MATERIALS AND METHODS CHAPTER-III

3.1 Area of the study

The explants for tissue culture were collected form Kokrajhar and Tamulpur districts of BTR, Assam. Kokrajhar district (89.46′E to 90.38′ E longitudes and 26.19″ N to 26.54″ N latitudes) is one of the 35 districts of Assam. The district is the gateway to the north-eastern states of India, located on the bank of Brahmaputra River from North. The district covers a total area of 3169.22km^2 according to 2011 census of India. Tamulpur district (longitude 91°34'25"E and Latitude 26°37'00"N) was completely considered as full-fledged district since 25th August 2023. According to the 2011 census Tamulpur district had a population of 389,150 out of which 1.45% (5631) falls in urban areas.

3.2 Sample collection and Identification

An ethno-botanical survey was conducted between January, 2020 to December 2022 for collection information of those medicinal plants used in the region. Another survey of collected medicinal plants was conducted by reviewing of literatures for selection of plants of which tissue culture protocol was not standardized till now in the region or comparative phytochemical and bioactive compound analysis is not yet conducted. After that the plants were selected for conducting the experiment. The voucher specimens of selected plants were prepared and deposited at Bodoland University Botanical Herbarium (BUBH) for identification.

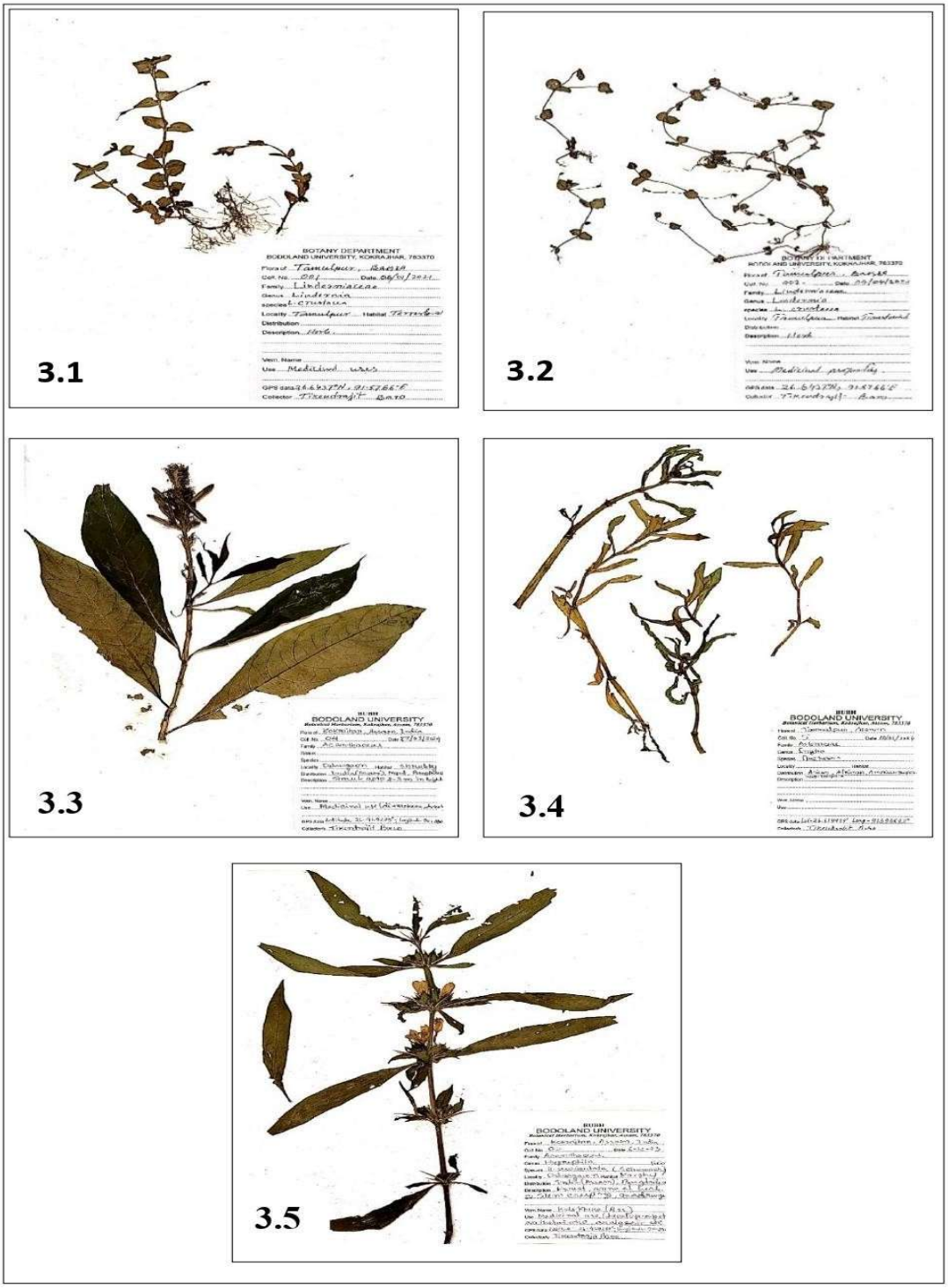

Fig: Photographs of Herbarium sheet samples of medicinal plants submitted at Bodoland University Botanical Herbarium. (3.1) ***Torenia crustacea,*** **(3.2)** ***Lindernia pusilla,*** **(3.3)** ***Phlogacanthus thyrsiformis,*** **(3.4)** ***Enydra fluctuans*** **(3.5)** ***Hygrophila auriculata.***

3.3 Explant collection and surface sterilization

The disease free, rapidly growing and healthy explants were selected from collected explants. These explants were selected for further surface sterilization step to minimize the rate of contaminations in the tissue cultured explants. Nodal explants were preferred for all the experiments. Initially the collected explants were washed carefully in tap water to remove excess dust particles and the leaves and root parts were removed. Only healthy and disease-free nodal explants were selected. Further the explants were dipped in distilled water containing 2-3 drops/litre of Tween 20 reagent for 30 min followed by trimming the explants to smaller size (1cm - 2cm in length) and washed in distilled water three times. The explants were taken inside the Laminar Air Flow (LAF) cabinet and treated in 0.5% Bavistin for 60 min and washed with sterile distilled water for three times. Finally, the explants were treated in mercuric chloride. 0.1% mercuric chloride for 0-5 min were tested for explant surface sterilization followed by immediate washing with sterile distilled water for three times (Baro & Das, 2022a).

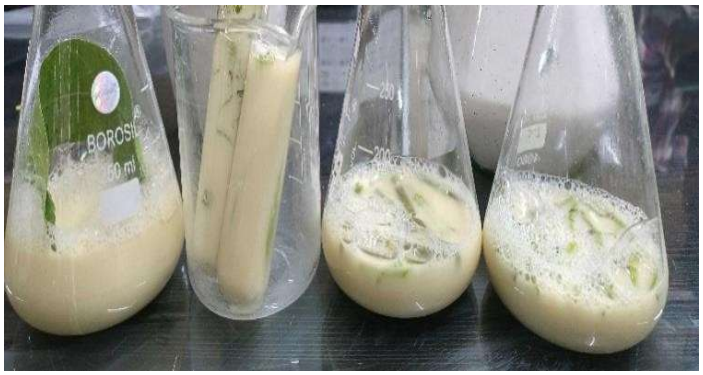

Fig 3.6: Explant surface sterilization in 0.5% bavistin

Fig 3.7: Explant surface sterilization in 0.1% mercuric chloride

3.4 Media preparation

Murashige and Skoog's (MS) media were used for all the tissue culture standardization experiments. Initially all the glass wares including phyta jar, beaker, conical flasks were washed thoroughly and autoclaved at 121ºC and 15PSI for 30 minutes. Stock solutions were prepared (Appendix Table 2).

After preparation of the stock solutions, MS media were prepared by adding all the components (Appendix: Table 2) into sterile distilled water in a conical flask and mixed well. 3% (w/v) sucrose were added and the pH of the solution was adjusted to 5.6 and finally 0.8% agar (w/v) was added and mixed by boiling inside the microwave oven. The above MS media was sterilized in an autoclave at 121 ºC and 15 psi for 15 min. 6- Benzyl amino purine (BAP), Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) were used as the plant growth regulators (PGR) for plant growth and development. The PGR's (Table 3.1) were added to the media using a 22 µm filter syringe after sterilization of the media in the autoclave. The media were poured into sterilized phyta jars and kept inside LAF under UV, overnight.

Table 3.1: Table showing basal medium with growth regulator concentration ration

Sl no	**Basal media**	**Growth regulators (mg/L)**		
		BAP	NAA	IAA
1	Control	0.0	0.0	0.0
2	BM1	1.0	0.0	0.0
3	BM2	1.0	0.2	0.0
4	BM3	1.0	0.4	0.0
5	BM4	1.0	0.5	0.0
6	BM5	1.0	1.0	0.0
7	BM6	2.0	0.0	0.0
8	BM7	2.0	0.5	0.0
9	BM8	2.0	1.0	0.0
10	BM9	3	00	0.0
11	BM10	00	00	0.5

12	BM11	0.0	0.0	1.0
13	BM12	00	00	2.0

3.5 Shoot induction, multiplication and Rooting

The surface sterilized explants were inoculated into the different concentrations and ratios of PGRs in different jars. The tissue explants were then incubated at 28^0C±2ºC temperature at 12 hours of photoperiod and 75%±5% relative humidity for 4 to 6 weeks inside the plant growth chamber and the jars were monitored regularly for contaminations and responses. The contaminated jars were removed immediately. After every 4 weeks the tissues were sub cultured to the MS media containing same PGR concentration as previous jars. Successfully grown tissue explants into multiple shoots and plant were taken out from the media and excised and again introduced into new fresh growth media for further mass propagation.

3.6 Hardening

For primary hardening vermicompost soil (50%) and coco pit mixture was prepared for planting the tissue cultured plantlets. the *in vitro* developed plants were taken out from the culture jars, the excessive stacked media was removed by washing and the plantlets were planted in the pots containing vermicompost and soil mixture. The plantlets were grown inside the green-house at the Technology Incubation Centre, Bodoland University at 28^0C±2ºC temperature and 70% relative humidity for 14-21 days. The well-established plantlets were gradually shifted out from the green-house and grown outside of the green house at environmental condition.

3.7 DNA extraction

The whole genome was extracted from the leaves of the wild plant and micropropagated plant using the DNeasy Plant Mini Kit (Qiagen) following the steps provided in the kit.

i. 100 mg of fresh leaves or 30mg of dry leave samples were disrupted using a mortar pestle.

ii. 400 µl of AP1 buffer (promotes cell membrane lysis, denaturation of protein, and macromolecules) were added followed by addition of 4 µl of RNase A and

vortexed. Incubated the mixture for 10 minutes at 65ºC, and inverted the tubes for 2-3 times during incubation.

iii. 130 µl of buffer P3 (neutralizes the lysate in the process of DNA extraction) were added and incubated on ice refrigerator for 5 min.

iv. After 5 min the lysate was centrifuged at 14000 rpm for 5 min.

v. The lysate was pipette out into new QIAshredder spin column placed in a 2ml collection tube and centrifuged at 14000rpm for 2min.

vi. The flow through was transferred to a new tube and 1.5 volumes of buffer AW1 was mixed by pipetting to remove the contaminants from the DNA.

vii. 650 µl of the above mixture was transferred to a new DNeasy Mini Spin column placed in a 2 ml collection tube and centrifuged for 1min at 8000rpm, the flow through was discarded. This step was repeated with the remaining sample.

viii. The spin column was placed in a new collection tube and 500µl of AW2 buffer was added and centrifuged for another 1 min at 8000rpm. The flow through was discarded.

ix. Again, 500µl of AW2 buffer (acts as washing of the DNA from other biomolecular materials) was added and centrifuged at 14000rpm for 2 min. The spin column was removed.

x. The spin column was removed carefully and placed in a new 2 ml collection tube.

xi. 100 µl of AE buffer was added for eluting the DNA, incubated at room temperature for 5 min and centrifuged at 8000rpm for 1 min. this step is repeated.

3.8 RAPD Assay

Random primers were synthesized from Eurofins genomics for RAPD assay. The OPC series OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9, OPC 10 RAPD, OPA01, OPA02, OPA04, OPA12, and OPA13 primers were purchased from Eurofins Scientific (Sharma et al., 2018). The PCR cycles consisted of initial denaturation, denaturation, annealing, extension, final extension and hold at 4ºC. The temperatures and durations of all steps in PCR were varied for different species (Zhang et al., 2019).

i) *Lindernia curstacea*

ii) *Lindernia pusilla*

iii) *Hygrophila auriculata*

iv) *Enydra fluctuans*

v) *Phlogacanthus thyrsiformis*

Table 3.2: Table showing the RAPD primer sequence of OPC1 to OPC 10

Sl no	Primer	Sequence 5'-3'	No of bases
1	OPC1	TTCGAGCCAG	10
2	OPC2	GTGAGGCGTC	10
3	OPC3	GGGGGTCTTT	10
4	OPC4	CCGCATCTAC	10
5	OPC5	GATGACCGCC	10
6	OPC6	GAACGGACTC	10
7	OPC7	GTCCCGACGA	10
8	OPC8	TGGACCGGTG	10
9	OPC9	CTCACCGTCC	10
10	OPC10	TGTCTGGGTG	10
11	OPA01	CAGGCCCTTC	10
12	OPA02	TGCCGAGCTG	10
13	OPA04	AATCGGGCTG	10
14	OPA12	TCGGCGATAG	10
15	OPA13	CAGCACCCAC	10

Table 3.3: Table showing the components used in PCR mixture for RAPD reaction

Components of PCR mix			
Sl no	Component	Amount in µl	Unit/ concentration
1	dNTP	2µL	10mM
2	Template (80ng)	5µL	100ng
3	RAPD primer	1µM	10pmol
4	Taq DNA polymerase	1U	Unit
5	PCR buffer(1X)	2.5µL	25X
6	Nuclease free water	13.5µL	
Total volume		**25µL**	

Table 3.4: Table showing different steps for PCR cycle

RAPD PCR Parameters			
Sl no	**Step**	**Temperature (in ºC)**	**Duration time (in min)**
1	Initial Denaturation	94-95	4-5 min
2	Denaturation	94-95	30 sec-1 min
3	Annealing	33-38	1 min- 2 min
4	Extension	72	2 min
5	Final Extension	72	7 min
6	Hold	4	∞

The amplified PCR products were separated in 1.5% agarose (containing 2 µl ethidium bromide to stain the PCR amplicon) gel electrophoresis at a constant voltage of 80V for 45 minutes. The amplified products were visualized in Gel-Doc E-Gel Imager camera hood (Life Technologies).

The random primers (synthesized from Eurofins genomics) used for amplification were OPA 02, OPA 13, OPC 01, OPC 02, OPC 03, OPC 04, OPC 05, OPC 06, OPC 07, OPC 08, OPC 09, OPA 01, OPA 04, and OPA 12.

The PCR cycle for *T. crustacea* consists of initial denaturation at 94 °C for 4-minute, denaturation at 94 °C for 30 seconds, annealing at 35 °C for 2 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min and hold at 4 °C.

The PCR cycle for *L. pusilla* consists of initial denaturation at 94 °C for 5-minute, denaturation at 94 °C for 30 seconds, annealing at 38 °C for 1 min 30 sec, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min and hold at 4 °C.

The PCR cycle for *P. thyrsiformis* consists of initial denaturation at 95 °C for 4-minute, denaturation at 95 °C for 1 min, annealing at 37 °C for 1 min 30 sec, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min and hold at 4 °C.

The PCR cycle for *E. fluctuans* consists of initial denaturation at 95 °C for 5-minute, denaturation at 95 °C for 2 min, annealing at 37 °C for 1 min 30 sec, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min and hold at 4 °C.

The PCR cycle for *H. auriculata* consists of initial denaturation at 95 °C for 4-minute, denaturation at 95 °C for 30 sec, annealing at 36 °C for 2 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min and hold at 4 °C.

The amplified products were separated at 1.5% ultrapure agarose gel electrophoresis at a constant voltage of 80V for 45 min. 2 µL of Et-Br (Ethidium Bromide) was added to the agarose to stain the DNA. The DNA bands were visualized in a Gel-Doc E-Gel Imager camera hood (Life Technologies). The samples were analysed based on the presence and absence of similar bands.

3.9 Antioxidant Assay (Extract preparation)

The successful *in vitro* propagated and wild plants were collected and dried inside hot air oven at 45ºC for 7 days and crushed up and ground to get homogeneous fine powder using grinder. The powder was stored in an air tight container. 10g of the powder was dissolved in 100 mL 70%(v/v) of methanol water. The mixture was sonicated in an ultrasonicator for 30 min and kept in normal room temperature for 48 hours for complete extraction.

Afte 48 h the solution was filtered using 42 number Whatmann's filter paper. The filtrated was lyophilized in lyophilizer to get completely dry crystals and stored in air tight container (Koleva et al., 2002).

3.9.1 Total Phenolic Content: For determination of total phenol content in the plant extract the folin-ciocalteau method (FCR) (Maheshwari et al., 2011) was used. In this test the plant extract was dissolved in 70% (v/v) methanol water solution was prepared in mg/mL concentration. 100µL of plant extract was taken in a test tube followed by adding 1600µL of double distilled water to it and 100µL of 0.25N FCR reagent was added and mixed thoroughly. After 3 min reaction of the above solution 150µL of 1N sodium carbonate solution was mixed and this mixture was incubated in dark condition at room temperature for 2 h. After 2 h absorbance was measured at 725nm at spectrophotometer. Gallic acid was used as standard for this experiment which was expressed as milligrams of gallic acid equivalent (GAE)/g of the dried extract.

3.9.2 Total flavonoid content: Aluminium chloride method (Hsieh et al., 2016) was used for the determination of total flavonoid content in the plant extracts. 1mg/mL extract was prepared in 70% methanol (v/v) water solution. In a test tube 2.8mL of double distilled water was taken and 100µL of extract was added to it followed by addition of 1.5mL of ethanol (95% v/v), again 100µL of aluminium chloride hexahydrate (10%) was reacted with the mixture and finally 100µL of 1M potassium acetate was allowed to react with the above mixture and incubated at room temperature for 40 min. Finally, the absorbance was measured at 415 nm, taking quercetin as the standard.

3.9.3 Total antioxidant capacity: Total antioxidant capacity test was conducted using the phosphomolybdate method (Jan et al., 2013). 300µL of 1mg/mL plant extract was added to 3 mL of phosphomolybdate reagent and incubated at 95 °C in a water bath for 90 min. After cooling, the absorbance was measured at 765 nm. Results were expressed in mg of ascorbic acid equivalent (AAE)/g of dried extract.

3.9.4 DPPH radical scavenging activity: The antioxidant capacity of the methanolic (70:30) water extracts was determined by their capacities to neutralize radicals of DPPH method described by (Baliyan et al., 2022). In this method approximately 1 mL of 100 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol was combined with an equal volume of extract in the methanol of various concentrations. The mixture was

incubated in dark for 30 min, while a pure solution of methanol served as the control. The colour change was observed through absorbance at 517 nm using spectrophotometer (Shimadzu, 1900 series). Ascorbic acid was used as standard for the reference. The free radical scavenging activity or radical inhibition percentage was calculated using the following equation.

DPPH Scavenging activity % = Abs. of control-Abs. of extract/ Abs. of control x 100

3.10 Quantitative HPLC analysis for detection of gallic acid and quercetin: For HPLC analysis for quantification of gallic acid and quercetin of wild and micropropagated *L. pusilla,* the following methods were used.

Extract Preparation: The collected plant sample of *L. pusilla* was dried inside hot air oven at 45^{o}C and ground to get homogenised fine powder. 5g of powder was dissolved in 70% methanol: water. Extraction was carried out inside ultrasonicator with heating overnight. Filtered through Whatman 41 filter paper. The process was repeated for second extraction. The filtrated sample was freeze dried inside Lyophilizer for 48h. After fully drying 1mg/mL extract was prepared for analysis.

Chromatographic parameters: The chromatographic column was a C-18 column, the mobile phase was methanol and water (70:30), the flow rate was 1 mL/min, and the injection volume was 20 µL. The detection wavelength was 280 nm at a temperature of 28 °C.

Precision: By administering a standard solution of gallic acid and quercetin (20 g/mL) six times, the method's accuracy was examined. Peak regions were identified and contrasted. Repetition and reproduction were done by injecting the standard solutions (1 mg/mL), each in six replicates, twice on the same day and once on the following day. Inter- and intra-day variation was done. Peak areas were measured and compared for the repeatability and reproducibility of the HPLC method (Dinakaran et al., 2018).

Calibration plots: The standard stock solution was diluted to create standards for gallic acid and quercetin in various quantities. To create the standard response curve for gallic acid and quercetin, a linear regression was fitted to triplicate values obtained at each of five concentrations (10 to 100 µg/mL). Each solution was chromatographed in a volume

of 20 µL, and the peak areas were calculated. The linear range of gallic acid and quercetin was then determined by plotting peak areas for each concentration.

Method validation: The method's repeatability, accuracy, precision, selectivity, and specificity were all validated. By spiking a standard of gallic acid and quercetin, accuracy was evaluated. The intra- and inter-day variation of peak area and retention time was used to gauge precision. All validation studies used the injection of standard and sample solutions in triplicate. Gallic acid and quercetin concentrations were determined for the intra-day study three times on the same day at intervals of two h, but for the inter-day study, they were determined on three different days. Injecting solutions containing the standard allowed for the evaluation of the method's selectivity and specificity.

HPLC Analysis: For the quantification of Gallic acid and Quercetin, 20 µL of the prepared samples were filtered through a membrane filter before injection and loaded in the HPLC for analysis. HPLC was carried out in a Waters 1525 binary HPLC pump with a Waters 2998 PDA detector using Empower software for data analysis. The column used is a watered spherisorb 5.0 µm ODS2. The column dimension was 4.6 mm å 250 mm**.** The absorbance was measured at 270 nm for Gallic acid and 370 nm for Quercetin.

3.11 Comparative GC-MS compound analysis in tissue cultured and wild extracts

GC-MS is an important tool in various fields of research, including pharmaceuticals, forensics, environmental science, and food safety. This method allows the identification and quantification of compounds present in any complex mixture. The 1mg/mL ethanolic plant extract of both wild and *in vitro* plants were used for the GC MS analysis (Gomathi et al., 2015). Volatile bioactive constituents of *L. pusilla* extract were identified by GC-MS analysis (Perkin Elmer Clarus 680 GC/600 MS, USA). The capillary column used was Elite-5MS (60 m'0.25 mm ID'0.25 mm), and the stationary phase was 5% diphenyl-95% dimethyl polysiloxane. Helium gas (99.99%) was used as carrier gas (mobile phase) at a flow rate of 1 mL/min. An injection volume of 1 mL was employed in split-less mode. The injector and ion-source temperatures were set at 280 °C and 180 °C, respectively. The oven temperature was programmed at 60 ºC (for 1 min), with an increase at the rate of 7 ºC/min to 200 ºC (hold for 3 min), then again increased at a rate of 10 ºC/min to 300 ºC (hold for 5 min). The total run time for GC was 39 min, and the solvent delay was kept at 7 min. MS Protocol Mass Spectra were taken in Electron Impact Positive (EI +) mode

at 70 eV. For the MS scan, a solvent delay of 8 min, the mass range (m/z) range was 50–600 amu. The volatile bioactive constituents in the extract were interpreted using the database software of the National Institute of Standards and Technology (NIST) (2014). The mass spectra of the unknown components were compared with the spectra of known components in the NIST library, and the compounds were identified.

Studies of the plant extracts using GC-MS mostly reveal the availability of important bioactive compounds in the plant. In the present study, the bioactive compounds found in wild and tissue-cultured plant extracts of *the collected medicinal plant extract* were studied using GC-MS analysis. The compound search was selected by retention time (RT), molecular formula, molecular weight, and peak area% (concentration).

Statistical analysis: Statistical analysis was conducted using SPSS program using one-way (ANOVA) analyses of variance test. All figures indicate means and standard errors of the means. $P < 0.05$ was regarded as statistically significant. The standard errors, standard deviation, regression was used for statistical analysis.