakkayam Kuruva Nadukani Thamarassery
4	***Hemidesmus indicus*:** Leaf spot and leaf blight: Spots initially small circular, later becomes irregular brown/rusty brown, coalesced to form blight surrounded by necrotic margin; measures 4x4 and 4x8 mm.	*Alternaria* sp. *Cladosporium* sp. *Colletotrichum* sp. *Curvularia lunata* *Fusarium* sp. *Pestalotiopsis* sp. *Phomopsis* sp.*	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thamarassery Thirunelli Tholpetty
5	***Ichnocarpus frutescens*:** Leaf spot: Circular to irregular brown spots surrounded by dark necrotic margin; measures 4x3 and 4×2 mm.	*Cladosporium* sp. *Colletotrichum* sp. *Fusarium* sp. *Pestalotiopsis* sp. *Phomopsis* sp.*	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thirunelli Tholpetty
6	***Pseudarthria viscida*:** Leaf spot: Circular to irregular dark brown spots; measures 2×1 mm and 5×2 mm.	*C. gloeosporioides** *Cladosporium* sp. *Fusarium* sp. *Pestalotia* sp. *Pestalotiopsis* sp.	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thamarassery Tholpetty
7	***Rauvolfia serpentina*:** Leaf spot and blight: Circular to irregular, dark brown spots with circular concentric rings surrounded by necrotic area; measures 9x15 and 15x20 mm.	*Alternaria alternata** *Fusarium* sp.	Kuruva Sulthan Bathery Thirunelli Tholpetty
8	***Rubia cordifolia*:** Leaf spot and leaf blight: Initially small circular brown lesions gradually enlarged and coalesce resulting in blight, severity leads defoliation and	*Colletotrichum* sp. *Fusarium oxysporum**	Sulthan Bathery Thamarassery Thirunelli Tholpetty

	caused stem infections; measures 2x3, 9x19 and 15x26 mm.		
9	***Solanum violaceum*:** Leaf spot: Small circular to irregular brown spots coalesced to form blights surrounded by chloretic margins and severity leads defoliation; measures 7x9 and 12x14 mm.	*C. gloeosporioides** *Cladosporium* sp. *Fusarium* sp. *Phomopsis* sp.	Kakkayam Kuruva Muthanga Nadukani Sulthan Bathery Thamarassery Thirunelli Tholpetty
10	***Strobilanthus ciliates*:** Leaf spot and blight: Circular to irregular brown lesions coalesce resulting in blight, severity leads defoliation; measures 6x8, 12x19 and 18x26 mm.	*Colletotrichum* sp. *Fusarium* sp.*	Kakkayam Kuruva Muthanga Nadukani

* Dominant fungus associated with naturally infected plant materials.

In the present study, among the dominating species, *Colletotrichum gloeosporioides* was found associated with disease samples of four medicinal plants *Centella asiatica, Desmodium gangeticum, Pseudorthria visida* and *Solanum violaceum*. *Phomopsis* sp. caused leaf infection in *Hemidesmus indicus* and *Ichnocarpus frutescens*. *Alternaria alternata* and *Pestalotiopsis versicolor* caused leaf spot and blight disease on *Rauvolfia serpentina* and *Cyclea peltata,* respectively. *Rubia cordifolia* and *Solanum violacenum* found affected by foliar disease caused by *Fusarium oxysporum* and *Fusarium* sp., respectively (Plate 1- 10).

Pathogenicity tests have been conducted only for those fungal species that were dominant and occurred in high percentage in the infected area. All dominant fungal species produced symptoms of disease very similar to that in plants growing in nature when inoculated with the spore concentrations of 2x, 4x and $6x10^4 ml^{-1}$ of spores (Table 3).

Plate 1

1. Apparantly healthy plants of *Centella asiatica*.
2. *Centella asiatica* showing leaf spot disease symptoms caused by *Colletotrichum gloeosporioides*.
3. Colony of *Centella asiatica* showing leaf spot disease symptoms caused by *C. gloeosporioides*.
4. Enlarged view of leaf spot disease caused by *C. gloeosporioides* in *C. asiatica.*

Plate 2

1. Apparantly healthy plants of *Cyclea peltata*.
2. *Cyclea peltata* showing leaf spot disease symptoms caused by *Pestalotiopsis versicolor*.
3. Elarged twine of *C. peltata* showing leaf spot disease symptoms caused by *P. versicolor*.
4. Enlarged view of leaf spot disease caused by *P. versicolor* in *C. peltata.*

Plate 3

1. Apparantly healthy plants of *Desmodium gangeticum*.
2. *Desmodium gangeticum* showing leaf spot and blight disease symptoms caused by *Colletotrichum gloeosporioides*.
3. Enlarged view of leaf spot caused by *C. gloesporioides* in *D. gangeticum.*

Plate 4

1. Apparently healthy plants of *Hemidesmus indicus.*
2. *Hemidesmus indicus* plants showing leaf spot and blight caused by *Phomopsis* sp..
3. Close view of *Hemidesmus indicus* plants showing severe infection of leaf spot and blight caused by *Phomopsis* sp..

Plate 5

1. Apparantly healthy plants of *Ichnocarpus frutescens*.
2. *Ichnocarpus frutescens* showing leaf spot disease symptoms caused by *Phomopsis* sp..
3. Extent of damage leaf spot disease symptoms caused by *Phomopsis* sp. in *I. frutescens*.
4. Enlarged view of leaf spot caused by *Phomopsis* sp. in *I. frutescens*.

Plate 6

1. Apparantly healthy plants of *Desmodium gangeticum*.
2. *Desmodium gangeticum* showing leaf spot symptoms caused by *C. gloeosporioides*.
3. Close view of leaf spot symptoms caused by *C. gloeosporioides* in *D. gangeticum*.
4. Close view of leaf spot in *D. gangeticum* by *C. gloeosporioides.*

Plate 7

1. Apparently healthy plants of *Rauvolfia serpentina*.
2. *Rauvolfia serpentina* plants showing leaf spot and blight caused by *Alternaria alternata*.
3. Enlarged view of *Rauvolfia serpentina* plants showing severe infection of leaf spot and blight caused by *A. alternata.*

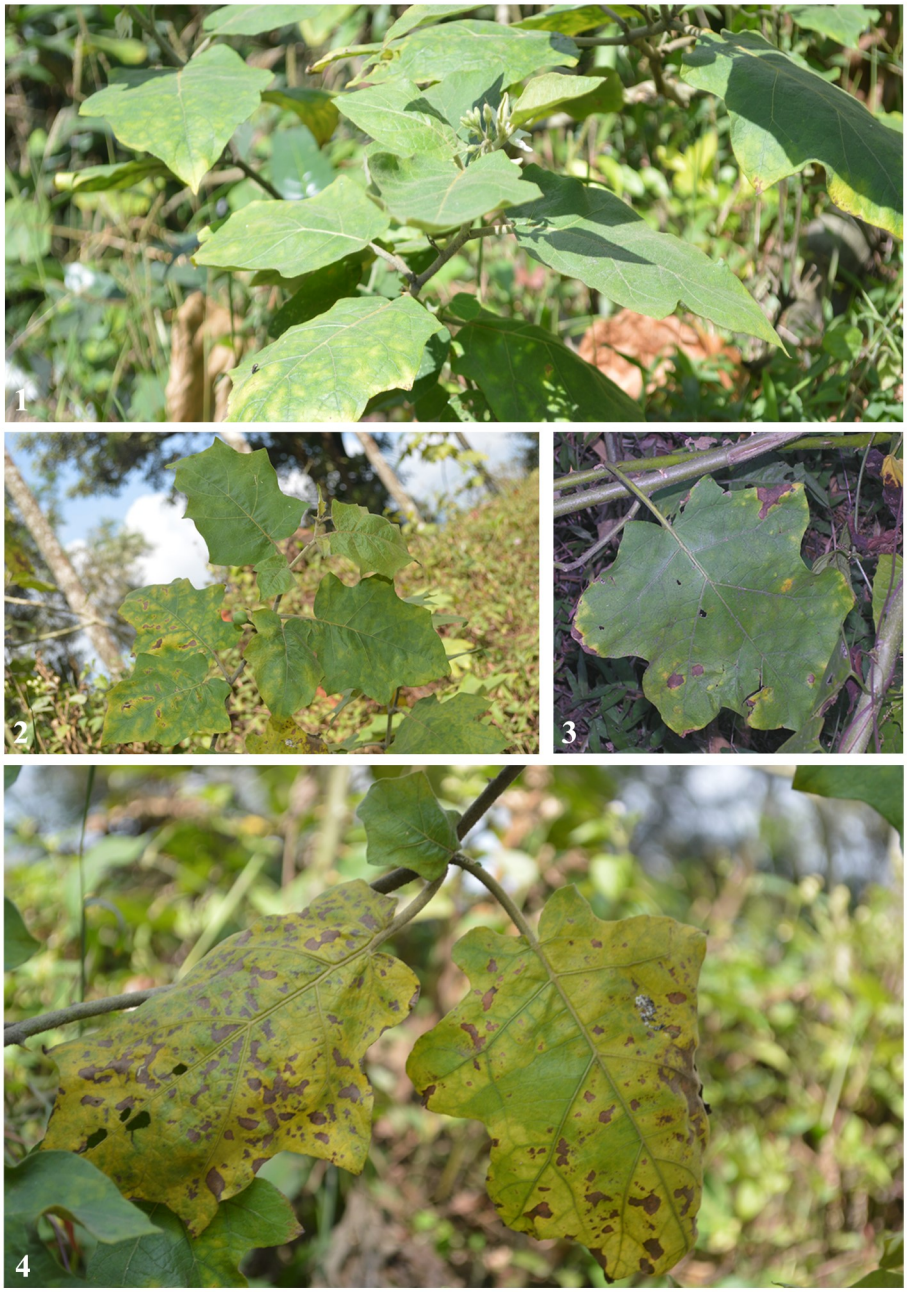
Plate 8

1. Apparantly healthy plants of *Rubia cordifolia*.
2. *Rubia cordifolia* showing leaf spot and blight disease symptoms and severity caused drying of stem caused by *Fusarium oxysporum*.
3. Closeup view of leaf spot & blight disease symptoms caused by *F. oxysporum* in *R. cordifolia*.
4. Enlarged view of leaf spot and blight caused by *F. oxysporum* in *R. cordifolia*.

Plate 9

1. Apparantly healthy plants of *Strobilanthes ciliatus*.
2. *Strobilanthes ciliatus* showing leaf spot and blight disease caused by *Fusarium* sp..
3. Enlarged view of leaf spot and blight caused by *Fusarium* sp. in *S. ciliatus*.

Plate 10



1. Apparantly healthy plants of *Solanum violaceum*.
2. *Solanum violaceum* showing leaf spot disease symptoms caused by *Colletotrichum gloeosporioides*.
3. Enlarged view of *S. violaceum* showing leaf spot disease caused by *C. gloeosporioides*.
4. Severity of leaf spot disease caused by *C. gloeosporioides* in *S. violaceum.*

Table 3. Expression of disease symptoms in plant species following artificial inoculation

Plant species, disease symptom and description	Associated fungal species	Inoculum load (Spores 10^6 ml^{-1})			Nature of disease symptom
		x2	x4	x6	
1. *Centella asiatica*	*C. gloeosporioides* *	+	+	+	Similar
Leaf spot: Circular to	*A. alternata*	-	-	-	-
irregular, dark brown spots	*Cladosporium* sp.	-	-	-	-
surrounded by chlorotic pale	*Curvularia lunata*	-	-	-	-
margin.	*Fusarium* sp.	-	-	-	-
	Phoma sp.	-	-	-	-
2. *Cyclea peltata*	*P. versicolor**	+	+	+	Similar
Leaf spot and blight:	*C. gloeosporioides*	-	-	-	-
Circular to irregular dark	*Cladosporium* sp.	-	-	-	-
brown lesions surrounded by	*Curvularia lunata*	-	-	-	-
yellow necrotic margin.	*Fusarium* sp.	-	-	-	-
3. *Desmodium gangeticum*	*C. gloeosporioides**	+	+	+	Similar
Leaf spot: Circular reddish	*Fusarium* sp.	-	-	-	-
brown spots later becomes	*Myrothecium roridum*	-	-	-	-
irregular dark brown					
surrounded by dark necrotic	*Pestalotiopsis* sp.	-	-	-	-
margin.					
4. *Hemidesmus indica*	*Phomopsis* sp.*	+	+	+	Similar
Leaf spot and leaf blight:	*Alternaria* sp.	-	-	-	-
Spots initially small circular,	*Cladosporium* sp.	-	-	-	-
later becomes irregular rusty	*Colletotrichum* sp.	-	-	-	-
brown surrounded by	*Curvularia lunata*	-	-	-	-
necrotic margin; measures	*Fusarium* sp.	-	-	-	-
4x4 and 4x8 mm.	*Pestalotiopsis* sp.	-	-	-	-
5. *Ichnocarpus frutescens*	*Phomopsis* sp.*	+	+	+	Similar
Leaf spot: Circular to	*Cladosporium* sp.	-	-	-	-
irregular brown spots	*Colletotrichum* sp.	-	-	-	-
surrounded by dark necrotic	*Fusarium* sp.	-	-	-	-
margin.	*Pestalotiopsis* sp.	-	-	-	-
6. *Pseudarthria viscida*	*C. gloeosporioides**	+	+	+	Similar
Leaf spot: Circular to	*Cladosporium* sp.	-	-	-	-
irregular dark brown spots.	*Fusarium* sp.	-	-	-	-
	Pestalotia sp.	-	-	-	-
	Pestalotiopsis sp.	-	-	-	-
7. *Rauvolfia serpentina*	*A. alternata**	+	+	+	Similar
Leaf spot and blight:	*Fusarium* sp.	-	-	-	-
Circular to irregular, dark	*Cladosporium* sp.	-	-	-	-
brown spots with circular	*C. lunata*	-	-	-	-
concentric rings with					
necrotic area margin					
8. *Rubia cordifolia*	*F. oxysporum**	+	+	+	Similar
Leaf spot and blight: Initially	*Cladosporium* sp.	-	-	-	-
small circular brown lesions	*Colletotrichum* sp.	-	-	-	-
gradually enlarged resulting	*Curvularia lunata*	-	-	-	-
in blight.	*Phoma* sp.	-	-	-	-

9. *Solanum violaceum* Leaf spot: Small circular to irregular brown spots surrounded by chloretic margins.	*C. gloeosporioides**	+	+	+	Similar
	Cladosporium sp.	-	-	-	-
	Fusarium sp.	-	-	-	-
	Phomopsis sp.	-	-	-	-
10. *Strobilanthus ciliates* Leaf spot and blight: Circular to irregular brown lesions.	*Fusarium* sp.*	+	+	+	Similar
	Colletotrichum sp.	-	-	-	-

* Dominant fungus associated with naturally infected plant materials, '+' – Present, '-' – Absent.

a. Seasonal occurrence of fungal diseases in medicinal plants

Disease symptoms on selected medicinal plants were observed mainly on foliages. The number of infected plants above five per cent were only taken for consideration to determine the disease incidence. The disease occurrence in different plants varied in different seasons depending on the pathogen, vegetation pattern in forest types and the host species. In some forests, plants of the same species were in high number but they were not at all infected, while, the same species although in small number were affected to the large extent in other forest areas. The disease incidence trend in most species of plants was almost similar during 2013-2015. However, a slight variation in disease incidence was observed in some plant species, possibly due to variations in the microclimatic conditions. Hence, for the sake of convenience, the data of the respective season of all the three years were averaged.

Disease Incidence

The disease incidence ranged from as low as 5% to 100%; this merely indicated the number of infected plants out of total number of plants in the region. In some study sites, the total number of plants of the same species was as low as five plants. Disease incidence, above 5%, in such a case would be 100%.

Centella asiatica was infected by *C. gloeosporioides* (Plate 1) which caused leaf spot disease in all the forest locations. The disease incidence was high in rainy season followed by winter and summer. A comparison of disease incidence in different seasons indicated that the number of infected plants was low in the summer season except in Thamarassery and Sulthan Bathery forests. The disease during August (rainy) was found in 77.80 to 95.5% of plants and during summer, the disease slightly decreased and ranged from 71 to 90%. The difference in disease incidence in these seasons in most forest regions were statistically significant ($P_{0.05}$) and disease incidence in different forest regions also varied significantly ($P_{0.05}$) in a particular season (Table 4).

Table 4. Incidence (%) of *Colletotrichum gloeosporioides* leaf spot disease in *Centella asiatica* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	86.00b C[2]	95.00a A	89.30c B
Kuruva	67.90e C	81.10c A	76.53f B
Muthanga	78.00c C	88.00b A	85.00d B
Nadukani	64.00f C	87.66b A	78.20ef B
Sulthan Bathery	90.00a C	95.33a A	92.00b B
Thamarassery	90.00a B	95.40a A	94.00a A
Thirunelli	87.30b B	94.50a A	94.50a A
Tholpetty	71.40d C	77.80d B	79.30e A
$LSD_{0.05}$	3.28	1.84	3.28
$LSD_{0.01}$	4.67	2.61	4.60

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column.

In case of *C. peltata*, disease incidence due to *P. versicolor* (Plate 2) was recorded in all the eight forest locations. The disease was most prevalent during the rainy season with large number of plants infected by this fungus in the range of 91.30 to 97%. The disease incidence during this season was almost similar in Kakkayam, Sulthan Batheri and Thirunelli, forest regions. The disease continued but decreased significantly ($P_{0.05}$) during winter in most of the regions except in Sulthan Batheri. Further, the disease incidence decreased significantly ($P_{0.05}$) when observations were made during summer (Table 5).

Table 5. Incidence (%) of *Pestalotiopsis versicolor* leaf spot disease in *Cyclea peltata* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	84.00b C[2]	94.00b A	90.00b B
Kuruva	80.40e C	92.10c A	88.60c B
Muthanga	70.33g B	91.90c A	71.80f B
Nadukani	71.90f C	91.30c A	85.70d B
Sulthan Bathery	85.30a B	94.30b A	94.00a A
Thamarassery	72.40f B	97.00a A	85.00d B
Thirunelli	82.50c C	94.80b A	85.80d B
Tholpetty	81.50d B	93.80b A	80.80e C
$LSD_{0.05}$	0.76	1.96	0.85
$LSD_{0.01}$	1.08	2.79	1.22

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column.

The disease incidence due to *C. gloeosporioides* in *D. gangeticum* (Plate 3) occurred in four forest regions. Here also, the disease was maximum during rainy season followed by that during winter and summer seasons. The decrease in disease incidence was noticed in winter as compared to the rainy season was significant ($P_{0.05}$). While, in all other forest locations, as compared to rainy and winter seasons, disease incidence in summer was low, it was still in the range of 51.9 to 78.33%. When the disease incidence in forest regions were taken in to consideration, in particular season the difference was not significant statistically ($P_{0.05}$) in at least two regions (Table 6).

Table 6. Incidence (%) of *Colletotrichum gloeosporioides* leaf spot disease in *Desmodium gangeticum* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	78.33b C[2]	95.00b A	84.40c B
Kuruva	70.30c C	92.30c A	83.00d B
Muthanga	-[3]	-	-
Nadukani	81.00a C	92.70c A	86.40b B
Sulthan Bathery	-	-	-
Thamarassery	51.90d C	99.00a A	89.33a B
Thirunelli	-	-	-
Tholpetty	-	-	-
$LSD_{0.05}$	0.95	0.72	0.62
$LSD_{0.01}$	1.38	1.05	0.91

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column. [3]Plants were not found in the site.

In case of *Hemidesmus indicus,* popularly known as 'Anantmul' a semi erect shrub widely distributed throughout the state, the disease incidence due to *Phomopsis* sp. (Plate 4) was high in the rainy season. The disease affected the plants in all the study locations. The disease incidence in the rainy, winter and summer seasons was almost similar in Kuruwa. But in the rest of the regions, the incidence was less in the winter than in the rainy season. The disease incidence in rainy season ranged from 80.80 to 95.00%. However, it ranged from 74.7 to 94.00% in winter season. Even during summer, the difference in disease incidence in all the three seasons was not significant ($P_{0.05}$) in Kuruva regions. However, in the rest of the state forest regions, the disease decreased significantly ($P_{0.05}$) (Table 7).

Another host plant, *I. frutescens* was affected by *Phomopsis* leaf spot disease (Plate 5) in seven of eight locations. Here also, the maximum disease production was

observed in rainy season, which occurred in the range of 88 to 95% which decreased gradually and significantly ($P_{0.05}$) in winter and summer seasons (Table 8).

Table 7. Incidence (%) of *Phomopsis* sp. leaf spot disease in *Hemidesmus indica* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	71.90[2]d C[2]	91.30b A	85.70d B
Kuruva	94.30a A	94.30a A	94.00a A
Muthanga	64.00f C	91.60b A	86.30c B
Nadukani	81.00b C	95.00a A	85.30de B
Sulthan Bathery	78.00c C	88.00c A	85.00e B
Thamarassery	67.90e C	81.10d A	76.53f B
Thirunelli	64.40 f C	80.80d A	74.70g B
Tholpetty	79.30c C	94.70a A	90.30b B
$LSD_{0.05}$	1.92	0.60	0.23
$LSD_{0.01}$	2.74	0.85	0.33

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column.

Table 8. Incidence (%) of *Phomopsis* sp. leaf spot disease in *Ichnocarpus frutescens* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	86.00a C[2]	95.00a A	89.30b B
Kuruva	78.00e C	88.00e A	84.00f B
Muthanga	84.00bc C	94.30b A	86.70e B
Nadukani	84.00bc C	94.00b A	90.00a B
Sulthan Bathery	80.40d C	92.10d A	88.60c B
Thamarassery	-[3]	-	-
Thirunelli	82.60c C	93.00c A	86.30e B
Tholpetty	85.00ab C	93.30c A	87.60d B
$LSD_{0.05}$	1.84	0.39	0.27
$LSD_{0.01}$	2.62	0.56	0.38

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column. [3] Plants were not found in the site.

The disease incidence due to *C. gloeosporioides* in *P. viscida* (Plate 6) occurred in all the forest regions except Thirunelli. During rainy season the disease was noticed maximum followed by that during winter and summer seasons. The decrease of disease incidence was noticed significantly ($P_{0.05}$) during winter as compared to the rainy season. While, in all other forest locations, as compared to rainy and winter seasons,

disease incidence in summer was significantly ($P_{0.05}$) low, it was in the range of 34.10 to 85.70% (Table 9).

Table 9. Incidence (%) of *Colletotrichum gloeosporioides* leaf spot disease in *Pseudarthria viscida* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	85.70a C[2]	97.00a A	92.20a B
Kuruva	85.60a C	94.20d A	89.90b B
Muthanga	70.90d C	95.50c A	91.50a B
Nadukani	72.40c C	97.00a A	85.00d B
Sulthan Bathery	84.00b C	96.70ab A	88.70c B
Thamarassery	70.60d C	92.80e A	80.16e B
Thirunelli	-[3]	-	-
Tholpetty	34.10e C	96.20b A	88.70c B
$LSD_{0.05}$	0.22	0.31	0.62
$LSD_{0.01}$	0.31	0.45	0.88

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column. [3] Plants were not found in the site.

Alternaria alternata caused leaf spot disease in *R. serpentina* (Plate 7) in four forest regions. *Alternaria alternata* also caused maximum disease in the range of 93.00 to 97% during rainy and 83.4 to 85.8% in winter and, in summer, the disease incidence decreased (72.4%) significantly ($P_{0.05}$) (Table 10).

Table 10. Incidence (%) of *Alternaria alternata* leaf spot disease in *Rauvolfia serpentina* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	-[2]	-	-
Kuruva	80.90b C[3]	93.00c A	84.56b B
Muthanga	-	-	-
Nadukani	-	-	-
Sulthan Bathery	81.20b C	95.00b A	83.40c B
Thamarassery	-	-	-
Thirunelli	72.40c C	97.00a A	85.00ab B
Tholpetty	82.50a C	94.80b A	85.80a B
$LSD_{0.05}$	0.57	0.65	0.49
$LSD_{0.01}$	0.84	0.95	0.72

[1]Data is an average of three years of 2013-2015. [2] Plants were not found in the site. [3]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column.

Fusarium oxysporum, which caused leaf spot and blight disease in *R. cordifolia* (Plate 8) in four forest regions. Here also, the disease was noticed to the maximum extent during rainy season followed by winter and summer season in most forest regions. In rainy, the disease ranged from 91.90 to 96.6% and in winter, it was 71.8 to 86.3 %. The disease during summer it was decreased ($P_{0.05}$) from 73.66% to 64.0 (Table 11).

Table 11. Incidence (%) of *Fusarium oxysporum* leaf spot disease in *Rubia cordifolia* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	-[2]	-	-
Kuruva	-	-	-
Muthanga	-	-	-
Nadukani	-	-	-
Sulthan Bathery	70.33b C[3]	91.90c A	71.80b B
Thamarassery	73.66a C	93.30b A	85.70a B
Thirunelli	70.50b C	95.50a A	72.40b B
Tholpetty	64.00c C	96.60a A	86.30a B
$LSD_{0.05}$	0.20	0.67	0.42
$LSD_{0.01}$	0.29	0.97	0.61

[1]Data is an average of three years of 2013-2015. [2] Plants were not found in the site. [3]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column.

In case of *S. violaceum*, disease incidence due to *C. gloeosporioides* (Plate 9) was recorded in all the forest locations except Thirunelli. During rainy season the disease was noticed maximum followed by winter and summer seasons. Also, in case of Kakkayam and Kuruwa, the disease incidence noticed was almost similar which may be due to the similar edaphic and climatic factors of the forest region. While, in all other forest locations, as compared to rainy season, disease incidence was decreased significantly ($P_{0.05}$) in winter and summer, and it was in the range of 35.33 to 91.16% (Table 12).

In case of *Strobilanthus ciliates*, *Fusarium* sp. caused leaf spot disease (Plate 10) and disease incidence was recorded in four of eight locations. Here also, the disease was noticed maximum during rainy season followed by winter and summer in most forest regions and drastic decrease ($P_{0.05}$) of disease was observed during summer season. In rainy season, the disease ranged from 94.30 to 96.6% and in winter, it was 80.8 to 86 %. The range of disease incidence during summer was 48.4 to 71.6% (Table 13).

Table 12. Incidence (%) of *Colletotrichum gloeosporioides* leaf spot disease in *Solanum violaceum* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	94.30a A[2]	94.30b A	94.00a A
Kuruva	93.30b B	94.70b A	94.30a A
Muthanga	35.33g C	96.20a A	88.70c B
Nadukani	72.40e C	92.00c A	85.00d B
Sulthan Bathery	70.60f B	90.80d A	91.16b A
Thamarassery	80.00c C	94.70b A	87.70c B
Thirunelli	-[3]	-	-
Tholpetty	75.40d C	96.93a A	85.00d B
$LSD_{0.05}$	0.40	0.66	0.93
$LSD_{0.01}$	0.57	0.95	1.33

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column. [3]Plants were not found in the site.

Table 13. Incidence (%) of *Fusarium* sp. leaf spot disease in *Strobilanthus ciliatus* in different forest regions during summer, rainy and winter seasons for the year 2013-2015

Forest regions	Disease incidence(%)[1]		
	Feb (Sum)	Aug (Rain)	Nov (Win)
Kakkayam	48.40d C[2]	95.30b A	85.00a B
Kuruva	69.30b C	94.30c A	81.80b B
Muthanga	61.10c C	96.00ab A	86.00a B
Nadukani	71.60a C	96.60a A	80.80b B
Sulthan Bathery	-[3]	-	-
Thamarassery	-	-	-
Thirunelli	-	-	-
Tholpetty	-	-	-
$LSD_{0.05}$	0.29	0.37	0.83
$LSD_{0.01}$	0.42	0.54	1.21

[1]Data is an average of three years of 2013-2015. [2]Means carrying same letters in a row to compare means of forest regions and column to compare means of seasons are not significantly different (DMRT, $P_{0.05}$). LSD value for comparing forest means under different season are given in the bottom of the column. [3]Plants were not found in the site.

b. Assay of Secondary Metabolites

Preliminary Screening of Secondary Metabolites

Preliminary screening for secondary metabolites by various tests indicated that solvent extracts of apparently healthy and diseased samples of different medicinal plants differed in the presence and the amount of secondary metabolites (Tables 14-23). All the tests for individual secondary metabolite were positive for some species of plants and for some other species, only a few tests were positive for secondary metabolite. This might depend on the amount of metabolite available in the extract and the sensitivity of the test. When the secondary metabolite was detected in healthy, it was also detected in diseased samples in most cases.

Eight out of ten plants tested positive for alkaloids in the healthy and diseased samples when extracted with all the solvents selected. While, aqueous extract of *D. gangeticum* (Table 16) and *P. visida* (Table 19) lacked alkaloids, petroleum ether fraction samples of *C. asiatica* (Table 14)*, H. indica* (Table 17)*, I. frutescens* (Table 18)*, P. visida* (Table 19) and *R. cordifolia* (Table 21) lacked alkaloids.

Table 14. Preliminary screening of apparently healthy and diseased leaf materials of *Centella asiatica* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H[5]	D[6]	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	-	-
	b. Wagner's test	-	-	-	-	-	-	-	-
	c. Dragendorff's test	-	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	+	+	+	+	+	+
	b. Ferric chloride test	-	-	-	-	+	+	+	+
	c. Lead acetate test	-	-	-	-	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	+	+	+	+	+	+
	b. Ellagic acid test	-	-	-	-	+	+	+	+
	c. Hot water test	-	-	-	-	+	+	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 15. Preliminary screening of apparently healthy and diseased leaf materials of *Cyclea peltata* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	+	+
	b. Wagner's test	-	-	+	+	+	+	-	-
	c. Dragendorff's test	+	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	+	+	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	-	-
	c. Lead acetate test	+	-	+	+	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	-	-	+	+	+	+
	b. Ellagic acid test	-	-	-	+	+	+	+	+
	c. Hot water test	-	-	+	+	+	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 16. Preliminary screening of apparently healthy and diseased leaf materials of *Desmodium gangeticum* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	-	-	+[8]	+	-	-
	b. Wagner's test	-	-	-	-	+	+	-	-
	c. Dragendorff's test	+	+	+	+	+	+	-	-
2	**Test for Flavonoids**								
	a. Flavonoid test	+	+	-	-	+	+	+	+
	b. Ferric chloride test	-	-	-	-	+	+	-	-
	c. Lead acetate test	-	-	+	-	-	-	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	-	-	+	+	+	+
	b. Ellagic acid test	+	+	-	-	+	+	-	-
	c. Hot water test	-	-	-	-	-	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 17. Preliminary screening of apparently healthy and diseased leaf materials of *Hemidesmus indica* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	-	-
	b. Wagner's test	-	-	+	-	+	+	-	-
	c. Dragendorff's test	-	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	-	-	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	-	-
	c. Lead acetate test	+	+	-	-	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	+	+	+	+	-	-
	b. Ellagic acid test	-	-	+	+	+	+	-	-
	c. Hot water test	+	+	-	-	+	+	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 18. Preliminary screening of apparently healthy and diseased leaf materials of *Ichnocarpus frutescens* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	+	+
	b. Wagner's test	-	-	-	-	-	-	-	-
	c. Dragendorff's test	-	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	-	-	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	+	+
	c. Lead acetate test	-	-	-	-	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	-	-	+	+	+	+
	b. Ellagic acid test	-	-	-	-	+	+	+	+
	c. Hot water test	-	-	-	-	-	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 19. Preliminary screening of apparently healthy and diseased leaf materials of *Pseudarthria viscida* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	-	-	+[8]	+	-	-
	b. Wagner's test	-	-	-	-	+	+	-	-
	c. Dragendorff's test	-	-	+	+	+	+	-	-
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	+	+	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	-	-
	c. Lead acetate test	+	+	-	-	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	-	-	+	+	+	+	+	+
	b. Ellagic acid test	-	-	-	-	+	+	-	-
	c. Hot water test	-	-	-	-	+	+	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 20. Preliminary screening of apparently healthy and diseased leaf materials of *Rauvolfia serpentina* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	-	+
	b. Wagner's test	-	-	+	+	+	+	-	-
	c. Dragendorff's test	+	+	+	+	+	+	-	+
2	**Test for Flavonoids**								
	a. Flavonoid test	+	+	-	-	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	+	+
	c. Lead acetate test	-	-	+	+	-	-	-	-
3	**Test for Phenolics**								
	a. Phenol Test	-	-	+	+	+	+	+	+
	b. Ellagic acid test	-	-	+	+	-	-	+	+
	c. Hot water test	+	+	-	-	-	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 21. Preliminary screening of apparently healthy and diseased leaf materials of *Rubia cordifolia* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	+	+
	b. Wagner's test	-	-	-	-	+	+	-	-
	c. Dragendorff's test	-	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	+	+	+	+	+	+
	b. Ferric chloride test	-	-	+	+	+	+	+	+
	c. Lead acetate test	-	-	+	+	+	+	+	+
3	**Test for Phenolics**								
	a. Phenol Test	+	+	-	-	+	+	+	+
	b. Ellagic acid test	-	-	+	+	+	+	+	+
	c. Hot water test	+	+	-	-	+	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 22. Preliminary screening of apparently healthy and diseased leaf materials of *Solanum violaceum* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	+[7]	+	+	+	+	+	-[8]	-
	b. Wagner's test	-	-	+	+	+	+	+	+
	c. Dragendorff's test	-	-	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	-	-	+	+	+	+	-	-
	b. Ferric chloride test	-	-	+	+	+	+	+	+
	c. Lead acetate test	-	-	-	-	+	+	-	-
3	**Test for Phenolics**								
	a. Phenol Test	-	-	+	+	+	+	+	+
	b. Ellagic acid test	-	-	+	+	+	+	+	+
	c. Hot water test	-	-	-	-	+	+	-	-

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

Table 23. Preliminary screening of apparently healthy and diseased leaf materials of *Strobilanthes ciliatus* for the presence of various secondary metabolites by different methods

Sl. No.	Tests	PE Extract[1]		$CHCl_3$ Extract[2]		EtOH Extract[3]		AQ Extract[4]	
		H[5]	D[6]	H	D	H	D	H	D
1	**Test for Alkaloids**								
	a. Mayer's reagent test	-[7]	-	+[8]	+	+	+	-	-
	b. Wagner's test	-	-	-	-	-	-	-	-
	c. Dragendorff's test	+	+	+	+	+	+	+	+
2	**Test for Flavonoids**								
	a. Flavonoid test	+	+	+	+	+	+	+	+
	b. Ferric chloride test	-	-	+	+	-	-	+	+
	c. Lead acetate test	+	+	+	+	-	-	+	+
3	**Test for Phenolics**								
	a. Phenol Test	+	+	+	+	+	+	+	+
	b. Ellagic acid test	-	-	+	+	-	-	+	+
	c. Hot water test	-	-	-	-	-	-	+	+

[1]PE = Petroleum ether, [2]$CHCl_3$ = Chloroform, [3]EtOH = Ethanol, [4]Aq = Aqueous extract, [5]H = Apparently healthy, [6]D = Diseased, [7]- = Absent and [8]+ = Present.

As for flavonoids (Table 14-23), all the fractions tested positive in healthy and diseased samples of selected species of plants. However, petroleum ether fraction of *C. asiatica* (Table 14), *I. frutescens* (Table 18) and *R. cordifolia* (Table 21) and *S. violaceum* (Table 22) lacked flavonoids. Flavonoids are derivatives of phenolic compounds. It comprises of two aromatic rings. These are one of the largest classes of plant phenolics based on the degree of oxidation of three-carbon bridge. Flavonoids are of 4 types - flavones, flavonols, isoflavones and anthocyanidins (Sinha, 2004).

The phenolics (Table 14-23) were detected in all the extracts of plant species. However, it was not detected in petroleum ether and chloroform fractions of *I. frutescens* (Table 18). On the other hand, the petroleum ether extracts of *C. asiatica* (Table 14), *C. peltata* (Table 15) and *P. visida* (Table 19) and chloroform extract of *D. gangeticum* (Table 16) did not show the presence of phenolics in all the three tests specific for the detection of phenolics.

Quantitative Estimation of Secondary Metabolites

Among the secondary metabolites, the contents of alkaloids (Table 24) increased ($P_{0.05}$) in both partially infected and heavily infected samples as compared to the healthy leaf samples. In the totally diseased samples, content of secondary metabolites further increased in comparison to the partially infected samples. Only in case of *S. violaceum* and *S. ciliatus* (Table 24), the totally disease sample had decreased

alkaloid content significantly ($P_{0.05}$) as compared to the healthy and partially infected samples.

As far as the content of flavonoids (Table 24) and phenols (Table 24) are concerned, the partially infected and heavily infected samples in comparison to uninfected samples differed in different plant species. The flavonoid and phenol contents decreased with increase in percentage of infection. For example, in three species (*I. frutescens, R. serpentina* and *R. cordifolia*), flavonoid content decreased significantly ($P_{0.05}$) in heavily infected samples in comparison to control. Similarly, phenols also decreased ($P_{0.05}$) in the same fashion in *C. asiatica*. However, this trend changed in certain other species of plants. In case of *I. frutescens* the content of phenols in partially infected samples showed high ($P_{0.05}$) content phenols (Table 24) in comparison to control. In contrast to this, in nine (*C. peltata, D. gangeticum, H. indicus, I. frutescens, P. visida, R. serpentina, R. cordifolia, S. violaceum* and *S. ciliatus*) and seven (*C. asiatica*, *C. peltata, D. gangeticum, H. indicus, P. visida, S. violaceum* and *S. ciliatus*) species, respectively, the decreased ($P_{0.05}$) content of phenol and flavonoid and was high in partially infected sample but in heavily infected sample, their contents were significantly ($P_{0.05}$) higher than in the partially infected samples. On the other hand, in two species (*I. frutescens* and *R. cordifolia*) the control samples always had maximum flavonoid contents and in both partially infected and heavily infected plant samples, these metabolites were always low.

Phenolic compounds are chemically diverse group of substances having diverse functions in plants such as protection against herbivores, pathogens, UV radiation and attraction to pollinators and seed dispersing and also serve as seed germination inhibitors (Sinha, 2004). In the present study, phenols increased in partially infected leaves in comparison to uninfected samples, which differed in different plant species. In the present study, the increase in phenols in partially infected plant materials could be due to its excess production as defence mechanism against pathogenic infection and decrease in totally infected leaves could be related to the degradation of phenolic compounds in to simple substances and its utilization by the host.

Table 24. Effect of fungal disease(s) on the phytochemical constituents (mg/g) of medicinal plant species

Plant species & Part used	Phytochemical Constituents[1]		
	Alkaloids	Phenolic	Flavonoids
Centella asiatica			
App.Healthy	0.0081b[2]	0.310b	2.60b
Partially (50%)	0.0041c	0.350a	1.60c
Heavily infected	0.0086a	0.070c	2.80a

Cyclea peltata			
App.Healthy	0.0007c	0.380c	0.220c
Partially (50%)	0.0480b	0.430b	0.320b
Heavily infected	0.1633a	0.510a	0.470a
Desmodium gangeticum			
App.Healthy	0.0013c	0.410c	2.280c
Partially (50%)	0.0090b	0.615b	2.550b
Heavily infected	0.0143a	0.720a	2.990a
Hemidesmus indicus			
App.Healthy	0.002c	0.600a	0.610c
Partially (50%)	0.012b	0.611a	0.839b
Heavily infected	0.027a	0.630a	1.300a
Ichnocarpus frutescens			
App.Healthy	0.007c	2.680c	1.670a
Partially (50%)	0.009b	4.609a	0.960b
Heavily infected	0.014a	4.220b	0.530c
Pseudarthria viscida			
App.Healthy	0.017a	2.460c	0.440c
Partially (50%)	0.010b	3.015b	0.461b
Heavily infected	0.0115ab	3.640a	0.530a
Rauvolfia serpentina			
App.Healthy	0.0039c	2.200c	0.380a
Partially (50%)	0.0044b	2.510b	0.330b
Heavily infected	0.0051a	2.900a	0.290c
Rubia cordifolia			
App.Healthy	0.0026c	0.100b	1.050a
Partially (50%)	0.0080b	0.125b	0.212c
Heavily infected	0.0104a	0.260a	0.650b
Solanum violaceum			
App.Healthy	0.060a	1.400b	0.180c
Partially (50%)	0.015c	0.935c	0.285b
Heavily infected	0.035b	2.310a	0.460a
Strobilanthes ciliatus			
App.Healthy	0.007a	0.152c	2.110c
Partially (50%)	0.004b	0.233b	2.860b
Heavily infected	0.001c	0.360a	3.612a

[1]Data is an average of three replication, [2] Means carrying same letters in a column to compare means of phytochemical constituents are not significantly different (DMRT, $P_{0.05}$).

Secondary metabolites are actively metabolized especially in growing tissues. All types of compounds are eventually degraded either in to various metabolic pool or are completely oxidized to carbon dioxide. Flavonoids and phenolic compounds are degraded to aliphatic acids, terpenoids to isoterpenoid units and alkaloids to amino acids (Sinha, 2004). This kind of biochemical estimation should be taken up in future to enable the understanding of the type of degraded products in infected materials. This might help in their quantification in crude herbal formulations.

Conclusion

Medicinal plants have various useful compounds and their use in traditional medicines has been known for a long time. Fungal disease survey of medicinal plants was carried out in eight locations of Northern Kerala parts of Westerns Ghats in three different seasons. The disease incidence of pathogenic fungal species was determined in medicinal plants species. The disease incidence in selected medicinal plants was high during the rainy followed by winter and summer seasons. The qualitative estimation of secondary metabolites showed the presence of alkaloids, phenols and flavonoids in most of the selected medicinal plants. The quantitative estimation of secondary metabolites indicated increased and decreased trend in both partially infected and heavily infected samples as compared to the healthy leaf samples of all the plant species tested. The increase in compounds in infected plant materials could be due to synthesis of metabolites as part defense mechanism against pathogen and decrease in totally infected leaves could be due to the degradation of compounds in to simple substances and its utilization by the host. Presence of metabolites tested positive in the present study indicates that increase of metabolites in plants may be the measure of total metabolite content. Biochemical estimation of diseased plants may be taken up in future to enable the understanding of the type of degraded products in infected materials. This might help in quantification of crude herbal formulations. The fungal degraded metabolites may have harmful or useful effects when consumed which needs to be evaluated in future experiments.

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