MATERIALS AND METHODS

3.1. Test plant species

During the present study ten plant species viz., *Acorus calamus*, *Cynarospermum asperrimum*, *Rotheca serrata*, *Curcuma leucorrhiza*, *Equisetum hyemale*, *Euphorbia hirta*, *Hyptis suaveolens*, *Melastoma malabathricum*, *Melilotus indicus* and *Tithonia diversifolia* were selected on the basis of their ethnobotanical importance in literature and their traditional uses, especially by different communities of Manipur. The plants were collected, identified and deposited in the Life sciences Department, M.U. The accession no. allotted to the plants are as follows:

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Scientific Name</th>
<th>Ac.No. M.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acorus calamus L.</td>
<td>001423</td>
<td></td>
</tr>
<tr>
<td>Cynarospermum asperrimum (Nees) Vollesen</td>
<td>000869</td>
<td></td>
</tr>
<tr>
<td>Rotheca serrata (L) Steane &amp; Mabb.</td>
<td>000770</td>
<td></td>
</tr>
<tr>
<td>Curcuma leucorrhiza Roxb.</td>
<td>003625</td>
<td></td>
</tr>
<tr>
<td>Equisetum hyemale L</td>
<td>001290</td>
<td></td>
</tr>
<tr>
<td>Euphorbia hirta L</td>
<td>000870</td>
<td></td>
</tr>
<tr>
<td>Hyptis suaveolens L. Poit.</td>
<td>004311</td>
<td></td>
</tr>
<tr>
<td>Melastoma malabathricum L.</td>
<td>000777</td>
<td></td>
</tr>
<tr>
<td>Melilotus indicus (L.) All.</td>
<td>000868</td>
<td></td>
</tr>
<tr>
<td>Tithonia diversifolia (Hemsl.) A. Gray</td>
<td>003624</td>
<td></td>
</tr>
</tbody>
</table>
The systematic position and brief description of these plants are given below:

1. *Acorus calamus* L.

   Plant (Kingdom)
   Phanerogams (Division)
   Angiosperms (Sub-division)
   Monocotyledons (Class)
   Nudiflorae (Series)
   Araceae (Family)
   *Acorus* (Genus)
   *Acorus calamus* (Species)

   *A. calamus* commonly known as Sweet flag, belongs to the family Araceae (Adoraceae). It is also called as *Acorus odoratus*. *A. calamus* is a native of eastern countries and also it is indigenous to the marshes of the mountains of India. It is cultivated throughout India, ascending to an altitude of about 2200 m. It is also found in marshy tracts of Kashmir, Shirmaur (Himachal Pradesh), Manipur and in Naga Hills. It is regularly cultivated in the Koratagere taluka of Karnataka state in peninsular India (Asolkar et al., 1992). *A. calamus* is a perennial herb bearing a long indefinite branched cylindrical rhizome with slender roots. The rhizome is about 3/4 inch in diameter, smooth, pinkish or pale green. It has brown, white and spongy leaf scars. It possesses few distichously alternate leaves.

2. *Cynarospermum asperrimum* (Nees) Vollesen

   Plant (Kingdom)
   Phanerogams (Division)
Cynarospermum asperrimum belongs to the family Acanthaceae, which is considered as one of the advanced and specialized families. Majority of the plants are herbaceous and medicinal (Nema et al., 2012). The plant is an annual prostrate herb and prefers moist hilly areas. The flowers are sessile, bi-lipped and blue. The hairy leaves are opposite and ovate-lancelike.

3. Rotheca serrata (L) Steane & Mabb.

Rotheca (Genus)

Rotheca serrata (Species)
R. serrata also known as Bharngi is a perennial shrub growing upto 3 m in height. The leaves are rough, serrated, tapering at the base and 7-15 cm long and 5 cm broad. The flowers are blue in colour and fragrant. The fruits are purple succulent drupes. The plant bears fruit during April and November. R. serrata is found all over India ascending upto an altitude of 1500 metres. It is found abundantly in Eastern Himalayas, Nepal, Kumaon, Bengal and Bihar.


Plant (Kingdom)
Phanerogams (Division)
Angiosperms (Sub-division)
Monocotyledons (Class)
Coronarieae (Series)
Gingiberaceae (Family)
Curcuma (Genus)
Curcuma leucorrhiza (Species)

C. leucorrhiza belongs to the family Gingiberaceae. The plant is an annual herb. Rootstock large, ovoid, sessile and cylindrical tubers nearly white inside, leaves oblong, large, broad at the middle, plain green and glabrous on both sides, spike vernal coma bracts pink, lobes of the corolla nearly white, flower bract green, flower pale-yellow rather shorter than bract, central lobe of the lip distinctly emarginated.

5. Equisetum hyemale L.

Plant (Kingdom)
Pteridophyta (Sub-Kingdom)
Equisetum hyemale is a pteridophytic perennial plant with branching rootstocks rooted at the internodes and growing upto 1 m in height. The aerial stems are cylindrical, hollow, simple and covered with silica. The cones are terminal. They prefer shady moist soils.

6. *Euphorbia hirta* L.

*E. hirta* is an annual herb belonging to the family Euphorbiaceae. Though a native of Central America it is common throughout India and other tropical and warm temperate regions frequently seen occupying open waste spaces and grasslands, road sides, and pathways. The herb is widely cultivated throughout the tropics, especially in
west, central and east Africa (Adedapo et al., 2005). It is usually erect, slender-stemmed, spreading up to 45 cm tall, though sometimes can be seen lying down (Burkill, 1994). The plant is lactiferous and bears oblong or oblong-lanceolate and elliptic leaves. The stem is hairy with many branches from the base to the top.

7. *Hyptis suaveolens* (L.) Poit.

Plant (Kingdom)

Phanerogams (Division)

Angiosperms (Sub-division)

Dicotyledons (Class)

Gamopetallae (Sub-class)

Bicarpellatae (Series)

Lamials (order)

Lamiaceae (Family)

*Hyptis* (Genus)

*Hyptis suaveolens* (Species)

The plant, *H. suaveolens* commonly known as Wilayati tulsi belongs to the family Lamiaceae and is an ethnobotanically important medicinal plant. Though a native to Tropical America, the plant has now been considered as an obnoxious weed worldwide (Azevedo et al., 2001). It is a fast growing perennial plant growing upto a height of 2 m. They are found growing along roadsides, in over-grazed pastures and around stock yards in dense clumps. When crushed, the plant gives off a characteristic minty smell (Santos et al., 2007). Almost all parts of this plant are being used in traditional medicine to treat various diseases.
8. *Melastoma malabathricum* L.

Plant (Kingdom)

Phanerogams (Division)

Angiosperms (Sub-division)

Dicotyledons (Class)

Polypetallae (Sub-class)

Calyciflorae (Series)

Myrtales (order)

Melastomaceae (Family)

*Melastoma* (Genus)

*Melastoma malabathricum* (Species)

*M. malabathricum* is a small shrub commonly found in previously cleared land, waste places, and roadside throughout the Southeast Asian countries, including Malaysia (van Valkenberg and Bunyapraphatsara, 2001). It is native to tropical and temperate Asia and the Pacific Islands (Ling *et al.*, 2009). The plant is one of the most common weeds that grow wildly and abundantly throughout the tropics including India, especially in the moist areas. They grow up to a height of 12-13 ft. occasionally even up to 20 feet (Burkill, 1966).


Plant (Kingdom)

Phanerogams (Division)

Angiosperms (Sub-division)

Dicotyledons (Class)

Polypetallae (Sub-class)

Calyciflorae (Series)

Rosales (order)
Fabaceae (Family)

*Melilotus* (Genus)

*Melilotus indicus* (Species)

*M. indicus* is an annual herb with erect, slender-branched stems. Leaves are 3-foliolate petiolate, leaflets ovovate or oblanceolate, glabrous, inciso-dentate. Flowers yellow, small in dense flowered axillary and terminal racemes. Calyx teeth deltoid. Stamens 2-adelphous. Pods glabrous obscurely reticulate, 1-seeded (Vardhana, 2008). It grows upto a height of 10 to 50 cm. (rarely to 1 m). Though a native of Northern America, Europe and Asia, the plant is naturalized throughout the world.

10. *Tithonia diversifolia* (Hemsl.) A. Gray

Plant (Kingdom)

Phanerogams (Division)

Angiosperms (Sub-division)

Dicotyledons (Class)

Gamopetallae (Sub-class)

Inferae (Series)

Asterales (order)

Asteraceae (Family)

*Tithonia* (Genus)

*Tithonia diversifolia* (Species)

*T. diversifolia*, commonly called Mexican sunflower, is a common perennial shrub (weed) belonging to the family Asteraceae. It is native to Central America but now it has become naturalized in many tropical countries, including the North Eastern parts of India. It is found in Manipur on road sides, crop fields and waste areas.

The medicinal and traditional uses of these test plant species are summarized in Table 3.1.
Table 3.1: Summary of traditional and medicinal uses of the test plant species.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of the plant</th>
<th>Parts used</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Acorus calamus</em> (Local name: Ok-hidak)</td>
<td>Rhizome</td>
<td>Rhizome is emetic, stomachic, used in dyspepsia, colic, remittent fevers, dysentery of children, insectifuge and in snake bite. Also used in epilepsy and other mental ailments, and glandular and abdominal tumours. Rhizome is also the source of calamus oil which is an essential oil containing asarone and its isomer. The essential oil free alcoholic extract of rhizome possesses sedative and analgesic properties</td>
</tr>
<tr>
<td>2</td>
<td><em>Cynarospermum asperrimum</em> (common name: Hill blepharis)</td>
<td>leaves</td>
<td>Some species of <em>Blepharis</em> are used in fever, malaria and inflammation.</td>
</tr>
<tr>
<td>3</td>
<td><em>Rotheca serrata</em> (Local name: Moirang khanam)</td>
<td>leaves</td>
<td>Leaf extract is given in cold and cough. Root is used in rheumatism and dyspepsia. Seeds are aperient, used in dyspepsia. Leaves used as febrifuge; also employed in external applications for cephalagia and opthalmia.</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Part</td>
<td>Use</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------------------</td>
<td>---------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4</td>
<td><em>Curcuma leucorrhiza</em></td>
<td>rhizome</td>
<td>The rhizome is used in Singbhum, Bihar for treating enlarged liver and spleen and ulcer in stomach. Rhizomes yield a form of arrowroot.</td>
</tr>
<tr>
<td></td>
<td>(Local name: Yai ngou)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Equisetum hyemale</em></td>
<td>shoot</td>
<td>They are highly efficient in treating urinary tract infection, cardiovascular diseases, respiratory tract infection and medical skin conditions.</td>
</tr>
<tr>
<td></td>
<td>(Local name: Lai uttong)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>Euphorbia hirta</em></td>
<td>shoot</td>
<td>Plant is used in worm diseases of children. Plant-juice is given in dysentery, bowel complaints, cough and colic; Plant decoction is used in bronchial affections and asthma. Latex is applied to warts. Plant is also used in diseases of genito-urinary tract.</td>
</tr>
<tr>
<td></td>
<td>(Local name: Pakhangba maton)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>Hyptis suaveolens</em></td>
<td>leaves</td>
<td>Plant is stimulant, carminative, sudorific and lactagogue. Infusion is used in catarrhal conditions, uterus affections and parasitical cutaneous diseases. Roots are chewed with betel nuts as a stomachic, and its decoction is used as an appetizer.</td>
</tr>
<tr>
<td></td>
<td>(Local name: Tukmaan)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8. *Melastoma malabathricum*  
(Local name: Nura khudonglei)  
leaves  
Bark and leaves are used for skin troubles. Plant is used in diarrhea, dysentery and leucorrhoea. Bark and root extract are antiseptic and used as gargle.

9. *Melilotus indicus*  
(Local name: Ingkhol yensil)  
shoot  
Plant is used as a discultient and emollient, externally is used as a fomentation, poultice or plaster for swellings. The seeds are useful in bowel complaints and infantile diarrhea.

10. *Tithonia diversifolia*  
(Local name: Lam numitlei)  
leaves  
Flower-heads are used for wounds and bruises.

### 3.2. Test fungal species

Ten plant pathogenic fungal species including one biocontrol agent (*Trichoderma viride* Pers.) were selected as the test fungi for the present study.

1. *Alternaria alternata* (Fr.) Keissler

   *A. alternata* has been recorded as a causal organism for leaf spot and other diseases on over 380 host species. It is an opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts. It is also pathogenic to human causing upper respiratory tract infections and asthma (Peter *et al.*, 1987).
2. *Alternaria solani* Sorauer

*A. solani* is a fungal pathogen that produces ‘early blight’ disease in tomato and potato plants. Despite the name "early," foliar symptoms usually occur on older leaves. If uncontrolled, early blight can cause significant yield reductions (Olanya *et al.*, 2009). The pathogen is responsible for stem lesions and fruit rot on tomato and tuber blight on potato. It also produces the distinctive "bullseye" patterned leaf spots.


*A. flavus* is one of the predominant fungus responsible for the aflatoxin contamination of crops prior to harvest or during storage (Yu *et al.*, 2004). It also causes diseases of agronomically important crops, such as corn and peanuts. It also causes human invasive aspergillosis and is second only to *A. fumigatus* and is the *Aspergillus* species most frequently reported to infect insects (St. Leger *et al.*, 2000).

4. *Aspergillus niger* Van Tieghem

*A. niger* is one of the most common species of the genus *Aspergillus*. It is a common contaminant of food and causes a disease called black mould on certain fruits and vegetables such as grapes, onions, and peanuts. It is ubiquitous in soil and is commonly reported from indoor environments. It forms black colonies which can be confused with those of *Stachybotrys* (Samson *et al.*, 2001).

5. *Curvularia lunata* (Wakker) Boedijn

*Curvularia lunata* is a predominant pathogen responsible for grain discoloration disease of rice. It affects quantity and quality of rice crop and even lowers drastically the milling recovery, cooking and nutritional qualities.

*Drechslera oryzae* is the causal organism of the brown spot disease of rice. The disease is also referred to as fungal blight. The disease is widely distributed throughout the country, especially in West Bengal, Orissa, Andhra Pradesh and Tamil Nadu (Rangaswami and Mahadevan, 2001).

7. *Fusarium oxysporum* (Schl.) emend. Snyder & Hansen

*F. oxysporum* is the causal organism of wilt diseases of tomato. This is a severe disease of tomato, occurring in many parts of the world including India, where it is found in every state. In some areas where tomato is grown repeatedly in the same soil, the disease is severe. (Rangaswami and Mahadevan, 2001).

8. *Penicillium expansum* Link ex. Fries

*P. expansum* is one of the fungus which causes considerable damage to fruits either on the tree and / or in the transit and storage. It causes soft rot disease of fruits (Rangaswami and Mahadevan, 2001). Among the various post-harvest mycopathogens of apple fruits, *P. expansum* is an important one that can cause infection even below 0°C. Infected fruits produce brown lesions covered by blue spore masses. The pathogen also produce toxic secondary metabolites in the diseased fruit tissue which are reported to exhibit mutagenic, neurotoxic, nephrotoxic and immunosuppressive effect in laboratory animals (Machinsky and Midio, 1995).
9. *Penicillium italicum* Wehmer

*P. italicum* is also an important species responsible for microbial decay of fruits during storage. It causes blue mould rot in citrus fruits (Rangaswami, and Mahadevan, 2001).


*T. viride* is a fungal species often used as a biocontrol agent against a number of plant pathogenic fungi. It is used for seed and soil treatment for suppression of various diseases caused by fungal pathogens. It is also a pathogen and can cause green mould rot of onion.

3.3. Isolation of fungi

The ten fungal species were isolated from different sources using different techniques. *A. alternata, A. solani, P. expansum, P. italicum* and *F. oxysporum* were isolated from the diseased plant parts. For the isolation of *A. alternata*, diseased leaf of bean (*Phaseolus vulgaris*) plant having characteristic young lesions were washed with tap water to clean the surfaces. Small pieces of tissue were then carefully cut out from the edges of the lesion using sterile scissors. Surface sterilization of the leaf pieces was done by dipping the leaf pieces in 10% sodium hypochloride for 1-3 min followed by several rinsing with sterile distilled water. After blotting dry with sterile blotter paper the leaf pieces were aseptically placed onto solidified Czapeck Dox Agar (CDA) plates and incubated for 5 days at 25±1°C. The plates were examined everyday for fungal growth. The fungal colonies observed were isolated in pure culture, identified and maintained on CDA slants. Similar method was employed for the isolation of *P. expansum* and *P. italicum* from infected apple and lemon fruits, respectively. In
case of *F. oxysporum* 1 cm root segments were prepared from tomato plants showing wilt symptoms. The root segments were surface sterilized and plated as described above. The fungal colonies which were identified as *F. oxysporum* were isolated in pure culture and maintained on CDA slants.

*T. viride* was isolated from field soil by soil dilution plate technique described by Parkinson *et al.* (1971). Ten gram field soil was suspended in 100 ml sterile distilled water kept in a 250 ml Erlenmeyer flask and was thoroughly shaken for 15 minutes on a horizontal shaker to get a homogeneous suspension. The suspension was further diluted up to $10^{-4}$ dilution by serial dilution method using sterile distilled water. One ml aliquots of $10^{-3}$ and $10^{-4}$ dilutions were inoculated separately into each of five replicated Petriplates. In each plate 20ml cooled liquefied CDA medium was poured. The Petriplates were rotated clockwise and anticlockwise to mix the homogenates and after solidification the plates were incubated at $25 \pm 1^0\text{C}$. The plates were observed for growth of fungal colonies after 2, 5 and 7 days of incubation. The fungal colonies which were identified as *T. viride* were isolated in pure culture and maintained in agar slants.

*D. oryzae* and *C. lunata* were isolated from the diseased seeds of rice using moist blotter technique as recommended by ISTA (1985). Petriplates (9 cm) lined with three circular moist blotting papers were sterilized by autoclaving. In each plate 25 rice seeds were placed aseptically with uniform spacing and incubated at $27 \pm 1^0\text{C}$ inside BOD incubator. Observation and identification of seed mycoflora up to species level were made after eight days of incubation. Pure culture isolation and maintenance of the test fungal species were made on CDA medium. *A. flavus* and *A. niger* were also isolated from the seeds of groundnut using the moist blotter technique.
The identities of some of the fungal cultures thus isolated from respective diseased plant parts were confirmed at ITCC, IARI, New Delhi and NFCCI, Pune. They are *Alternaria alternata* ITCC Id no. 8246.11, *Aspergillus flavus* NFCCI 2791, *Aspergillus niger* ITCC Id no. 8241.11, *Drechslera oryzae* ITCC Id no. 8240.11, *Fusarium oxysporum* NFCCI 2790 and *Trichoderma viride* ITCC Id no. 8229.11.

3.4. Test bacterial species

The bacterial strains used for testing antimicrobial activities of plant extracts include two Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 and two Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The cultures of the bacterial strains were obtained from the Department of Microbiology, Regional Institute of Medical Sciences (RIMS), Imphal, Manipur, India. The bacterial cultures were maintained on nutrient agar medium (NA) at 37°C and sub-cultured periodically. Stock cultures of the bacteria were also maintained on semi-solid agar at 4°C for further use.

*S. aureus* form part of the normal flora of various parts of the body which includes skin, conjunctiva, nose mouth and oropharynx. It is also a potential pathogen. *E. feacalis* is also an opportunistic pathogen forming part of the gastrointestinal and skin flora. *E. feacalis* may survive in environments due to its intrinsic resistance to commonly used antibiotics. Infections of *S. aureus* and *E. feacalis* may arise endogenously from the patient’s own flora and/or through transmission of direct contact (Rajan, 2007).

*P. aeruginosa* is reported as one of the most common potential wound pathogens causing infection in burn wounds (Ugburo *et al.*, 2004; Mayhall, 2003;
Collier, 2004). *P. aeruginosa* is also an opportunistic and environmental pathogen. Burn wounds infected with *P. aeruginosa* may be difficult to control and eradicate, due to multiresistance to antimicrobial agents (Mayhall, 2003; Arslan *et al.*, 1999). *E. coli* is one of the most common member of the normal flora of the large intestine. It is one of the causal organisms of acute watery diarrhoea and gastroenteritis. They are gram-negative and facultatively anaerobic (Rajan, 2007).

3.5. Microbiological media

(a) Fungal media

Czapeck Dox Agar medium (PH- 5.5)

(i) Sodium Nitrate (NaNO₃) 2.0g
(ii) Magnesium Sulphate (MgSO₄·7H₂O) 1.0g
(iii) Potassium Chloride (KCl) 0.5g
(iv) Potassium dihydrogen phosphate (KH₂PO₄) 1.0g
(v) Ferrous Sulphate (FeSO₄·7H₂O) 0.1g
(vi) Sucrose 30.0g
(vii) Agar agar 20.0g
(viii) Distilled water 1000ml.

(b) Bacterial media

Nutrient Agar Medium (PH-7.5±0.1)

(i) Peptone 10.0g
(ii) Meat extract 10.0g
(iii) NaCl 5.0g
(iv) Agar 24.0g
(v) Distilled water 1000ml.
Mueller Hinton Agar (PH-7.3±0.1)

(i) Mueller Hinton Agar (MHA) 38.0g.
(ii) Distilled water 1000ml.

Semi Solid Media

(i) Agar agar 0.5g
(ii) Distilled water 100ml.

3.6. Preparation of plant extracts

Plant parts of the test plants which were selected for the study were collected, separately washed with tap water and cut into small pieces, dried in the shade and powdered with the help of a blender. The powdered plant material was filled in the thimble and extracted successively with three organic solvents viz., petroleum ether (PE), chloroform (CH) and methanol (ME) using a Soxhlet extractor at 40-60°C. All the extracts were concentrated using rotary flash evaporator, collected separately and preserved at 5°C in airtight brown bottles until further use. These extracts were used for assaying *in-vitro* antimicrobial activities against the test microorganisms.

3.7. Bioassay of plant extracts for antimicrobial activities

3.7.1. Screening for antifungal activity

The *in-vitro* antifungal activity was carried out by following the poisoned food technique as described by Nene and Thapliyal (1979). In the preliminary screening each extract was first dissolved in dimethyl sulphoxide (DMSO) and amended to CDA medium to get the final concentrations of 0.1, 0.5 and 1.0mg/ml of the culture medium. In each Petriplate (9 cm dia) 25ml of CDA medium amended with a plant extract having a particular concentration was poured. Plate containing medium without plant extract was used as control and plate containing 0.1% Carbendazim served as
fungicide control while plate containing respective quantity of DMSO was treated as solvent control. After solidification of the medium an agar culture disc (5mm dia) aseptically removed from 2-3 days old culture of a test fungus was placed at the center of each plate. Three replicated plates were maintained for each treatment. The inoculated plates were incubated at 25\(^{0}\)C for 72 hours. The radial growths of the test fungi were measured and the percent growth inhibition was calculated using the following formula (Vincent, 1927).

\[
I = \frac{(C - T) \times 100}{C}
\]

Where, I= percent growth inhibition

\[ C = \text{Radial growth in control medium – disc diameter (mm)} \]

\[ T = \text{Radial growth in treated medium– disc diameter (mm)} \]

After the preliminary screening the concentration of 0.5mg/ml was found to be suitable and further assessments of antifungal activity were performed using this concentration.

3.7.2. Determination of MIC

Based on the preliminary screening the extracts having potent antifungal activity against certain fungal species were further subjected to the MIC assay. Various concentrations ranging from 0.25mg/ml to 16 mg/ml of the extracts were prepared by adding appropriate quantities of each extract to pre sterilized molten CDA medium and thoroughly mixed with the medium. Twenty milliliter of each treated medium was poured in each 90 mm diameter sterilized Petriplate. Plates were inoculated with each fungal species, incubated and observed for fungal growth as described above. Each treatment was replicated thrice. Minimum inhibitory concentration was recorded as the concentration at which no visible mycelial growth was observed after the incubation period.
3.7.3. Screening for antibacterial activity

Antibacterial activities of the plant extracts were determined using disc diffusion method. The bacterial culture grown in NA medium was suspended in fresh normal saline. The turbidity of the resulting suspension was adjusted to 0.5 McFarland turbidity standard. Sterile MHA plates were swabbed with the culture of the respective bacterial species using sterile cotton swabs and kept for 15 min in laminar chamber for absorption to take place. The sterile filter paper (Whatmann No.1) discs (6mm dia) were impregnated with 20µl of a plant extract solution to achieve desired concentration of 1mg/disc and placed on the inoculated agar plates. Streptomycin (10µg/disc) was used as positive control and DMSO was used as negative control. The antibacterial assay plates were incubated at 37°C for 24h. Each experiment was carried out in 5 replicates and the mean diameter of the inhibition zone of bacterial growth was recorded in mm.

3.7.4. Determination of MIC and MBC

Determination of the minimum inhibitory concentration (MIC) was carried out using the Broth dilution method (Sahm and Washington, 1990; Adesokan et al., 2007; Oyeleke et al., 2008). One ml of reconstituted plant extract solution having a concentration of 100 mg/ml was added to another test tube containing 1 ml of sterile broth so as to obtain a concentration of 50 mg/ml. One ml of this dilution was transferred to another test tube in the same manner till the 8th test tube was reached. The 9th test tube did not contain any extract, but a solution of pure solvent and served as negative control. Then 1 ml of an 18 hr old culture of each of the bacteria earlier adjusted at 10^8 cfu/1ml was inoculated into each tube and thoroughly mixed on a vortex mixer. The tubes were incubated at 37°C for 24 hr and observed for bacterial growth in
form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC.

The MBC values were determined by removing 0.10 ml of bacterial suspension from the MIC tubes that did not show any growth and sub-cultured in MHA plates and incubated at 37°C for 24 hr. The concentration at which no visible growth was observed was recorded as the MBC.

3.8. Effect of plant extracts treatment on rot of apples caused by *Penicillium expansum*.

Based on the results of the preliminary screening, the extracts which were effective against *P. expansum* were further tested against *P. expansum* rot of apples following the methods described by Singh and Sumbali (2007). Rhizomes of *A. calamus* and *C. leucorhiza* were collected and their aqueous extracts were used for study. For preparation of extracts, fresh rhizomes (80g) were cleaned with tap water, followed by sterilized distilled water and separately macerated in 400 ml of sterilized distilled water. The slurry was filtered through double layered cheese cloth to obtain the extract that was used for giving pre- inoculation and post- inoculation dip treatments of the apple fruits.

Fresh and healthy fruits of apple cv. Red Delicious were given pre-inoculation and post-inoculation dip treatments of the plant extracts. In pre- inoculation treatment, weighed fruits prior to inoculation were given a dip in the respective test extracts for 10 min and kept for 12 hr in sterile beakers covered with sterile Petriplates at 28±2°C. After the lapsed of this period, the fruits were bored with a sterile cork borer 5×10mm deep and then inoculated with the spore suspension (10^5 spores ml⁻¹) of the pathogen.
In case of post-inoculation treatment, weighed fruits were first inoculated with the pathogen and incubated at 28±2°C for 12 hr and treated with the plant extract by dipping for 10 min. Same procedure was followed for the fruits which were kept as control, except that in place of different extracts, sterilized water was used for dipping of fruits for the same period. All the treated fruits were separately kept sterile 500ml beakers. Observations based on average of seven replicates for each test extract, were recorded 20 days after inoculation.

At the end of the incubation period, decayed portion of each fruit was removed and were then reweighed. From these observations, % rot and % control were calculated by the following formulae:

\[
\text{% rot} = \left(1 - \frac{w}{W}\right) \times 100
\]

Where, \( W \) = weight of the fruit before inoculation; \( w \) = weight of the fruit after removal of the rotten tissue.

\[
\text{% control} = \frac{\text{% decay in untreated} - \text{% decay in treated}}{\text{% decay in untreated}} \times 100
\]

3.9. Effect of plant extracts on seed mycoflora of rice

The plant extracts which were effective against the storage mycoflora of rice in the earlier experiment (PE extract of \( C. \) leucorrhiza, ME ether extract of \( C. \) leucorrhiza, PE extract of \( A. \) calamus, CH extract of \( A. \) calamus, PE extract of \( H. \) suaveolens, PE extract of \( T. \) diversifolia, CH extract of \( T. \) diversifolia, ME extract of \( T. \) diversifolia, ME extract of \( E. \) hirta, CH extract of \( R. \) serrata, ME extract of \( R. \) serrata) were further tested for their effect against storage mycoflora in rice seeds.
Two hundred gram seeds of rice (var KD-2-6-3) were randomly collected from ten farmers and mixed together and 200g portions were separated for treatment with different plant extracts separately. Seeds were mixed thoroughly with the plant extracts at the rate of 1.5g/kg (wt./wt.) and the treated seeds were sealed separately in sterilized self locking polythene bags (300 gauge) and stored under ambient conditions for three months. Untreated seeds sealed in sterilized polythene bags were kept as control. Seed mycoflora were examined after three months of storage. The method adopted for isolation of seed mycoflora was standard moist blotter technique as recommended by International Seed Testing Association (ISTA, 1985). Petriplates lined with three circular moist blotting papers were sterilized by autoclaving. One plate was treated as one replication and four replications were kept for each treatment. In each plate, 25 seeds were placed aseptically with uniform spacing. The Petriplates were then incubated at 25±1°C inside BOD incubator. Observations and identification of seed mycoflora upto species level were made after eight days of incubation. Pure culture isolations were made on Czapek-Dox-Agar medium whenever necessary. The fungal population was expressed in terms of per cent occurrence for each fungal species with the following formula:

\[ \text{Percent occurrence} = \frac{\text{No. of seeds on which growth of the particular fungal species were detected}}{\text{Total no. of seeds examined}} \times 100 \]

Statistical Analysis

Angular transformations and square root transformations of the values for the data were done where necessary. Data were also expressed as the means ± standard deviations where appropriate. Statistical analysis of the data was carried out by analysis of variance (ANOVA). In all cases P values <0.05 were considered statistically significant.