Materials and Methods

Study area

The Western Ghats, one of the hotspots of biodiversity, is located along the western coast of the Indian subcontinent. It is an ecologically important province covering about five percent of India's land area. Phytogeographically, the vegetation in this region is rich with high species diversity (Menon and Bawa, 1997) and is the center of endemism (Anon., 1978). The vegetation pattern ranges from moist evergreen to scrub jungle. Several major rivers take their origin in the Western Ghats. The Ghats extends into 12 districts in Karnataka, within which seven National Parks (2073 sq km) and 39 Wildlife Sanctuaries (13,862 sq km) have been established.

Bhadra Wildlife Sanctuary, earlier known as 'Jagar Wildlife Sanctuary' (200 sq km, Est. 1951), was established on 25th September 1974 on an area of 492.46 sq km. Subsequently, it was declared as the protected area under the 'Project Tiger' (25th Tiger Reserve) on 19th November 1988.

Bhadra Wildlife Sanctuary in the Western Ghats of Karnataka lies between 13° 21' and 13° 50' north latitude and 75° 15' and 75° 50' east longitude. It extends over two taluks of Chikkamagalore (Chikkamagalore and Narasimharaja Pura) and Bhadravathi taluk of Shimoga district. Sanctuary has valleys and hillocks with varied altitudes (65 to 1975 m) and is well drained by river Bhadra. The mean annual rainfall varies from 1200 to 2600 mm and much of the rain is received during July and September from southwest monsoon winds. The temperature ranges between 9° and 35°C. The relative humidity in the sanctuary varies from 68 to 95%.
The vegetation in the sanctuary is of moist deciduous to dry deciduous type and the forest soil type is red to heavier black soil. The vegetation included more than 100 species of herbs, shrubs and trees and more than 25 species of orchids, several species of grasses and ferns (Anon., 2000). An exclusive area marked as medicinal plant conservation area (MPCA) at Kemmannugundi has been established within the sanctuary for the *in situ* conservation and maintenance of germplasm of naturally growing medicinal plants.

Many plants growing in the sanctuary are reported to have medicinal properties (Table 3). The people living in 35 villages inside the sanctuary (relocated since 2002) have been reported to use 60 plant species for medicinal purposes (Parinitha *et al.*, 2004).

**Study sites**

Bhadra Wildlife Sanctuary is divided into four forest ranges (Lakkavalli, Muthodi, Thanigebylu and Hebbe) by the State Forest Department of Karnataka, Government of Karnataka. Four forest ranges consisted of 12 forest regions viz., Aldara, Gangegiri, Hebbegiri, Kagemanegiri, Kakanahosudi, Kemmannugundi, Lakkavalli, Madla, Mudhuguni, Muthodi, Singanamane and Thammadihalli (Fig. 1). Photographs of certain forest ranges are shown in Plate 1 and 2. The loss of vegetation in Muthodi state forest region is shown in Plate 2. Thirty-six study sites were selected within these state forest regions (Table 4). In each of the state forest region, three study sites were selected at random (Fig. 1). Each of the study sites consisted of a minimum of three replicates and in each replicate, three quadrates (10 sq m) were established.
Fig. 1. Location map of the Bhadra Wildlife Sanctuary showing forest ranges and the state forests regions. The study sites have been marked as black triangles.
PLATE - 1

A. Hebbegiri state forest region in Bhadra Wildlife Sanctuary

B. Lakkavalli state forest region in Bhadra Wildlife Sanctuary

C. Muthodi state forest region in Bhadra Wildlife Sanctuary

D. Lakkavalli state forest region showing bamboo plantation in the back waters of Bhadra Wildlife Sanctuary

E. Medicinal plant conservation area at Kemmannugundi state forest region in Bhadra Wildlife Sanctuary

F. Medicinal plant conservation area at Kemmannugundi state forest region in Bhadra Wildlife Sanctuary

G. Madia state forest region in Bhadra Wildlife Sanctuary

H. Madhuguni state forest region in Bhadra Wildlife Sanctuary
PLATE - 2

A. Thammadihalli state forest region in Bhadra Wildlife Sanctuary

B. Rampant forest fire in Muthodi state forest region

C. Devastating effects on vegetation following forest fire in Muthodi region
Table 3. Important medicinal herbs growing in the Western Ghats and their therapeutic applications

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Plant species</th>
<th>Family</th>
<th>Therapeutic value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Abrus precatorius L.</td>
<td>Papilionaceae</td>
<td>Diuretic and is given for cough, cold(^1), tonic emetic, stiffness of shoulder joints, jaundice, paralysis, leprosy(^1,2), antitumor activity, aphrodisiac, purgative, leucoderma, analgesic, anthelmintic, uterine stimulant, antifertility property(^3).</td>
</tr>
<tr>
<td></td>
<td>(Gulaganji) En</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Achyranthes aspera L.</td>
<td>Amaranthaceae</td>
<td>Diuretic(^1), renal dropsies(^2), astringent, antiperiodic, purgative, piles, boils, skin eruptions, stomach ache, diarrhoea, dysentery, anthelmintic, syphilitic, sores(^3); scorpion sting(^6).</td>
</tr>
<tr>
<td></td>
<td>(Prickly chaff flower, Uttarani)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Aloe barbadensis Mill.</td>
<td>Liliaceae</td>
<td>Cathartic, refrigerant, used in liver, spleen ailments and eye troubles, x-ray burns, dermatitis, cutaneous jeishmaniasis(^1,3).</td>
</tr>
<tr>
<td></td>
<td>(Indian aloe, Lole-sara) LRnt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Alpinia galanga L.</td>
<td>Zingiberaceae</td>
<td>Rheumatism, bronchial catarrh, stimulant, carminative(^1).</td>
</tr>
<tr>
<td></td>
<td>(Rasna) Vu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Asparagus racemosus Willd.</td>
<td>Liliaceae</td>
<td>Tonic, diuretic, galactogogue, dyspepsia and used for nervous and rheumatic complaints(^1,3), menstrual disorders(^6).</td>
</tr>
<tr>
<td></td>
<td>(Satamuli) Dwpc/Vu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Biophytum sensitivum DC.</td>
<td>Oxalidaceae</td>
<td>Gonorrhoea and lithiasis(^3); tonic and stimulant used in chest complaints, diabetes, asthma and inflammatory tumors(^1).</td>
</tr>
<tr>
<td></td>
<td>(Lajalu)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Centella asiatica L.</td>
<td>Apiaceae</td>
<td>Brain tonic, leprous wounds(^1,2), refrigerant, hair growth promoter(^3), fever, eczema, psoriasis, and epilepsy, bronchial asthma and rejuvenator, skin diseases(^2); menstrual disorders, jaundice(^6).</td>
</tr>
<tr>
<td></td>
<td>(Vondelaga)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Clitoria ternatea L.</td>
<td>Papilionaceae</td>
<td>Cathartic, diuretic, nerve tonic, useful in chronic bronchitis, irritation of the bladder and urethra and as antidote(^1,3).</td>
</tr>
<tr>
<td></td>
<td>(Aparajit)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Contd...
Materials and Methods

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Family</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td><em>Curcuma amada</em> Roxb.</td>
<td>Zingiberaceae</td>
<td>Skin diseases, blood purifier, and bruises, carminative, stomachache, sprains and convulsions&lt;sup&gt;3,4&lt;/sup&gt;.</td>
</tr>
<tr>
<td>10</td>
<td><em>Cyclea peltata</em> (Lam) hook f.&amp; Thomson (Padavala balli) Lrvt</td>
<td>Menispermaceae</td>
<td>Febrifuge, stomachic, tonic&lt;sup&gt;1,3&lt;/sup&gt;.</td>
</tr>
<tr>
<td>11</td>
<td><em>Cynodon dactylon</em> Pers.</td>
<td>Poaceae</td>
<td>Astringent, anti epileptic, antidiarrhoeal, anticatarrhal, ophthalmia, cuts and wounds, secondary syphilis, bleeding piles&lt;sup&gt;3&lt;/sup&gt;, diuretic&lt;sup&gt;1&lt;/sup&gt;, ulcers&lt;sup&gt;6&lt;/sup&gt;.</td>
</tr>
<tr>
<td>12</td>
<td><em>Dioscorea bulbifera</em> L.</td>
<td>Dioscoriaceae</td>
<td>Piles, dysentery&lt;sup&gt;1,3&lt;/sup&gt;, sores, syphilis&lt;sup&gt;3&lt;/sup&gt;.</td>
</tr>
<tr>
<td>13</td>
<td><em>Eclipta alba</em> (L.) Hassk</td>
<td>Asteraceae</td>
<td>Tonic, wounds in cattle and for darkening hair&lt;sup&gt;1&lt;/sup&gt;; emetic, purgative, promotes hair growth, scalding of urine, conjunctivitis, prevents abortion, piles&lt;sup&gt;2,3&lt;/sup&gt;, used in spleen enlargement, skin troubles, deobstruent&lt;sup&gt;1,2&lt;/sup&gt;.</td>
</tr>
<tr>
<td>14</td>
<td><em>Elephantopus scaber</em> L.</td>
<td>Asteraceae</td>
<td>Emollient in dysuria and other urethral discharges&lt;sup&gt;1&lt;/sup&gt;; dysentery, swelling, stomach pain, tooth ache, diarrhoea, eczema, ulcer, and arrests vomiting&lt;sup&gt;1,3&lt;/sup&gt;; intestinal worms in cattle&lt;sup&gt;6&lt;/sup&gt;.</td>
</tr>
<tr>
<td>15</td>
<td><em>Gloriosa superba</em> L.</td>
<td>Liliaceae</td>
<td>Tonic, used of neuralgic pains, skin troubles, gout, and rheumatism and to promote labour pain&lt;sup&gt;1&lt;/sup&gt;; febrifuge, purgative, cancer, malaria, leprosy, piles, colic, gonorrhoea&lt;sup&gt;2,3&lt;/sup&gt;; stomachic, anthelmintic, abortifacient&lt;sup&gt;1,3&lt;/sup&gt;.</td>
</tr>
<tr>
<td>16</td>
<td><em>Gymnema sylvestre</em> R.Br.</td>
<td>Asclepiadaceae</td>
<td>Expectorant, asthma, amenorrhoea, and dysmenorrhoea&lt;sup&gt;1,3&lt;/sup&gt;, snakebite, emetic, rheumatism&lt;sup&gt;3&lt;/sup&gt;.</td>
</tr>
<tr>
<td>17</td>
<td><em>Hemidesmus indicus</em> R.Br.</td>
<td>Asclepiadaceae</td>
<td>Diuretic, biliousness, sore eye, emetic, expectorant, astringent, stomachic, antidote, stimulates heart and circulatory system, activates the uterus and used in furunculosis, laxative&lt;sup&gt;2,3&lt;/sup&gt;, antidiabetic, stimulates heart&lt;sup&gt;1,3&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>

Contd...
<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Family</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td><em>Ichnocarpus frutescens</em> (L.) R. Br. (Syamalatha)</td>
<td>Apocynaceae</td>
<td>Tonic, demulcent, diaphoretic, diuretic, dyspepsia, stone in bladder; antisyphilitic, antibilious, antiemetic, diabetes, febrifuge; diabetes.</td>
</tr>
<tr>
<td>19</td>
<td><em>Naravelia zeylanica</em> DC. (Neend mali/Vatakodhi)</td>
<td>Ranunculaceae</td>
<td>Astringent, anthelmintic, depurative, anodyne, anti inflammatory, leprosy, rheumatism, colic, wounds, ulcer.</td>
</tr>
<tr>
<td>20</td>
<td><em>Nervilia aragoana</em> Gaud. En</td>
<td>Orchidaceae</td>
<td>Decoction of leaves after parturition.</td>
</tr>
<tr>
<td>21</td>
<td><em>Oxalis corniculata</em> L. (Huli soppu)</td>
<td>Oxalidaceae</td>
<td>Piles and anemia, warts; cooling, antiscorbutic, appetizing, fevers, biliousness, corns, inflammations, corneal diseases, chronic dysentery, liver disorders, haemorrhage and to remove cancerous growth from lips.</td>
</tr>
<tr>
<td>22</td>
<td><em>Phyllanthus amarus</em> Schumm &amp; Thonn. (Nelanelli)</td>
<td>Euphorbiaceae</td>
<td>Astringent, deobstruent, stomachic, diuretic, febrifuge, diarrhoea; dysentery, dyspepsia, colic, dropsy, jaundice, galactogogue and sores, to cure genito-urinary affection.</td>
</tr>
<tr>
<td>23</td>
<td><em>Plumbago zeylanica</em> L. (Chitramula) Vu</td>
<td>Plumbaginaceae</td>
<td>Abortifacient, skin diseases, diarrhoea, piles, influenza, anasarca; leprosy, scabies, ulcers, dyspepsia, flatulence and rheumatic pains; head ache.</td>
</tr>
<tr>
<td>24</td>
<td><em>Rauwolfia serpentina</em> Benth. (Sarpagandha) En/R/Vu</td>
<td>Apocynaceae</td>
<td>Nervous disorders, excitement, psychosis, schizophrenia, insanity, insomnia and epilepsy, uterine contraction, opacity of cornea; snake bite.</td>
</tr>
<tr>
<td>25</td>
<td><em>Rubia cordifolia</em> L. Indian madder</td>
<td>Rubiaceae</td>
<td>Tonic, antidysenteric, antiseptic, deobstruent, vermifuge, used for rhinosinal infections.</td>
</tr>
<tr>
<td>26</td>
<td><em>Ruta graveolens</em> L. (Garden rue, Nagadala)</td>
<td>Rutaceae</td>
<td>Anthelmintic, antispasmodic, antiepileptic, rubefacient, emmenagogue, used in hysteria, ammenorrhoea; snake bite.</td>
</tr>
<tr>
<td>27</td>
<td><em>Sida acuta</em> Burm. (Bala)</td>
<td>Malvaceae</td>
<td>Stomachic, diaphoretic, antipyretic, gonorrhoea; Bitter tonic, bowel complaints, rheumatic affections, and chronic dysentery.</td>
</tr>
</tbody>
</table>

Contd...
<table>
<thead>
<tr>
<th>No.</th>
<th>Scientific Name</th>
<th>Family</th>
<th>Description</th>
</tr>
</thead>
</table>
| 28  | *Solanum nigrum* L.  
(Kakmachi) | Solanaceae | Fever, headache and giddiness, gonorrhoea; rheumatism, gouty joints, skin diseases, dropsy, heart diseases, piles, inflammatory, painful and swollen testicles, chronic cirrhosis of the liver and spleen, antiseptic, antidysenteric, anthrax, pustules, diuretic, laxative, eye trouble; ulcers. |
| 29  | *Spilanthes acmella* Murr.  
(Vanamugali) | Asteraceae | Throat affections, tongue paralysis, stammering, stimulant, lithnoriptic, diuretic, scabies, psoriasis; sialagogue and caries, inflammation of jaw, dysentery, root purgative, tooth ache and periostites. |
| 30  | *Tinospora cordifolia* (Willd.)  
Miers  
(Amrutha balli) | Menispermaceae | General debility, dyspepsia, emetic, leprosy, antispasmodic, anti-inflammatory, tonic, gout, urinary diseases; gonorrhoea, skin diseases, visceral obstruction, diarrhoea, stomachic, stimulant, diuretic, antiabetic, aphrodisiac, antiperiodic, vaginal and urethral discharges, piles, anemia, jaundice, antirheumatic, fever, cough, burning sensation, delirium and vomiting. |
| 31  | *Tylophora indica* (Burm. f.)  
Merriel.  
Syn. *T. asthmatica* W&A.  
(Adumuttada gida) | Asclepiadaceae | Febrifuge, demulcent, diaphoretic, urinary complaints; antileucorrhoea, antisyphilitic, diuretic, tonic, galactogenic, antidote for scorpion sting and snake bite, blood purifier, antirheumatic, aperitive, eye inflammation. |
| 32  | *Withania somnifera* Dunal.  
(Ashwaghanda) | Solanaceae | Hiccup, febrifuge, scabies, sore eyes, seminal debility, rheumatism; Anthelmintic, carbuncles, obstunaste ulcers, nervous exhaustion, spermatorrhoea, cure sterility of Women, leucorrhoea, lumbago pains, skin diseases. |


**LRnt** – Lower risk near threatened, **En** – Endangered, **Vu**- Vulnerable, **Dwpc**- Declared in wild but progressively cultivated, **R** – Rare and **CEn** – Critically endangered (Anon., 1998a and b).
**Materials and Methods**

Table 4. Forest ranges, state forest regions and study sites and their area in Bhadra Wildlife Sanctuary in Karnataka

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Forest range</th>
<th>State forest regions</th>
<th>Area of the state forest region (sq km)</th>
<th>No. of study sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lakkavalli</td>
<td>Lakkavalli state forest</td>
<td>174.34</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kakanahosudi</td>
<td>2.02</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aldara</td>
<td>33.57</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thammadihalli</td>
<td>11.95</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Singanamane</td>
<td>1.29</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Muthodi</td>
<td>Muthodi</td>
<td>97.07</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kagémangiri</td>
<td>33.54</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Thanigebylu</td>
<td>Kemmannugundi</td>
<td>55.68</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Hebbe</td>
<td>Madhuguni</td>
<td>10.42</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Madla</td>
<td>8.04</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hebbegiri</td>
<td>21.15</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gangegiri</td>
<td>43.39</td>
<td>3</td>
</tr>
</tbody>
</table>

Field visits were undertaken, to these study sites, during January 2001 to December 2002. Each of the study sites was surveyed for herbaceous medicinal plants and their diseases were recorded at least once in each season of the year.

**Survey of herbaceous plants in the sanctuary**

Herbaceous plants (excluding grasses, orchids and ferns) growing in the quadrates of each study site were collected and identified and data were recorded for all the three seasons during the first year. These plants were characterized based on the identification keys given in the standard identification manuals - Flora of Madras Presidency, Hassan and Chikkmagalore districts (Saldana and Nicolson, 1976; Yoganarasimhan *et al.*, 1981 and Gamble, 1997). Information on the therapeutic value of these herbaceous plants, if any, was collected by referring to the authentic literature on medicinal values of
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Screening herbs in the sanctuary for diseases

After identification of herbs, they were screened for diseases that may be expressed on leaves, stems and other plant parts. The study sites having diseased plants were labeled for data collection in other seasons. Infected plant materials were collected in moist polyethylene covers and brought to the laboratory for the study of disease symptoms and causal organisms. The disease symptoms were examined by the naked eye and stereo-binocular microscope for their association with fungal species.

Incubation of disease materials

Since the study sites are parts of the sanctuary area, plant materials were collected only by non destructive harvesting method by the permission of forest officials. The root samples were collected from outside the boundary of the sanctuary for the isolation and identification of the associated fungal pathogens.

The infected plant materials were washed in slow running tap water (10 to 15 min), surface disinfected in 0.5% sodium hypochlorite solution (2-3 min) and rinsed in distilled water three to four times. The diseased materials were excised into one-centimeter area fragments under aseptic conditions and plated on 9-cm dia. glass petriplates containing three layers of moistened sterile blotter discs. Similarly, the surface disinfected and washed leaf materials were blotted dry with sterilized blotters and placed on Potato Dextrose Agar (PDA) contained in 9-cm dia. glass petriplates under aseptic conditions. In both the tests, the plates were incubated under 12/12 h alternating cycles of
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light/darkness at 23±2° C for three to five days and examined for fungal expression in diseased materials. The fungal colonies and their sporulation in the infected materials were observed under a stereo binocular microscope and identification of the fungus was established based on the characteristics like the habit of fungal colony, fruiting body, sporulation and spore size and with the help of identification manuals (Tandon, 1968; Booth, 1971; Ellis, 1971; Ingold, 1971; Barnett et al., 1972; Sivanesan, 1983 and Subramaniyam, 1983).

Since more than one fungal species were expected to occur on incubated plant materials, the fungal species that occurred abundantly in the infected area was determined as the dominant species and those that occurred less abundantly as non-dominant fungal species. The dominance of the fungal species was calculated by counting their presence in the segments of incubated plant materials as compared to the total number of segments of diseased materials placed for incubation.

Pathogenicity test

Pathogenicity of all the fungal species associated with the disease symptoms was conducted on selected plant species. The plant species were selected based on the severity of the disease in most of the seasons and their importance as medicinal plants in pharmaceutical industries. The importance of the herbal species for medicinal purpose was determined based on the literature / information from the pharmacopoeia and their commercial value in the local market (Anon, 1948-60 and 1987; Nadakarni, 1976; Kirthikar and Basu, 1995; Grewal, 2000; Shakti et al., 2002 and Prajapati et al., 2003).
Pure culturing

The fungal colonies produced on incubated diseased plant materials on blotter or on PDA around the material were tested under the microscope and after confirmation of identity (as described earlier), the particular fungal species was transferred aseptically into sterile glass petriplates containing PDA amended with antibiotic streptomycine (25 mg/L⁻¹). The plates were incubated as described previously. The test fungus was then tested for its pathogenicity by leaf or stem bioassay or tooth prick method.

Leaf/stem bioassay

For the purpose of bioassay, fully expanded apparently healthy leaves as well as young stem segments (3-4 cm) were collected, washed and surface disinfected as earlier. Excess moisture was blotted out and placed in petriplates containing sterile moistened blotter discs. The leaf or stem segments were artificially inoculated by either using the spore suspension or PDA disc colonized by the fungus.

Spore suspension method

Spore suspension was prepared by placing one milliliter of sterilized distilled water on 5-day-old sporulated colony culture of fungus on PDA and agitated with the camel hair brush. The spore suspension obtained was filtered through three layers of muslin cloth to remove the hyphal fragments and the filtrate was tested for spore density by using a haemocytometer. Spore load of the suspension was adjusted to $10^6 \times 2$ or $10^6 \times 4$ or $10^6 \times 6$ spores ml⁻¹ by adding sterile distilled water. One hundred microliter of the spore suspension was taken with a micropipette and placed on the upper surface of the leaf lamina and stem segments with or without pricks (pricks were made using a
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sterilized needle by simple incision). In case of failure of disease expression even after 5 days after incubation, inoculum load per unit volume was increased.

**Agar disc method**

In this method, agar discs of 5-day-old colony culture of the fungus on PDA was prepared by using a sterilized 5-mm dia. cork borer, which was used to pierce the growing margin of the colony culture. The discs were placed on detached and surface disinfected leaves and stem segments with or without pricks with fungal culture on discs facing the leaf surface.

**Tooth prick method**

This method was used only in case of a fungus lacking the ability to produce sporulation on PDA but produced mycelial mat and/or tiny sclerotia on the media. Commercially available wooden tooth pricks were soaked in distilled water for 24 h and autoclaved at 100° C, 120 lb for 20 min. The sterile pricks were placed on 5-day-old colony culture on PDA contained in sterile screw caped bottles (6x9 cm) with their pointed tips touching the culture. The tooth pricks were incubated until they were colonized with the fungus or for 10 to 12 days. These fungal infested tooth pricks were used for pricking the surface of the disinfected stem segments.

The artificially inoculated leaf and stem segments were incubated as described earlier. Disease symptoms were recorded and compared with that of plants growing in the study sites. The diseased plant materials (through artificial inoculation) were incubated further for colony growth and sporulation. The fungal colonies and
sporulations were compared with that of the culture on PDA (used for artificial inoculation) and the causal agent of disease was confirmed.

**Determination of disease incidence**

Following the study of disease symptoms and characterization of the associated fungal pathogens, the incidence of disease due to particular pathogen(s) in each plant species was assessed in all the three seasons and throughout the study period. The per cent incidence of a disease in a population of the species for a specific area was calculated based on the number of infected plants out of the total number plants of the same species.

**Disease calendar / map**

Disease calendars were prepared for each of the fungal diseases that occurred in 13 selected medicinal plants based on the data of disease severity and incidence in different seasons. A disease map of the particular pathogen of the entire study area was prepared based on the prevalence of disease(s) in each of selected medicinal plants.

**Assay of phytochemical compounds**

Healthy and diseased leaves of 13 selected medicinal plants (*Centella asiatica* Urb; *Clitoria ternatea* L., *Cyclea peltata* (Lam.) Hook. f. and Thomas., *Dioscorea alata* L., *D. bulbifera* L., *Elephantopus scaber* L., *Hemidesmus indicus* L., *Ichnocarpus frutescens* R.Br., *Naravelia zeylanica* DC., *Rauwolfia serpentina* (L.) Benth., *Rubia cordifolia* L., *Tinospora cordifolia* Miers. and *Withania somnifera* (L.) Dun.) were collected from the study sites during maximum disease seasons. The disease incidence was not uniform and it varied depending on the pathogen, host and the environment. The
infected as well as healthy plant materials including leaves and stems (in certain plant species) were brought to the laboratory and assessed for disease incidence and graded into three categories depending on the per cent incidence of diseases. i). Sample containing only apparently healthy leaves (no visible symptoms when collected or zero), ii). Sample containing infected leaves having specific disease incidence or partially diseased, and iii). Sample containing only infected leaves or totally diseased (100% disease incidence). Details of the disease severity due to fungal pathogens in these plants are described. The second sample, in some cases, also contained infected petiole and stem. Samples were air-dried under shade (ambient conditions of the laboratory) for about 2-3 days and the dried samples were stored in the air-tight zipper lock polyethylene covers in dark cooler places until further use. The dried samples were powdered to 0.5-mm size particles using a coffee grinding hand mill and stored in zipper lock polyethylene covers. At the time of analysis the samples were again powdered using a pestle and mortar. The primary and secondary metabolites of the healthy and diseased samples of medicinal plants, were determined by following standard methodologies. Reagents were prepared fresh and stored in amber coloured bottles under laboratory conditions and used immediately for carrying out the analyses.

Assay of primary metabolites

The above samples were tested for primary metabolites like total carbohydrates, proteins, proline and lipids.
Total carbohydrates

This was estimated by the method of Hedge and Hofreiter (1962). Here, the anthrone reagent was used which was prepared fresh by mixing 200 mg of anthrone in 100 ml of ice cooled 95% H₂SO₄.

One hundred milligrams of the sample was taken in a test tube and hydrolyzed in a boiling water bath by adding 5 ml of 2.5N HCl for 3 h, and cooled to room temperature. Then, it was neutralized with solid sodium carbonate until effervescence stops and the volume was made up to 100 ml with distilled water. The mixture was centrifuged at 1000 rpm for 2 min and the supernatant was collected for quantitative estimation.

To 100 ml of the aliquot taken in a test tube, 1 ml of distilled water and 4 ml of anthrone reagent was added. The mixture was heated in a boiling water bath for 8 min, and cooled rapidly. The green to dark green solution obtained was read at 630 nm. A standard glucose solution was prepared by dissolving 2 g glucose in 2 ml of distil water, dilutions were made and diluted samples were estimated as described earlier. A standard graph was prepared by plotting the concentration of the standard on X axis and the absorbance on Y axis. Using the standard graph, the amount of carbohydrate present in the sample was determined. The amount of carbohydrate was expressed as mg/g sample.

Proteins

Proteins in the samples were estimated quantitatively by Lowry’s method (1951). The important reagents used here are 2% Na₂CO₃ in 0.1 N NaOH, (Reagent A), 0.5% CuSO₄ in 0.1% potassium sodium tartarate (Reagent B) and alkaline copper solution was prepared by mixing 50 ml of A and 1 ml of B prior to use.
Materials and Methods

Folin-Ciocalteu reagent (FCR) : One hundred grams of sodium tungstate with 25 g sodium molybdate and 700 ml water and 50 ml 85% phosphoric acid and 100 ml concentrated HCl are taken in 1.5 liter flask and reflexed for 10 h. To this, 150 g lithium sulphate and 50 ml water and a few drops of bromine water were added and boiled for 15 min to remove excess bromine. The mixture was cooled, and the volume was made up to 1 liter and filtered.

Standard protein solution was prepared by dissolving bovine serum albumin (BSA, fraction V) in distilled water and the final volume made up to 50 ml using distilled water in a standard flask.

A 500 mg of sample was taken with 5 ml of phosphate buffer (0.2 M, pH 7.5), agitated and centrifuged, and the supernatant was used for protein estimation. One hundred microliter of the extract was added to 0.9 ml distilled water to make it up to 1 ml. To this test tube, 5 ml of alkaline copper solution was added and incubated for 10 min at room temperature. To this mixture 0.5 ml of FCR reagent was added and mixed thoroughly and incubated at room temperature in darkness for 30 min. The blue colour solution that developed following incubation was read at 660 nm. Using the BSA solution, a standard graph was generated and the amount of protein in the sample was calculated. The amount of protein was expressed as mg/g sample.

Proline

Proline in the samples were estimated by Mahadevan and Sridhar (1982). Here, a 500 mg of sample was homogenized with 10 ml of 3% sulfosalicylic acid in a mortar with pestle. Filter the homogenate through Whatmann No. 2 filter paper and pool the filtrates make up volume to 10/50 ml. Two ml of the filtrate is pipetted into a test tubes
and add 2 ml of acid ninhydrine and 2 ml of glacial acetic acid. Incubate the tubes for 1 h at 100° C in a hot water bath and then tubes were transferred to an ice bath to terminate the reaction. Add 4 ml of toluene and mix the contents vigorously using test tube stirrer for 15-20 sec, collect toluene fraction and measure the absorbance at 520 nm in a colorimeter, maintain a reagent blank and calculate its amount on fresh weight basis using the following formula

$$\text{Proline (mg/g fresh weight)} = \frac{36.23 \times 1000 \times \text{vol. made (10/50 ml)} \times \text{O.D.}}{\text{Vol. taken (2) x wt. of tissue in mg (500)}}$$

**Lipids**

The total crude lipids in the sample was determined by procedures detailed in the Manual of Laboratory Techniques, ICMR, Hyderabad (Anon., 1957). The most important reagent required for the estimation was 2:1 chloroform-methanol mixture.

One milligram of the powdered sample and 5 g of anhydrous sodium sulphate were taken in a mortar and ground with a pestle. To this, 20 ml of chloroform-methanol mixture was added and transferred to iodometric flask and shaken for 1 h. The mixture was filtered through a glass sintered funnel. The extraction with chloroform methanol was repeated. Solvents were evaporated under vacuum. The residue left containing crude lipids, was extracted again with 10 ml chloroform-ethanol mixture containing 20 ml of saline solution (1% sodium chloride solution). The fractions were poured into a separating funnel, shaken thoroughly and allowed to stand for 5 min. The lipids recovered in lower chloroform layer were separated out. The upper layer was added with 10 ml of chloroform-ethanol mixture again and extracted to remove any residual lipid
in it. This was repeated for 2 to 3 times. The entire lipid containing fraction was poured into a pre-weighed beaker and the solvent evaporated by keeping the beaker in warm water bath (50°C, 10 min). Weight of the crude lipids in the sample was estimated.

**Assay of secondary metabolites**

The secondary metabolites of samples of selected plants were first subjected to preliminary screening for qualitative analyses and later for quantitative analyses following the conformation of the presence of secondary metabolites.

The 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. These extracts were first subjected to preliminary screening for the presence of phenols, flavonoids, alkaloids, steroids, glycosides, saponins and tri terpinoids.

**Preliminary screening for secondary metabolites (Fiegel, 1960 and Gibbs, 1974)**

**Testing for phenolics**

i. **Phenol test** - This test was done by adding 0.5 ml of ferric chloride solution to the plant extract. The presence of phenolics was indicated by the formation of the intensive colour in the solution.

ii. **Ellagic acid test** - This test yielded muddy yellow, olive brown, niger brown or deep chocolate colour upon reaction of plant extract with few a drops of 5% mixture containing glacial acetic acid and 5% (w/v) sodium nitrate solution.
iii. **Hot water test** - In this test, leaf and stem pieces, when partially dipped in hot water, produced intense colour at the junction.

**Testing for tannins**

i. **Sodium chloride test** - The formation of precipitate with the addition of few drops sodium chloride solution to the extract indicated the presence of tannins.

**Testing for lignans**

i. **Furfuraldehyde test** - When extracts treated with furfuraldehyde (2% v/v), formation of red colour indicates the presence of lignans.

**Testing for flavonoids**

i. **Flavonoid test** - In this test, the plant extract containing a few magnesium turnings when added with concentrated H₂SO₄ through the sides of the test tube produced magenta colour indicating the presence of flavonols, scarlet colour indicating flavones or deep cherry colour indicating flavonoids.

ii. **Ferric chloride test** - When neutral ferric chloride solution was added to the extract, blackish green colour was produced indicating the presence of flavonoids.

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

**Testing for alkaloids**

i. **Mayer’s reagent test** - When 2 ml of Mayer’s reagent and 1 ml of diluted HCl was added to plant extract, yellow precipitate was produced indicating the presence of alkaloids.
ii. **Wagner's reagent test** - Production of a white precipitation by the plant extract when added to it with 2 ml of Wagner's reagent and 1 ml of diluted HCl indicating the presence of alkaloids.

iii. **Dragendorff's reagent test** - In this test, addition of 2 ml of Dragendorff's reagent and 1 ml of diluted HCl to the plant extract produced orange precipitation indicating the presence of alkaloids.

**Testing for steroids**

i. **Salkowski test** - The production of wine red colour in the extract when it was added with concentrated H$_2$SO$_4$ indicated the presence of steroids.

ii. **Liebermann-Burchardt's test** - When the extract was added with a few drops of acetic unhydride and mixed with 1 ml of concentrated sulphuric acid (dropped from the sides of the test tubes), the mixture produced a red ring at the junction of two layers, which indicated the presence of steroids.

**Testing for glycosides**

i. **Kellar Kiliani test** - In this test, the plant extract is added with a few drops of glacial acetic acid and boiled for a minute and cooled. Into this solution, two drops of Ferric chloride was added which was then transferred to another test tube containing conc. H$_2$SO$_4$. The formation of reddish brown ring at the junction of layers indicated the presence of glycosides.

ii. **Molisch's Test** - In this test, one ml of Molich's reagent was added to the plant extract and when 1 ml conc. H$_2$SO$_4$ was poured through the sides of the test tube, a reddish violet ring was formed at the junction of two layers indicating the presence of glycosides.
Testing for triterpenoids

i. Salkowski test - Here in this test, golden yellow colour of the lower layer with the addition of few drops of concentrated sulphuric acid to the extract indicated the presence of triterpenes.

ii. Liebermann-Burchard’s test - This test also answers the detection of triterpenes by the production of red rings at the junction of two layers. With the addition of few drops of acetic anhydride and 1 ml of concentrated sulphuric acid to the extract.

Quantitative estimation of secondary metabolites

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

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

Into a 50 ml 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 absorbance was read at 660 nm. A standard graph was prepared using different dilutions of tannic acid and the amount of phenol (mg/g) was calculated.
Estimation of flavonoids by Swain and Hillis (1959)

A 500 mg of the sample was added with 10 ml methanol, homogenized and centrifuged at 3000 rpm for 10 min. The supernatant was used for the estimation of flavonoids.

To a 25 ml conical flask, 1 ml supernatant was taken and diluted to 2 ml with distilled water, 4 ml of vanillin reagent was added rapidly from a burette (within 10-15 sec) to flask A. 4 ml of sulphuric acid was added to flask B into flask C and 4 ml of vanillin reagent and 2 ml of water was added and this was considered as blank. The content of flasks A and B were shaken in a water bath bellow 30° C and they were transferred to room temperature for exactly 15 min. The absorbance was measured at 500 nm against 47% sulphuric acid (Flask D). The absorbances of the contents of flasks B and C were subtracted from that of A. Alternatively, the absorbance of the content of A + D against B + C could be used. A standard curve was prepared using phloroglucinol and amount of flavonoid (mg/g) was calculated.

Estimation of sterol by Sanchez et al. (1972)

A 500 mg of sample was hydrolyzed by reflexing with 25 ml of 3 N HCl at 60° C for 4 h. The solid matter was filtered through Whatman filter paper No. 1 and washed with dilute ammonia until the washing was neutral (pH 6.8-7.00). The sterol was extracted from the residue in soxhlet extractor using chloroform.

The 1 ml of chloroform extract was evaporated to dryness and redissolved in 4 ml of sulphuric acid-methanol reagent. After the reaction was initiated (2 min) the absorbance was noted at 405 nm against blank (the optimum time required for a stable
optical density is 2 min). Standard graph was generated using the proper dilution of cholesterol and the quantity of sterol (mg/g) presented was determined.

**Estimation of alkaloids by Ikan method (1969)**

A 500 mg of sample was extracted with methanol, and the methanol extract was condensed and 20 ml dilute acetic acid (1:5) was added and shaken well in a separating funnel. The acetic acid layer was collected and 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 collected and filtered 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,

\[
\text{Total alkaloid} = \frac{\text{The weight of alkaloid residue (X)}}{\text{Weight of sample (W)}} \times 100
\]

- X - Weight of the residue
- Y - Weight of the empty evaporating dish
- Z - Weight of the empty dish + alkaloid residue

Total (X) = Z - Y