3. Materials and Methods

3.1 Survey, collection and identification of plants

A survey was carried out during the year 2006-07 in the foothills of Assam-Arunachal Pradesh border and its adjoining areas to collect medicinal plants (Fig. 2). Ethnobotanical evidences were used as lead for collecting plants having antidermatophytic activity (Das, 2008; Gajurel, et al., 2001; Vagasiya et al., 2007; Tamuli and Saikia, 2004; Tambekar and Kharate, 2005; Bhardwaj and Gakhar, 2005; Parekh and Chanda, 2006, Sarmah and Joshi, 2004). Emphasis was also given on the recommended selection criteria such as season, time, environmental conditions, information on traditional uses, easy availability etc. in collecting the plant materials (Dubey et al., 2004). Twenty one plant species were accordingly selected for the present study. Identification of the collected plant species was done on the basis of their morphological and floral characters. The identity of the selected plant species was further authenticated at Botanical Survey of India, Kolkata, India.

3.2 Extraction of plant materials

The fresh leave samples were washed thoroughly with water and dried under shade and crushed into coarse powder for extraction of crude extract.

3.2.1 Extraction

For preliminary screening, each powdered plant material (1000 g) was immersed separately in methanol for 7 days with intermittent shaking (Hayet 2008). The extracts were then filtered through Whatman filter paper No-1 (pore size-11μm). The process was
Fig. 2: Foothills of Assam-Arunachal Pradesh border, the plants collection site
repeated three times to assure exhaustive extraction. The filtrates were pulled in and concentrated to dryness at 40°C under reduced pressure using rotary vacuum evaporator (Heidolph Instruments GmbH & Co. KG, Germany) and were finally lyophilized (The Benchtop FreeZone plus Cascade 4.5L Freeze Dry System, Labconco, USA) to get the crude methanol extracts (Fig. 3). The extracts were tested for antidermatophytic activity by agar well diffusion (Kaushik and Goyal, 2008) and agar dilution (Garcia et al., 2003; Ali-Shtayeh and Ghdeib, 1999) techniques.

![Diagram of steps of extraction]

Fig. 3: Steps of extraction of crude extracts of plants using methanol as solvent
3.2.2 Sequential extraction

Based on the results of preliminary screening of methanol extracts for antidermatophytic activity, *Piper longum* was selected for further study. Powdered sample of *P. longum* leaves was sequentially extracted with petroleum ether, chloroform, methanol and water in their increasing polarity as shown in Fig. 4. Solvents were evaporated under reduced pressure at 40°C and finally lyophilized to get the extracts and recorded their extractive values. All the extracts were kept in air tight glass bottles at -20°C till further use.

![Diagram of sequential extraction](image)

*Fig. 4: Steps of sequential extraction of *Piper longum* using different solvents*
3.2.3 Preparation of plant sample for antidermatophytic assay

Test extracts / isolated fractions / components of desired concentrations were prepared by dissolving the sample in dimethyl sulphoxide (DMSO, w/v), filtered (Millipore filter MILLEX® GP, Ireland, pore size 0.22 μm) and used for *in vitro* assay for their efficacy against a range of dermatophytes as mentioned below employing standard protocols. (Kaushik and Goyal, 2008; Garcia, 2003; Ali-Shtayeh and Ghdeib, 1999; Hammer, 2002)

3.3 Dermatophyte culture and inoculum

3.3.1 Culture medium

Sabouraud dextrose agar (SDA) and sabouraud dextrose broth (SDB) were used for culturing the dermatophytes. The culture media were sterilized by autoclaving at 121°C (15 psi) for 15 minutes.

3.3.2 Dermatophyte culture

The cultures of dermatophyte species namely *Trichophyton mentagrophytes*, *T. rubrum*, *T. tonsurans*, *Microsporum fulvum* and *M. gypseum* were obtained from the School of Tropical Medicine, Kolkata. The cultures were maintained by sub culturing them regularly (1-2 months interval) on SDA and SDB. They were stored at 4°C till further use. A set of the dermatophyte cultures was deposited at MTCC (Microbial Type Culture Collection) and Gene bank, Institute of Microbial Technology, Chandigarh, India. Accordingly the identity of the dermatophyte species was confirmed and obtained the MTCC number of the deposited dermatophyte species.
3.3.3 Preparation of inoculum

Inoculum was prepared on SDA slants by incubating at 28±2°C for 15-20 days depending on the growth rate of the pathogens. Broth inoculum was prepared using SDB with final concentration of 2.5x10⁴ CFU ml⁻¹ was made for the experiments (Hammer et al., 2002).

3.4 In vitro screening for antidermatophytic activity

The antidermatophytic activity of the extracts / fractions was tested by agar well diffusion (Kaushik and Goyal, 2008) and agar dilution method (Garcia et al., 2003; Ali-Shtayeh and Ghdeib, 1999). Five replicates were maintained in each case. The minimum inhibitory concentration of the active extracts and column fractions was determined by agar dilution method. Broth microdilution method was used to determine the MIC of the isolated antidermatophytic components. DMSO was used as negative control while clotrimazole or griseofulvin was used as positive control.

3.4.1 Agar well diffusion method

In agar well diffusion method, the SDA plates (80 mm dia.) were swabbed with 150 µl of the inoculum (2.5x10⁴ CFU ml⁻¹). A well of 8 mm diameter was made in the center of the plate and loaded with 150 µl of the respective test extract / fraction and allowed to diffuse at room temperature for 2 hours. The plates were incubated at 28±2°C for 15-20 days. The activity was determined by measuring the diameter of the zone of inhibition caused by the test sample.

**Percentage inhibition:** Percentage of inhibition was calculated as follows (Vyas et al., 2006)

\[
\text{% Inhibition} = \frac{\text{Inhibition zone in treatment}}{\text{Control}*} \times 100
\]

*Growth zone is equal to plate diameter i.e., 80mm as growth occurs all over the agar plate.
3.4.2 Agar dilution method

Plant extracts / fractions were incorporated separately in sterilized molten medium at desired concentration and inoculated with the dermatophytes and incubated at 28±2°C for 15-20 days. The growth of the mycelia was categorized as:

- : no mycelial growth
+ : growth like a pinhead
++ : growth covers less than 50%
+++ : more than 50% growth

\[ \text{Inhibition\%} = \left( \frac{d_c - d_t}{d_c} \right) \times 100 \]

\( d_c \) = Colony diameter in control
\( d_t \) = Colony diameter in treatment

3.5 Determination of minimum inhibitory concentration value

The minimum inhibitory concentration (MIC) values of the active extracts and column fractions obtained from chloroform extracts of \( P. longum \) were determined by agar dilution method as described above (Garcia et al., 2003). The MIC of the isolated components and the column fractions obtained from methanol extract of \( P. longum \) were determined by broth microdilution assay as described by Hammer et al., (2002) with some modifications.

3.5.1 Broth microdilution method

Filter sterilized stock solution of each sample, prepared in DMSO was serially diluted in 96-well microtiter plate with RPMI 1640 (Rosewell Park Memorial Institute, Himedia) to
obtain a concentration ranging from 39 to 5000 µg ml⁻¹. Inoculum concentration of 2.5×10⁴ CFU ml⁻¹ approximately was adjusted in each well. The plates were incubated at 28 ±2°C for 15-20 days. Clotrimazole and griseofulvin were used as standard. The MIC was interpreted as the lowest concentration of the test samples resulting no visible growth.

3.6 Determination of longevity of the active extracts

The longevity of the chloroform and methanol extract was observed by storing the extract at 4°C and room temperature (25-34°C) for 180 days. The efficacy of the extract (0.5×10⁴ µg ml⁻¹) was tested at one month interval by determining the radial growth of *T. mentagrophytes*.

3.7 Phytochemical analysis

Phytochemical analysis of chloroform and methanol extracts of *P. longum* leaves was performed for the presence of alkaloids, phenolic compounds, tannins and saponins (Harborne, 1998; Falodun et al., 2008; Edeoga et al., 2005). Test solution of 50 mg/ml was prepared in acetone to perform the analysis.

3.7.1 Test for alkaloids, phenolic compounds, tannins and saponins

Presence of alkaloid in the chloroform and methanol extracts of *P. longum* was tested by following the method of Falodun et al. (2008). One ml of the test solution was treated with a few drops of Dragendorfs reagent. Presence of alkaloid was also tested by treating 1 ml of test solution with Mayer’s reagent. The presence or absence of alkaloids in the extracts was observed on the basis of change of colour of the test solution.
For the presence of phenolic compounds, one ml of the test solution was treated with 1% ethanolic ferric chloride. Change of colour to blue green / dark blue indicated the presence of phenolic compounds in the extract (Harborne, 1998).

Presence of tannins in the extract was tested by treating the extract with a few drops of 0.1% ferric chloride and observed for brownish green or a blue-black colouration that indicated the presence of tannins (Edeoga et al., 2005).

Test solution (1 ml) was mixed with 5 ml water and shaken for 2 minutes to observe any stable froth formation and further mixed with 3 drops of olive oil and shaken vigorously. Formation of emulsion indicated the presence of saponins (Falodun et al., 2008).

3.8 Activity guided fractionation of P. longum extract

Based on the results obtained from in vitro study, the chloroform and methanol extracts of P. longum were further fractionated into semi purified fractions through chromatographic techniques like thin layer chromatography (TLC) and column chromatography (Sasidharan 2008; Lee et al., (2005); Hassan et al., 2007).

3.8.1 Thin layer chromatography

Thin layer chromatography was used to select the appropriate mobile phase for column chromatography and to analyze the bioactive molecules. Slurry was prepared by mixing silica gel G with double distilled water in a ratio of 5 : 13 (w/v). TLC plates of 0.5 mm thickness were prepared by spreading the silica gel slurry, by using applicator (JSGW, India). Plates were air dried followed by activation at 120°C for 1 hour. TLC sample was prepared by dissolving the extract/fraction in suitable solvent (ethyl acetate / chloroform)
at a concentration of 10-30 mg/ml. About 2µl of the TLC sample was spotted on TLC plates using a glass capillary. Mobile phases such as petroleum ether-ethyl acetate, hexane-chloroform and hexane-ethyl acetate in different combinations were used in TLC study (Table 1 a, b, c). Silica gel F254 aluminium backed sheet (Merck) was used for TLC study of isolated column fractions / active components using different mobile phases such as petroleum ether, hexane, dichloromethane, ethyl acetate, methanol either alone or in different combinations to suit TLC fingerprint (Table 1 d). Developed plates were assessed at 254 and 365 nm in an ultraviolet fluorescence inspection cabinet (JSGW, Ambala Cantt, India). Iodine vapour was also used for visualization of the spot.
<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Solvent ratio</th>
<th>Mobile phase</th>
<th>Solvent ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Petroleum ether: Ethyl acetate</td>
<td>90 : 10 to 10 : 90 (10% increase in each step)</td>
<td>d. Petroleum ether: dichloromethane Ethyl acetate Methanol</td>
<td>100 % 90 : 10 to 10 : 90 100 % 90 : 10 to 60 : 40</td>
</tr>
<tr>
<td>b. Hexane: Chloroform</td>
<td>90 : 10 to 10 : 90 (10% increase in each step)</td>
<td></td>
<td>Dichloromethane: Ethyl acetate Methanol</td>
</tr>
<tr>
<td>c. Hexane: Ethyl acetate</td>
<td>90 : 10 to 70 : 30 (10% increase in each step)</td>
<td></td>
<td>Ethyl acetate: Methanol</td>
</tr>
</tbody>
</table>
3.8.2 Column chromatography

Chloroform and methanol extracts were separated by column chromatography using glass columns. Silica gel (60-120 mesh / 230-400 mesh, Merck, Mumbai) slurry prepared in the respective solvent (hexane / petroleum ether) was loaded in the column. Plant sample was dissolved in minimum quantity of solvent (chloroform / ethyl acetate) and adsorbed on silica gel. Then solvent was air dried and introduced into the column. Chloroform extract was separated using petroleum ether-ethyl acetate-methanol as mobile phase while methanol extract was separated with two different mobile phases, a) hexane–chloroform and b) hexane-ethyl acetate-methanol. The fractions were collected under gravitational flow. The first few fractions were collected with non polar solvents (hexane / petroleum ether). The solvents were used as a gradient system with 10% increase in each step (Table 2 a, b, c). The fractions obtained from first step column chromatography from choloform extract were further fractionated in the same way using mobile phase petroleum ether-dichloromethane-ethyl acetate-methanol as a gradient system with 10% increase in each step (Table 2 d).

The eluted fractions were analyzed using TLC method and the fractions having similar TLC profiles were combined together. Collected fractions were concentrated to dryness at 40°C under reduced pressure. The dry weight of the fractions was recorded and stored in airtight bottles at -20°C till further analysis.
Table 2 Different solvents and their combinations used as mobile phases in column chromatography

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Solvent ratio</th>
<th>Mobile phase</th>
<th>Solvent ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. For chloroform extract</td>
<td></td>
<td>b. For methanol extract</td>
<td></td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>100%</td>
<td>Hexane</td>
<td>100%</td>
</tr>
<tr>
<td>Petroleum ether : Ethyl acetate</td>
<td>90 : 10 to 10 : 90</td>
<td>Hexane : Chloroform</td>
<td>90 : 10 to 10 : 90</td>
</tr>
<tr>
<td>(10% increase in each step)</td>
<td></td>
<td>(10% increase in each step)</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>100%</td>
<td>Chloroform</td>
<td>100%</td>
</tr>
<tr>
<td>Ethyl acetate : Methanol</td>
<td>90 : 10 to 60 : 40</td>
<td>Ethyl acetate in Methanol</td>
<td>90 : 10 to 60 : 40</td>
</tr>
<tr>
<td>(10% increase in each step)</td>
<td></td>
<td>(10% increase in each step)</td>
<td></td>
</tr>
<tr>
<td>c. For methanol extract</td>
<td></td>
<td>d. For 2nd Step Column chromatography</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>100%</td>
<td>Petroleum ether</td>
<td>100%</td>
</tr>
<tr>
<td>Hexane : Ethyl acetate</td>
<td>90 : 10 to 10 : 90</td>
<td>Petroleum ether : Dichloromethane</td>
<td>90 : 10 to 10 : 90</td>
</tr>
<tr>
<td>(10% increase in each step)</td>
<td></td>
<td>(10% increase in each step)</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>100%</td>
<td>Dichloromethane</td>
<td>100%</td>
</tr>
<tr>
<td>Ethyl acetate : Methanol</td>
<td>90 : 10 to 60 : 40</td>
<td>Ethyl acetate in Methanol</td>
<td>90 : 10 to 60 : 40</td>
</tr>
<tr>
<td>(10% increase in each step)</td>
<td></td>
<td>(10% increase in each step)</td>
<td></td>
</tr>
</tbody>
</table>
3.8.2.1 Chloroform extract

Chloroform extract was separated by column chromatography (column length x diameter - 400 x 40 mm). Preactivated silica gel, 230-400 mesh (125 g) was loaded as slurry prepared in petroleum ether. The chloroform extract (1.25 g) was dissolved in minimum quantity of chloroform and adsorbed on 12.5 g preactivated silica gel (230-400 mesh). The solvent was air dried and loaded into the column. Mobile phase petroleum ether-ethyl acetate-methanol was used as a gradient system with 10% increase (Table 2 a). The first few fractions were collected with petroleum ether. Forty five fractions were collected and concentrated. An aliquot of each concentrated fraction was loaded on the TLC plate. The TLC was developed using hexane: ethyl acetate (70:30). Developed plates were air dried for 30 minutes. The spots were observed under UV at 254 and 365 nm. Fractions having similar TLC profile were combined into nine major fractions (F₁…. F₉). The dry weight of each fraction was recorded after evaporating the solvents under reduced pressure at 40°C and stored in airtight bottles at -20°C till further analysis. The process was repeated four times to obtained sufficient amount of active components.

All the combined fractions were tested for antidermatophytic activity against *T. mentagrophytes* by agar well diffusion method. From the activity point of view four fractions, fraction-1, fraction-4, fraction-5 and fraction-7 were selected for further purification. The first column fraction was subjected to chromatographic and spectroscopic analysis and the fraction-4, Fraction-5 and Fraction-7 were further purified by column chromatography as described below.
3.8.2.1.1 Column fraction-1

TLC profile was observed with mobile phase hexane-ethyl acetate (3:1) and hexane (100%). Chromatographic and spectroscopic analysis such as UV, HPLC and GC-MS were performed to find out the nature of the active compounds present in the fraction.

3.8.2.1.2 Column fraction-4

The column fraction-4 was dissolved in dichloromethane and TLC was performed (Silica gel F_{254} aluminium backed sheet) using dichloromethane and various combinations of hexane, dichloromethane and ethyl acetate as mobile phase to select the mobile phase for further column chromatography. Further separation was done through column chromatography using glass column (column length x diameter - 150 x 20 mm). Preactivated silica gel, 230-400 mesh (33.0 g) was loaded as slurry prepared in petroleum ether. The fraction (0.330 g) was dissolved in minimum quantity of dichloromethane and adsorbed on 3.3 g of preactivated silica gel. The solvent was air dried and loaded into the column. Petroleum ether- dichloromethane-ethyl acetate-methanol (Table 2d) was used as mobile phase with 10% increase in their combinations in each subsequent step. Sub fractions (20 ml each) were collected and pooled according to their TLC profiles. Of these, sub fraction-4 was analyzed by GC-MS to identify the compounds.

3.8.2.1.3 Column fraction-5

Column fraction-5, obtained from the first step column chromatography was further subjected to purification. TLC screening of the fraction with various combinations of solvents as mobile phase, such as, hexane-ethyl acetate, dichloromethane-ethyl acetate
and dichloromethane (100%) was done. Further purification of the fraction was done through silica gel column chromatography (column length x diameter-150 x 20 mm, silica gel 230-400 mesh) was performed using petroleum ether-dichloromethane-ethyl acetate-methanol as mobile phase (Table 2d). Column was packed with slurry of preactivated silica gel (18.0 g) prepared in petroleum ether. The fraction (0.18 g) was dissolved in minimum quantity of dichloromethane and adsorbed on 1.8 g of preactivated silica gel. The solvent was air dried and introduced into the column. The collected fractions were pooled into 27 major fractions based on their TLC profile. Sub fraction 10, 17 and 24 were studied further. Sub fractions-10 and 24 were recrystallized from methanol and petroleum ether respectively. Finally the purified components from the sub fraction 10, 17 and 24 were subjected to GC-MS analysis.

3.8.2.1.4 Column fraction-7

Column fraction-7 obtained from the first step column chromatography was further purified through column chromatography. The fraction was dissolved in dichloromethane and TLC was performed (Silica gel F254 aluminium backed sheets) using dichloromethane and various combination of hexane, dichloromethane and ethyl acetate as mobile phase to select appropriate mobile phase for column chromatography. Separation of the components was done using glass column (column length x diameter -150 x 20 mm). Preactivated silica gel, 230-400 mesh (32.0 g) was loaded as slurry prepared in petroleum ether. The fraction (0.32 g) was dissolved in minimum quantity of dichloromethane and mixed with 3.2 g of preactivated silica gel. The solvent was air dried and loaded into the column. Mobile phase petroleum ether-dichloromethane-ethyl acetate-methanol was used
with 10% increase in their combinations in each subsequent step. Sub fractions (20 ml each) were collected and pooled according to their TLC profiles. Total 26 sub fractions were collected, of which sub fraction-7, eluted with 20% petroleum ether in dichloromethane was recrystallized from methanol to obtain a crystal like compound.

3.8.2.1.5 Determination of MIC of the isolated fractions / components from chloroform extract

MIC values of the isolated components (1, 4, 5a, b & c, and 7) obtained from repeated column chromatography were determined employing broth microdilution assay.

3.8.2.2 Methanol extract

Column chromatography was performed using two different mobile phases- a) hexane–chloroform and b) hexane-ethyl acetate-methanol to purify active components present in methanol extract. Methanol extract (1 g for each column) was eluted through a column of silica gel (60-120 mesh, 100 g, column length x diameter- 300 x 40 mm) using mobile phases, a) hexane-chloroform and b) hexane-ethyl acetate-methanol as a gradient system with 10% increase in their combinations in each subsequent step.

3.8.2.2.1 Mobile phase: Hexane–chloroform

Silica gel was loaded as slurry prepared in hexane. The extract (1.0 g) was dissolved in minimum quantity of chloroform and introduced into the column. The fractions were eluted with mobile phase hexane-chloroform in their increasing polarity with 10% increase in their combinations in each subsequent step (Table 2b). The fractions having similar TLC profiles were combined together. All the fractions were concentrated to
dryness at 40°C under reduced pressure using rotary vacuum evaporator. The dry weight of the fractions was recorded and stored in airtight bottles at -20°C till further analysis.

3.8.2.2 Mobile phase: Hexane-ethyl acetate-methanol

Preactivated silica gel (60-120 mesh, 100 g) prepared in hexane was loaded in the column. The extract (1.0 g) was dissolved in minimum quantity of ethyl acetate and loaded into the column. The solvent system hexane-ethyl acetate-methanol was used in their increasing polarity as mentioned above (Table 2c) and fractions were collected. Based on TLC profiles, similar fractions were combined. The solvents were evaporated under reduced pressure at 40°C and the dry fractions stored in airtight bottles at -20°C till further analysis.

3.8.2.3 Antidermatophytic evaluation of column fractions from methanol extract

Antidermatophytic activity of the column fractions obtained from methanol extract was performed against *T. mentagrophytes* by agar well diffusion method. MIC was determined by broth microdilution assay (Hammer 2002).

3.9 Characterization of the isolated fractions / components

3.9.1 High performance liquid chromatography (HPLC)

The isolated fractions were tested for purity using reverse-phase HPLC (Waters Delta 600 Pump with Inline Degasser, Waters 2996 Photodiode Array Detector), injecting the sample (20 μl, 1000 ppm) into C18 Hypersil ODS column and detected at 280 nm. The gradient programme used for mobile phase with hexane: ethyl acetate was as follows, 100:0, 80:20, 50:50 and 0:100 (v/v) with a flow rate of 1ml/min.
3.9.2 UV spectroscopy (UV)

UV-Visible spectrum of the isolated fractions was recorded on a UV-Visible spectrophotometer (Thermo Electron Corporation, Model-UV1) at room temperature. Test sample of 250 ppm, prepared in acetone, was used to record the spectrum (200-800 nm).

3.9.3 Gas chromatography mass spectroscopy (GC-MS)

The GC-MS analysis was performed in EI mode on a GCMS, Perkin Elmer, Turbomass gold, GC-Autosample xL (Perkin Elmer International, Boesch, Huenenberg, Switzerland) system with fused capillary column Elite-1, dimethylpolysiloxane, 30 m x 0.25mm x 0.25µm directly coupled to mass detector. The mass spectrometer was operated at 70 eV. Injection conditions were as follows: Column temperature 40 - 250°C at a rate of 4°C/1 min; carrier gas was He: 1ml/min; sample injection volume 1 µL. The bioactive constituents were identified based on the comparison of mass spectra with those of data available in the National Institute of Standards and Technology libraries (NIST/EPA/NIH mass spectral library).

3.9.4 Fourier-transform infrared spectroscopy (FT-IR)

IR spectrum of the sample was recorded on FT-IR spectrophotometer (Model-Nicolet-Impact 1-410). The sample was mixed with IR grade KBr powder using agate mortar and pestle and pressed into pellet before measurement. A blank was prepared with IR grade KBr with out of sample. The pellets were placed in the pellet holder of the instrument and a region from 400 to 4000 cm\(^{-1}\) was used for scanning (Kemp, 1991).
3.9.5 Nuclear magnetic resonance spectroscopy (NMR)

$^1$H NMR and $^{13}$C NMR spectra of the isolated samples were recorded on a NMR spectrophotometer at 400 MHz (Model-400 MHz NMR Spectrophotometer, Jeol). Sample was dissolved in CDCl$_3$ and used for recording the spectra.

3.9.6 Authentication of *Piper longum* by DNA fingerprinting

3.9.6.1 Plant materials and DNA extraction

Twenty *Piper* samples belonging to *P. longum*, *P. acutistigmum*, *P. betle*, *P. sylvaticum* and *P. betleoides* were collected from foothills of Assam-Arunachal Pradesh border and its adjoining areas. Genomic DNA was isolated from the young tender leaves, using Qiagen DNeasy Plant Mini Kit according to manufacturer’s instructions.

All centrifugation steps were performed at room temperature (15-25°C). The plant samples were ground in liquid nitrogen and 100 mg of the powdered material was added to 400 µl of Buffer AP$_1$ and 4µl RNase. The mixture was incubated at 65°C for 10 minutes after vortexing. Buffer AP$_2$ (130 µl) was added to the homogenate and incubated for 5 minutes on ice. The sample was centrifuged at 14000 rpm for 5 minutes (Sigma 3-30K) and the supernatant was pipetted into a QIA shredder Mini spin column in 2 ml collection tube and again centrifuged at 14000 rpm for 2 minutes. The flow through fraction was transferred into a new tube without disturbing the pellet. 1.5 µl of buffer AP$_3$/E was added to the fraction. The mixture (650 µl) was transferred into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged for 1 minute at 8000 rpm. The
flow through liquid was discarded. The step was repeated with the remaining samples. The spin column was placed into a new collection tubes and mixed with 500 µl of Buffer AW and centrifuged for 1 minute at 8000 rpm. Then another 500 µl of Buffer AW was added to spin column and centrifuged for 2 minutes at 14000 rpm. The spin column was transferred to a new 1.5/2.0 ml mini centrifuge tubes, added 100 µl Buffer AE to it for elution, incubated for 5 minutes at room temperature and centrifuged for 1 minute at 8000 rpm to collect the genomic DNA. Purity of the isolated DNA was checked by resolving it on 0.8% agarose gel.

3.9.6.2 PCR amplification

The components of the PCR amplification reaction was optimized as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>0.5 mM dNTPs</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>0.2 mM MgCl₂</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (3u)</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>Primer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>DNA (50 ng/µl)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>16.2 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>25.0 µl</td>
</tr>
</tbody>
</table>

PCR amplifications for seven random primers (series of OPA, OPC and OPN) were performed using thermal cycler (Applied Biosystem thermal cycler). The PCR amplification condition was optimized as follows:-
**PCR amplification condition**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Annealing</td>
<td>40°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Total cycles</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

The resulting products were separated electrophoretically in 1.2% agarose gel stained with ethidium bromide (5 μg/ml) in TAE buffer and the resultant amplified DNA profile, called RAPD fingerprint of the individual samples were photographed and analyzed.

### 3.9.6.3 RAPD analysis

Presence or absence of amplified band was scored for each primer. Faint or poorly amplified fragments were excluded from the analysis. Percentage of polymorphism was calculated as the proportion of amplification products which were polymorphic across all the samples to the total number of amplified products.