3. MATERIALS AND METHODS

3.1. Direct organogenesis

3.1.1. Direct organogenesis from nodal segments

3.1.1.1 Establishment of aseptic culture

*A. lanata* were collected from the basin of river Cauvery during January, 2012 and the plants were raised in the medicinal plant garden of Jamal Mohamed College, Tiruchirappalli, India. Nodal segments from *ex vitro* mother plants were used as initial explants. They were washed in running tap water for 10 min, soaked in 5% (v/v) teepol for 2 min, surface sterilized with 0.2% mercuric chloride for 10 min, and rinsed 3 times with sterile distilled water. After that, they were blotted using sterilized Whatman filter paper and allowed to dry naturally. They were cut into small pieces (0.5 cm in size) and inoculated on MS basal medium (Murashige and Skoog, 1962), supplemented with 1.0 mg L⁻¹ BAP and 0.25 mg L⁻¹ NAA. The polarity of the shoots was maintained during inoculation.

3.1.1.2. Culture media and conditions

Culture medium containing MS salts supplemented with macro and micro-elements, 3% (w/v) sucrose and 0.8% (w/v) agar was used as gelling agent. The pH of the medium was adjusted to 5.6 to 5.8 using 1N NaOH before being autoclaved at 121°C for 20 min. All the cultures were maintained at 25 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 40 μmol m⁻²s⁻¹ light intensity provided by white fluorescent lamps and a relative at 55-65% relative humidity.
3.1.1.3. Effect of PGRs for shoot induction and multiplication

For multiple shoot induction, the nodal segments were placed on MS medium supplemented with various cytokinins TDZ and BAP at different concentration (0.5, 1.0, 2.0 and 3.0 mg L\(^{-1}\)) either singly or in combination with NAA (0.25, 0.5 and 1.0 mg L\(^{-1}\)). After shoot induction, they were transferred to MS medium containing 2.0 mg L\(^{-1}\) TDZ in combination with 0.5 mg L\(^{-1}\) NAA for shoot multiplication. The percentage of explants forming shoots and the number of shoots per explants were recorded after 7 weeks of inoculation.

3.1.1.4. Rooting and establishment of plantlets

Elongated shoots from each explant (4–5 cm long) were excised and transferred to full strength MS as well as 1/2 strength MS medium containing different concentration of auxins IAA or IBA (0.5, 1.0, 1.5 and 2.0 mg L\(^{-1}\)) for root induction. The percentage of rooting and the mean number of roots per shoot were recorded after 4 weeks.

3.1.1.5. In vitro flowering

For in vitro flowering, nodal derived shootlets from 4 weeks culture were transferred to MS basal medium containing 1.0 mg L\(^{-1}\) TDZ in combination with 0.5 mg L\(^{-1}\) NAA.

3.1.1.6. Statistical analysis

Twenty tubes with media of each concentration were taken. All experiments were repeated thrice. The results are expressed as a mean ± SE of three independent experiments. Data were analyzed by analysis of variance (ANOVA) to detect significant differences at \( P < 0.05 \) using Duncan’s multiple range tests, using SPSS (version 16.0) software.
3.1.2. Effect of explants on direct organogenesis

3.1.2.1 Explant source

Different explants such as nodes of vegetative stage, just before flowering and after mature flower bud node (length 0.5-1 cm) from actively growing branches of *ex vitro* plants were collected and used for inoculation. These explants were properly cleaned with running tap water for 10 min and soaked in 5 % (v/v) teepol for 2 min. Finally, surface sterilization of explants was done in a laminar flow cabinet with 0.2 % (w/v) mercuric chloride solution for 7–10 min and washed repeatedly with sterile distilled water to remove all traces of sterilizing agents. After surface sterilization, they were used for direct organogenesis. Similarly, same type of explants (nodes of vegetative stage, just before flowering and after mature flower bud node) from *in vitro* raised plants were also excised and subjected to direct organogenesis.

3.1.2.2. Effect of explant and growth regulators on shoot induction and multiplication

MS medium supplemented with various concentrations of plant growth regulators BAP (0.2, 0.5, 1.0 mg L⁻¹) and KIN (0.2, 0.5, 1.0 mg L⁻¹) was used for induction and multiplication of shootlets. Cultures were sub-cultured onto fresh media after every 3 weeks. The frequency of explants producing shoots, number of shoots per explants and shoot length were recorded after 4 weeks.

3.1.2.3. Effect of auxins for rooting

Regenerated shoots (4-5 cm long) were excised and placed vertically into rooting media composed of full or 1/2 strength MS basal salt supplemented with different concentrations (0.25, 0.5 and 1.0 mg L⁻¹) of the
auxins (IBA, NAA), 3 % sucrose and solidified with 0.8 % agar. Cultures were maintained in a 16-h photoperiod in the conditions described above. After 4 weeks, rooting percentage and the number of roots per responding shoot were recorded.

3.1.2.4. Histological analysis

Nodal segments, young flower bud node and mature flower bud node explants both from ex vitro and in vitro plants were used for histological studies. Standard procedures were followed for histological studies (Nakano et al., 2006). For anatomical analysis, free hand sections were performed using a razor blade. The sections were mounted on glass slides and allowed to dry for at least 5 min before staining. All the sections were stained using 0.025% Safranin O and Toluidine blue for 5 min. The prepared slides were examined under a bright field microscope and they were photographed using digital camera (Olympus, Japan).

3.1.2.5. Statistical analysis

The statistical analysis was performed according to the V 16.0 SPSS system. Mean and standard errors were used throughout, and the statistical significance between the mean values was assessed applying a Duncan’s multiple range tests. A probability of $P < 0.05$ was considered significant.

3.1.3. Effect of liquid and solid medium on shoot proliferation

3.1.3.1. Effect of cytokinins for shoot induction

Three month old in vitro raised node and shoot bud segments of *A. lanata* were used in this experiment. In order to optimize the concentration
of cytokinins for shoot induction and proliferation, MS solid medium with different concentrations of cytokinins TDZ (0.3, 0.6, 0.9 mg L⁻¹) and BAP (0.3, 0.6, 0.9 mg L⁻¹) were used. All media were adjusted to pH 5.6-5.8 before autoclaving for 20 min at 121°C. The cultures were incubated at 24 ± 2°C under 16/8 (light and dark cycle) photoperiod (40 μmol m⁻² s⁻¹) and irradiance provided by cool-white fluorescent (Philips, India). Further, after 2 weeks shoot induction cultures were recorded.

3.1.3.2. Effect of multiple shoot proliferation

The comparative increments in shoot multiplication rates in both solid and liquid MS medium supplemented with concentration of 0.6 mg L⁻¹ TDZ and 0.6 mg L⁻¹ BAP in combination of 0.3 mg L⁻¹ NAA, 0.2 mg L⁻¹ IBA were observed. Different growth parameters such as shoot length, root length, number of shoots/explants, fresh weight and dry weight (60°C) were recorded. Observations of clusters of plantlets were recorded after 4 weeks of culture.

3.1.3.3. Optimization of liquid medium volume for shoot proliferation

In order to optimize the liquid medium conditions for growth and shoot proliferation, different volumes of MS basal liquid medium (10, 20, 30, 40 and 50 ml) were supplemented with concentrations of 0.6 mg L⁻¹ TDZ combination with 0.3 mg L⁻¹ NAA, 0.2 mg L⁻¹ IBA and sucrose (3.0 %) were tested in conical flask. Mean number of shoots produced per explants were recorded at regular intervals of 14, 21, 28 and 35 days. These same medium after 14 days root development and various parameters like root induction period, root length and branching of root were recorded after 4 weeks of culture. The type and concentration of auxin were optimized subsequently to induce rooting in both solid and liquid medium.
3.1.3.4. Statistical analysis

All the experiments were repeated three times and each with three replicates. Data were recorded after 4 weeks of culture for multiple shoot formation in liquid culture. Data were statistically analyzed using of variance (ANOVA). Data were presented as mean ± standard error (SE). The mean separations were carried out using Duncan’s multiple range test and significance was determined at \( p < 0.05 \) was performed.

3.1.4. Direct organogenesis from leaf explants

3.1.4.1. Plant material and culture condition

*In vitro* raised leaf explants were used for this experiment. The pH of media was adjusted 5.7 ± 0.1 before autoclaving at 121°C and 104 kPa for 15 min. All experiments were performed using MS solid medium with 3 % (w/v). Cultures were maintained at 25 ± 2°C, 16 h photoperiod under 40 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) light intensity provided by white fluorescent tubes and a relative humidity at 55-65%.

3.1.4.2. Influence of TDZ on organogenesis

To study the role of TDZ on shoot organogenesis, young leaves were sectioned (approximately 0.5 x 0.5 cm) from 20 days old *in vitro* raised plants and incubated on MS medium supplemented with 0.25-2.0 mg L\(^{-1}\) TDZ. The frequency of shoot regeneration and the number of shoots per leaf explants were recorded after 7 weeks (starting from the initial day of inoculation).
3.1.4.3. Influence of in vitro flowering

To test the influence of in vitro flowering, shootlets from 4 weeks culture were transferred to MS basal medium containing 1.0 mg L\(^{-1}\) TDZ alone or in combination with 0-1.0 mg L\(^{-1}\) indole-3-acetic acid (IAA) or NAA.

3.1.4.4. Rooting and establishment of plantlets

For root development, regenerated shoots (4-5 cm long) were excised and cultured on half strength MS medium containing 0.5-2.0 mg L\(^{-1}\) IBA for 1 week, then plantlets were moved to pots filled with soil: perlite: vermiculate (1:1:1; v/v/v) mixture and acclimatized for 2 weeks under higher humidity before transferring to garden pots (Soundar Raju et al., 2013).

3.1.4.5. Histological analysis

The origin of the adventitious shoots was studied using histological analysis. Standard procedures were followed for histological studies (Nakano et al., 2006). The samples were fixed for 24 h in FAA (70 % ethanol: formalin: acetic acid = 90:5:5; v/v/v), dehydrated in a graded ethanol series, and embedded in paraffin (58 °C). Sections (~10 μm thick) were stained with toluene blue O. The prepared slides were examined through a light microscope (Leica®, Switzerland), and all the images were photographed using digital camera (Nikon®, Japan).

3.1.4.6. Statistical analysis

All experiments were conducted using a completely randomized design and each experiment consisted of five explants per culture tube and 15 replicate tubes per treatment. Percentage of shoots producing roots and the
numbers of roots formed per shoot were recorded 3 weeks after inoculation in IBA media. Data were subjected to a one way ANOVA followed by statistical significance test. The significant differences among the mean ± standard error were carried out using Duncan’s multiple range tests and significance level of $P < 0.05$. (IBM® SPSS ver. 19).

3.2. Indirect organogenesis

3.2.1. Induction of callus

Four-five weeks old in vitro raised leaf and nodal explants of 1.0 cm in length were inoculated on MS medium supplemented with different concentration of 2,4-D (0.0 - 2.0 mg L$^{-1}$), NAA (0.1 - 1.0 mg L$^{-1}$) either alone in combinations for callus induction results were recorded after 3 weeks of culture initiation.

3.2.2. Shoot proliferation and maintenance

Callus regenerated from the explants on callus induction medium was separated and cut into small pieces and transferred to the MS basal medium supplemented with different combination and concentration of BAP (0.2 - 2.0 mg L$^{-1}$), KIN (0.2 - 2.0 mg L$^{-1}$) and NAA (0.1 - 1.0 mg L$^{-1}$) for shoot development and elongation. Elongated shoots were subcultured after every 3 weeks. The percentage of shoots per callus culture, shoot length and root length were recorded after 5 weeks.

3.2.3. In vitro Rooting and acclimatization

Healthy shoots of 2-4 cm in height were transferred for root induction on MS medium supplemented with different concentrations of IBA (0.25 - 2.0 mg L$^{-1}$). The percentage of shoots with roots, number of roots per shoots and
roots length were recorded after 4 weeks of culture. Plantlets with well developed roots were removed from the culture medium, washed gently under running tap water, and transferred to soil : perlite : vermiculate (1 : 1 : 1; v/v/v) mixture in paper cup under hardening conditions. The percentage of plant survival rate 86.0 % was recorded after 5-7 weeks.

3.2.4. Histological analysis

Explant pieces before and after differentiation were selected for histological studies and fixed in FAA (2:1:17, v/v/v) for 24 hrs serial grades of alcohol were followed by embossing in paraffin wax (Dam et al., 2010). Thin sections (4-10 μm) were using hand section. The sections were mounted on glass slides and allowed to dry for at least 10 min before staining. Finally they were stained with 0.025 % toluidine blue O or safranin O and mounted in DPX (BDH). The prepared slides were examined through a light microscope (Leica®, Switzerland) and photographed.

3.2.5. Statistical analysis

The callus induction, shoot regeneration and rooting experiment were conducted with a total of twenty replicate per treatment and were repeated thrice. The data were subjected to analysis of variance (ANOVA) and statistical significance between mean values followed by Duncan’s multiple range test at a significance level of P ≥ 0.05 (IBM SPSS ver. 19).

3.3. Synthetic seed technology

3.3.1. Explant source

Nodal segments (3-5 mm) excised from 3-5 week old in vitro grown plantlets were used as explants for the production of synthetic seeds.
3.3.2. Encapsulation matrix and complexing agent

Sodium alginate was used as gelling agent and prepared in different strength of MS liquid medium containing 3 % sucrose and 0.5 mg L\(^{-1}\) BAP. Different concentrations (1, 2, 3, 4 and 5 % (w/v)) of sodium alginate were used. For complexion, 25, 50, 75, 100 and 200 mM calcium chloride (CaCl\(_2\).2H\(_2\)O) solution was prepared in distilled water. The pH of the gel matrix and the complexing agent and pH was adjusted to 5.6-5.8 prior to autoclaving at 121°C for 20 min.

3.3.3. Encapsulation of explants

Encapsulation was accomplished by mixing the nodal segments with sodium alginate solution and dropping them in CaCl\(_2\).2H\(_2\)O solution using a pipette. The droplets containing the explants were held at least for 20–25 min to achieve polymerization. The alginate beads containing the nodal segments were retrieved from the solution and rinsed twice with sterilized double distilled water (DDW) to remove the traces of CaCl\(_2\).2H\(_2\)O and transferred to sterile filter paper in Petri dishes for 5 min under the laminar airflow cabinet to eliminate the excess of water and thereafter transferred to culture vials containing nutrient medium.

3.3.4. Media and culture conditions

The encapsulated nodal segments (alginate beads) were transferred to the petriplate (Borosil, India) containing MS basal medium without plant growth regulator (PGR) and MS solid and liquid medium supplemented with BAP at various concentrations (0, 0.25, 0.5, 0.75 and 1.0 mg L\(^{-1}\)) either alone or in combination of IBA (0.25 mg L\(^{-1}\)). The culture medium was gelled with 0.8% (w/v) agar and pH was adjusted to 5.6-5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 24 ± 2°C under 16/8 h light–dark condition.
3.3.5. Low temperature storage

The encapsulated nodal segments were kept in two sterile beakers properly covered with aluminum foil and stored in refrigerator at 4°C in dark in different duration (0, 15, 30, 45, 60 and 90 days) were evaluated for conversion of synseeds into plantlets. After each storage period, ten encapsulated nodal segments were transferred to MS medium containing optimal concentration of PGRs for conversion into plantlets. During storage period the beads were sprayed with sterile DDW after every 2 weeks to ensure the moist conditions so that the beads may not shrink by losing water. The percentage of shoot regeneration of encapsulated nodal segment was recorded after 5 weeks of culture. The plantlets developed from encapsulated nodal segments were hardened and acclimatized as specified below.

3.3.6. Ex vitro germination of encapsulated seeds

Encapsulated nodal segments were also transferred to sterile soilrite a mixtures of soil: vermicompost (1:1), sand: soil: vermicompost (1:2:1) and sand: soil: coconut fiber: vermicompost (1:1:1:1) for ex vitro conversion and recovery of complete plantlets. The soilrite was regularly moistened with 1/2-strength MS salt solution (without vitamins and sucrose) after every 4 days. The conversion response was recorded after 5 weeks of sowing.

3.3.7. Acclimatization

Well-developed plantlets regenerated from encapsulated nodal segments were transferred to polythene cover containing three different types of mixtures such as soil:vermicompost (1:1), sand:soil:vermicompost (1:2:1), sand:soil:coconut fiber:vermicompost (1:1:1:1) moistened with MS liquid.
medium. After 2-4 weeks, the polythene cover was transferred to sunlight, initially for a short time, and gradually the time was increased. Percentage of survival was recorded after the development of 1-2 months.

3.3.8. Statistical analysis

The frequency of plantlet regeneration was calculated as the percentage of encapsulated nodal showing well-developed shoot and root out of total number of nodal explant encapsulated. For each experiment 24 replicates were used and each experiment was repeated thrice. The mean standard error and one-way ANOVA were calculated using SPSS (version 16.0) software. The mean separations were carried out using Duncan’s multiple range tests and significance was determined at $P < 0.05$.

3.4. Adventitious root culture

3.4.1. Plant material

The in vitro raised leaf explants were cut into approximately 0.5-1.0 cm pieces for adventitious root induction.

3.4.2. Induction of adventitious roots from leaf explant

The leaf explants were inoculated onto MS solid media under different concentrations of IBA (0.5, 1.0, 2.0 and 3.0 mg L$^{-1}$), NAA (0.5, 1.0, 2.0 and 3.0 mg L$^{-1}$) and IAA (0.5, 1.0, 2.0 and 3.0 mg L$^{-1}$) with 3 % sucrose and 0.8 % agar, the pH 5.6-5.8 of the medium was adjusted to before autoclaving (121°C, 15 min). Each Petri dish (9 cm in diameter and 1.5 cm in height) was sealed with a plastic wrap and cultured after 3 weeks, adventitious roots induction at 25 ± 2°C in the dark.
3.4.3. Optimization of culture conditions

Adventitious roots (50 mg L\(^{-1}\), 1 cm long) were transferred to 150 ml shake flasks containing 30 ml MS liquid with 3 % (w/v) sucrose and different concentrations (0.5, 1.0, 2.0 and 3.0 mg L\(^{-1}\)) of indole-3-acetic acid (IBA). The second experiment involved optimum concentrations of 2.0 mg L\(^{-1}\) IBA combinations with 0.25 and 0.5 mg L\(^{-1}\) IAA for root proliferation. The initial concentration of root inoculum was adjusted to 30, 50, 70 and 100 mg L\(^{-1}\) FW to determine the optimal amount for root growth. Different strength of MS medium (1/4, 1/2, 3/4 and Full strength) were also tested. The cultures were incubated in an orbital cooling shaker in the dark at 25 ± °C and 75 rpm for 3-5 weeks.

3.4.4. Determination of the root biomass

The adventitious roots were separated from the medium by filtration through a 1-mm stainless steel sieve, and the fresh weight (FW) was measured after rinsing once with sterile water and blotting away the surface water. The roots were dried at 60°C for one day and the root dry weight (DW) was recorded. Growth ratio was calculated as follows: [harvested dry weight (g) - inoculated dry weight (g)] / inoculated dry weight (g) after 5 weeks of culture.

3.4.5. Statistical analysis

Data were subjected to a one way ANOVA followed by statistical significance test. The significant differences among the mean ± standard error were carried out using Duncan’s multiple range tests and significance level of \( P \leq 0.05 \). (SPSS ver. 16).
3.5. Hairy root culture

3.5.1. Plant material

In the present study, the leaf explants were excised from 3 month old in vitro grown plants. All experiments were done by MS salts supplemented with 3.0 % sucrose and 0.8 % agar. The pH of the media was adjusted to 5.6-5.7 before solidification. Media were autoclaved at 121°C and 104 KPa for 15 min. culture were maintained at 25 ± 2°C for 16 hrs photoperiod with light intensity 40 µmol m⁻² m⁻¹ provide by white fluorescent tubes and a relative humidity at 55-65 %.

3.5.2. Bacterial strains and culture condition

A. rhizogenes strains R1000, MTCC 2364 and MTCC 532 were used for hairy root induction. For the transformation experiments each strain was inoculated into liquid YEB medium (sucrose 5 g L⁻¹, beef extract 1 g L⁻¹, yeast extract 1 g L⁻¹, peptone 5 g L⁻¹), (Himedia laboratories, India). A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (80 rpm) at 26°C for 16 h till the OD₆₀₀ was about 1.0. The bacterial suspension was centrifuged at 10000 rpm for 10 min and the pellet was resuspended in 15 ml MS liquid medium containing 200 mg L⁻¹ acetosyringone before dilution to different concentrations for infection

3.5.3. Hairy root induction

Leaf explants were pre-cultivated on MS medium solidified with 0.8% (w/v) agar at an optimum pH 5.7 and for 2 days. The explants, which measured about 0.5 cm in length were removed from the medium and placed
for 25 min in conical flasks containing the bacterial suspension with concentrations of acetosyringone (0, 50, 100, 200, 400 and 500 mg L\(^{-1}\)), after which they were blotted dry on sterile filter paper (Whatman No. 1) and transferred to the MS medium. After 3 days, these explants were transferred to MS medium containing 250 mg L\(^{-1}\) cefotaxime so as to kill the residual \(A.\ rhizogenes\). Controls consisted of explants treated similarly except that they were not co-cultivated with \(A.\ rhizogenes\). Cefotaxime concentration was then halved each week from 450 to 50 mg L\(^{-1}\) and finally cultures free of \(A.\ rhizogenes\) were transferred to MS medium solidified with 0.8 % agar. After two weeks the hairy roots induction were found from wounding leaf segments.

3.5.4. Influence of sonication on hairy root induction

For sonication treatment, the tubes containing leaf explants were immersed in MS liquid medium containing strain R1000 suspension (5ml) in 50 ml sterilized falcon tube were placed on sonicator (Ultrasonic bath sonicator, PCI Analytics Pvt Ltd®, Mumbai, India) for 10, 20, 30, 40, 50 and 60 s sonication. All transformation procedures were performed in dark, at 25 ±2°C. After 3 days all explants from co-cultivation, direct infection and sonication were transferred to solid media, supplemented with 30 mg L\(^{-1}\) sucrose, 250 mg L\(^{-1}\) cefotaxime and 0.8% agar and subcultured at an interval of 3 days to induce hairy roots. The efficiency of transformation was recorded after 3 weeks.
3. Materials and Methods

3.5.5. PCR analysis

DNA was extracted using the CTAB method (Giri et al., 2000) from hairy root line as well as from control non-transformed roots (*in vitro* germinated roots). PCR primers were used for amplification of a 423 bp fragments of the *rolB* gene. The sequence of each primer was as follows (forward primer 5’ GCTCTTGCAGTGCTAGATT 3’ and reverse primer 5’ GAAGGTGCAAGCTACCTCTC 3’). The PCR reactions were carried out in a total 50 μl volume and consisted of 200 ng of DNA, 10 pm/μl primer, 200 μM dNTP, 1 U of Taq DNA polymerase, 1x PCR buffer and 2 Mm MgCl₂. PCR conditions were 94°C for 5 min (initial denaturation), 42 cycles of 94°C for 1 min, 52.5°C for 1.5 min and 72°C for 2 min and a final extension at 72°C for 10 min.

3.5.6. Establishment of hairy root culture and root biomass accumulation

Hairy roots emerged from the wound sites of the explants within 3 weeks. Then hairy roots were excised from the explant tissues and subcultured on hormone free MS liquid medium (different strength) containing 3.0 % sucrose. The cultures were maintained at 75 rpm in an orbital shaker (LM-570RD, YIHDER® Thiwan) for further growth. All the cultures were maintained in complete darkness at 25 ± 2°C. The hairy roots were subcultured to fresh media for every weeks of culture. The roots were separated from the media by passing them through the forceps. Their fresh weight (FW) was determined after they were washed with distilled water and the excess surface water blotted away. Dry weight (DW) was recorded after
the roots were dried at 60°C till constant weight is recorded. Growth ratio was calculated as follows: \[ \frac{\text{harvested dry weight (g)} - \text{inoculated dry weight (g)}}{\text{inoculated dry weight (g)}} \] after 5 weeks of culture.

### 3.5.7. Statistical analysis

All the experiments were performed in triplicates and each experiment was repeated twice. All subjected to one-way ANOVA followed by the statistical significance test. The significant difference among the mean ± standard error (SE) was carried out using Duncan’s multiple range test and significance level of \( P < 0.05 \).

### 3.6. Biochemical analysis

#### 3.6.1. Sample preparation

The aerial parts (2.0 g DW) and roots of both \textit{in vivo} and \textit{in vitro} derived \textit{A. lanata} were air dried (for 3 days) and completely grinded by using pestle and mortar. The extraction was carried out using methanol (20 ml) and the sample was collected by sterile filter paper (Whatman No 1).

#### 3.6.2. GC-MS analysis

The samples were analyzed using a Shimadzu gas chromatography apparatus (Model - TRACE ULTRA VER: 5.0) using a MS DSQ II capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a gas at a flow rate of 1ml/minute. The column temperature was programmed at 50°C for 1 min and then heated to 300°C and kept for 2 min at late of 5°C min. Injector temperatures of 280°C, detector temperature of 310°C and split rate of
1: 5 carrier gas (helium) with a flow rate of 1 ml/min were applied based on GC peak areas without F10 response factor. The components were identified by computer search, followed by matching the mass spectral data with those held in the database library.