RESULT CHAPTER IV

4.1 Authentication and identification of selected plants: All the collected plants were identified in the Bodoland University botanical herbarium the accession number of identified plants are-

a. *Torenia crustacea (*L) Charm. & Schltdl- BUBH0000576
b. *Lindernia pusilla* (Willd.) Bold- BUBH0000897
c. *Phlogacanthus thyrsiformis* Nees- BUBH0000577
d. *Enydra fluctuans* Lour- BUBH0000903
e. *Hygrophila auriculata* (Schumach.) Heine- BUBH0000875

4.2 *Torenia crustacea*

4.2.1 Explant selection and surface sterilization*:* The collected explants of *T. crustacea* were sterilized using 0.1% mercuric chloride. The selection of disease free, healthy and rapidly growing explants is very important step to obtain disease free cultures. Most of the contaminations in the culture were observed in the culture after 7-20 days of inoculation. Sometimes it may observe in the callus also. Most effective explant survival was observed in the explant treatment using 0.1% mercuric chloride for 2 min, 63.33±4.71% of the cultured explant was survived after 21 days of culture (Fig 4.2.1). 1 min 0.1% mercuric chloride treatment showed only 23.33±4.71% of explant survival rate, the 63.33±4.71% of the explants got contaminated after 5-10 days of explant initiation. 46.67±4.71% of the explants survived while the explants were treated with 0.1% mercuric chloride after 21 days of explant initiation. Only 23.33±4.71% explants were survived after 21 days of explant initiation using 0.1% mercuric chloride. Finally, 5 min treatment using 0.1% mercuric chloride resulted only 6.67±4.71% explant survival rate after 21 days of explant initiation.

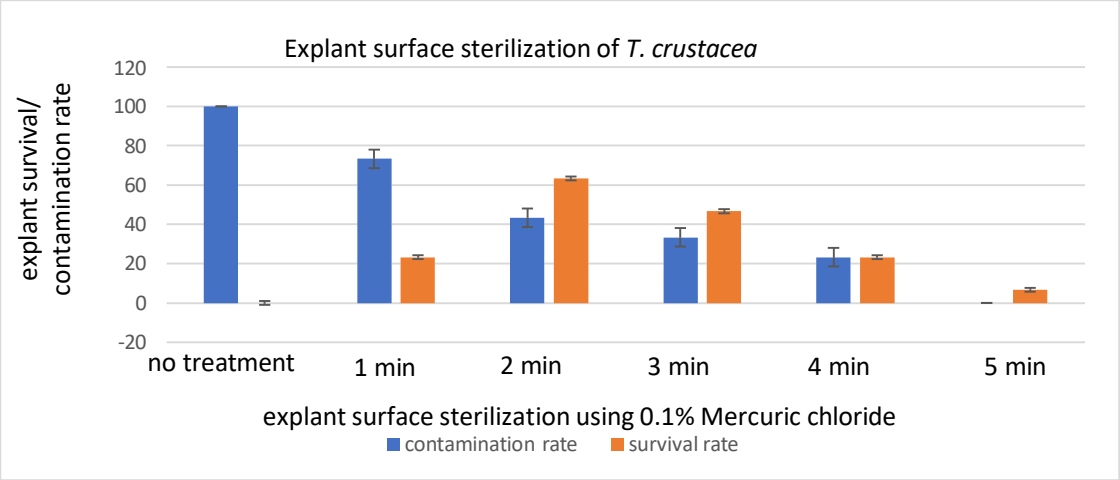


Fig 4.2.1: Explant survival rate of *in vitro* propagated *T. crustacea* after 21 days of explant initiation using 0.1% mercuric chloride. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

4.2.2 Explant inoculation and shoot multiplication and rooting

In the present study, BAP (6 benzyl amino purine), IAA (Indole-3 acetic acid) and NAA (naphthalene acetic acid) were used as growth regulators. Although explants in the control medium without growth regulators also showed response but, the rate of response was low. All the explants cultured in the different MS media successfully established, though the response of all the explants were varied accordingly to the different concentrations of growth regulators used in the MS media. The most effective shoot proliferation and multiplication were observed in the MS media supplemented with 1mg/L BAP and 0.2 mg/L NAA and the highest average shoot length was observed. Maximum average number of rooting were observed in the MS media supplemented with 1mg/L IAA (Fig 4.2.2). In the control media an average of 6.9±0.81 shoots per explant, 7.4±0.73 cm average shoot length and an average of 14.2±1.21 roots per explant were observed. In the BM1 medium where 1mg/L BAP was supplemented with the MS medium an average of 12.09±0.68 shoots per explant, .4±0.73cm average shoot length, and an average of 13.2±1.21 number of roots per explant were observed. An average of 13.39±0.74 shoots per explant with an average of 14.8±0.68 cm shoot length per explant, and 14.6±1.23 number of roots per explant were observed in the BM2 media where 1mg/L BAP and 0.2mg/L NAA was supplemented. In the BM3 media (MS+1mg/L BAP+ 0.4mg/L NAA)

an average of 10.58±1.06 shoots per explant, 12.2±1.06cm average shoot length, and 14.4±0.44 number of roots per explant were observed. An average of 10.33±1.34 shoots per explant, 13.2±0.68 cm average shoot length, and an average of 15.2±0.68 roots per explant were observed in the BM4 medium (MS+1mg/L BAP+ 0.5mg/L NAA). In the BM5 media (MS+1mg/L BAP+1mg/L NAA) 8.65±1.57 average shoots per explant, 12.4±0.44cm average shoot length, and 15.6±0.73 number of roots per explant. An average of 10.40±0.81 shoots per explant, 13±1 cm average shoot length, with an average root number of 12.8±1.77 were observed in the BM6 medium (MS +2mg/L BAP). In the BM7 medium (MS+2mg/L BAP+0.5mg/L NAA) an average of 8.81±0.68 shoots per explant, 11.2±0.68cm shoot length, and an average of 16.6±1.23 roots per explants were observed. In the BM8 medium (MS+2mg/L BAP+ 1mg/L NAA) an average of 8.81±0.68 shoots per explant, 11.4±0.93cm average shoot length, and an average of 13.2±2.54 roots per explant were observed. An average of 8.68±0.81 shoots per explant, 12.6±0.73cm shoot length with an average of 12.6±1.54 roots per explant were observed in the BM9 medium (MS+3mg/L BAP). In the BM10 medium where MS was supplemented with 0.5mg/L IAA, an average of 8.27±0.95 shoots per explants, 9±0.57 cm length per shoot, and an average of 25.4±1.23 roots per explant were observed. In the BM11 medium (MS+1mg/L IAA) an average of 7.72±1.06 shoots per explant, 8.8±0.68 cm average shoot length, with an average 31.2±2.54 number of roots and in the BM12 medium (MS+2mg/L IAA) an average of 6.5±0.95 shoots per explant, 7.8±0.68cm average shoot length, and 29±2.76 average root numbers were formed (Table 4.2.1).

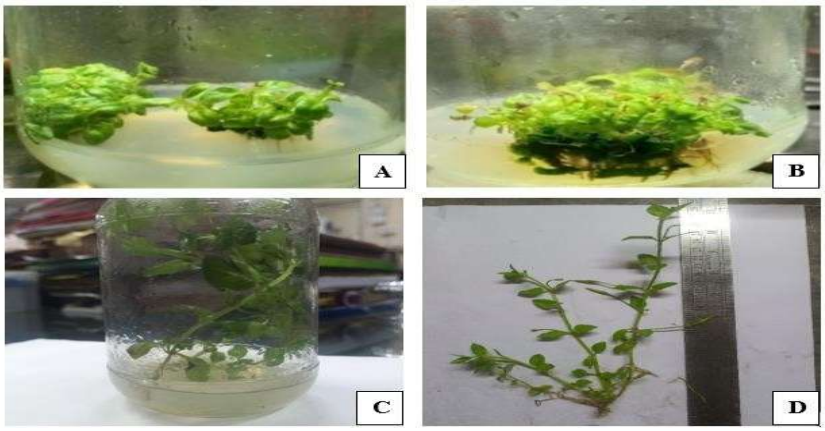


Fig 4.2.2*: In vitro* propagation of *T. crustacea*, A; explant initiation, B&C; shoot multiplication and, D; Hardening.

Table 4.2.1: Effect of different growth regulators on *in vitro* propagation of *T. crustacea.* Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Sl no	**Basal media**	**Growth regulators (mg/L)**			**Number of explant culture initiation**	**Rate (%) of explant producing shoots**	**Number of shoots per explant**	**Shoot length (cm)**	**Number of roots**
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	05	100	6.9±0.81	7.4±0.73	14.2±1.21
2	BM1	1.0	0.0	0.0	05	100	12.09±0.68	8.4±0.73	13.2±1.21
3	BM2	1.0	0.2	0.0	05	100	13.39±0.74	14.8±0.68	14.6±1.23
4	BM3	1.0	0.4	0.0	05	100	10.58±1.06	12.2±1.06	14.4±0.44
5	BM4	1.0	0.5	0.0	05	100	10.33±1.34	13.2±0.68	15.2±0.68
6	BM5	1.0	1.0	00	05	100	8.65±1.57	12.4±0.44	15.6±0.73
7	BM6	2.0	0.0	0.0	05	100	10.40±0.81	13±1	12.8±1.77
8	BM7	2.0	0.5		05	100	8.81±0.68	11.2±0.68	16.6±1.23
9	BM8	2.0	1.0		05	100	8.81±0.68	11.4±0.93	13.2±2.54
10	BM9	3	00	00	05	100	8.68±0.81	12.6±0.73	12.6±1.54
11	BM10	00	00	0.5	05	100	8.27±0.95	9±0.57	25.4±1.23
12	BM11	0.0	0.0	1.0	05	100	7.72±1.06	8.8±0.68	31.2±2.54
13	BM12	00	00	2.0	05	100	6.5±0.95	7.8±0.68	29±2.76

4.2.3 Hardening:

The primary hardening 80% of the *T. crustacea* was successfully done on the 50% vermicompost soil mixture inside the green house at a temperature of $28^0C \pm 2^0C$ and a relative humidity of 70%. After 21 days grown in the greenhouse the explants were taken out to the shed net house in natural environment with varying temperature $28^0C \pm 5^0C$. all the plantlets survived in the natural condition in this step. Finally, the plantlets were successfully grown in the natural climate condition.

4.2.4 Genomic DNA extraction: The whole genome of both wild and tissue cultured *T. crustacea* was extracted using Qiagen DNeasy Plant Mini kit followed by steps mentioned in the kit. Lane 1 and lane 2 is the genomic DNA extracted from leaf explant of tissue cultured *T. crustacea.* Lane 3 and lane 4 is genomic DNA extracted from leaf explant of wild *T. crustacea* (Fig 4.2.3).

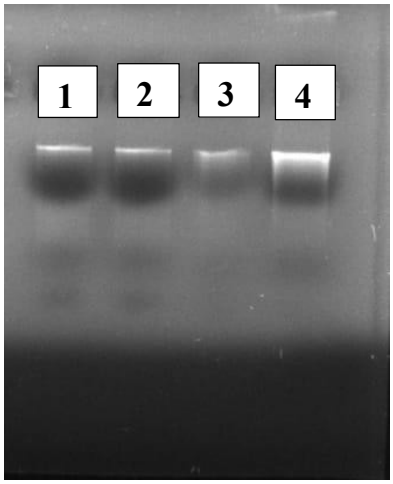


Fig 4.2.3: Isolated Genomic DNA of *T. crustacea*, where **1&2** – Tissue cultured (using leaf explant), **3&4** – Wild Plant (using leaf explant)

4.2.5 RAPD assay: When compared to the mother plant, the phenotypic and genetic makeup of the in vitro produced plants may undergo a variety of alterations. Once these variations have spread, it is crucial to find them. Using molecular markers to identify somaclones can be a helpful method. For the detection of somaclonal variation in the micropropagated *T. crustacea* nine distinct RAPD primers were used (OPC 01, OPC 02, OPC 03, OPC 04, OPC 05, OPC 06, OPC 07, OPC 08, and OPC 09).

In the visualization of amplified RAPD product in 1.5% agarose gel electrophoresis (Fig 4.2.4), the OPC-1 RAPD primer formed a total of 3 polymorphic DNA bands in wild and

4 polymorphic DNA bands in tissue cultured *T. crustacea.* In the OPC-2 RAPD primer both the wild and micropropagated *T. crustacea* plant formed a total of 7 polymorphic DNA band. A total of two polymorphic DNA bands were formed by the OPC-4 RAPD primer in both the wild and micropropagated *T. crustacea.* Again, the OPC-5 RAPD primer formed single band in the wild and two polymorphic DNA bands in the micropropagated *T. crustacea* plant. A total of seven polymorphic DNA bands were visualized in the agarose gel in both the wild and micropropagated plant. Finally, the OPC-8 RAPD primer formed a total of seven polymorphic DNA bands in wild and a total of four polymorphic DNA bands in micropropagated *T. crustacea* plant. The four RAPD primer (OPC-3, OPC-7, OPC-9, and OPC-10) did not formed any DNA bands. From the result the three RAPD primers (OPC-2, OPC-4, and OPC-6) formed similar monomorphic DNA bands in both the wild and micropropagated *T. crustacea* plant and the other three RAPD primers (OPC-1, OPC-5, and OPC-8) formed different polymorphic DNA bands in wild and micropropagated *T. crustacea* plant, hence the somaclonal variation is confirmed in the micropropagated *T. crustacea* plant (Table 4.2.2).

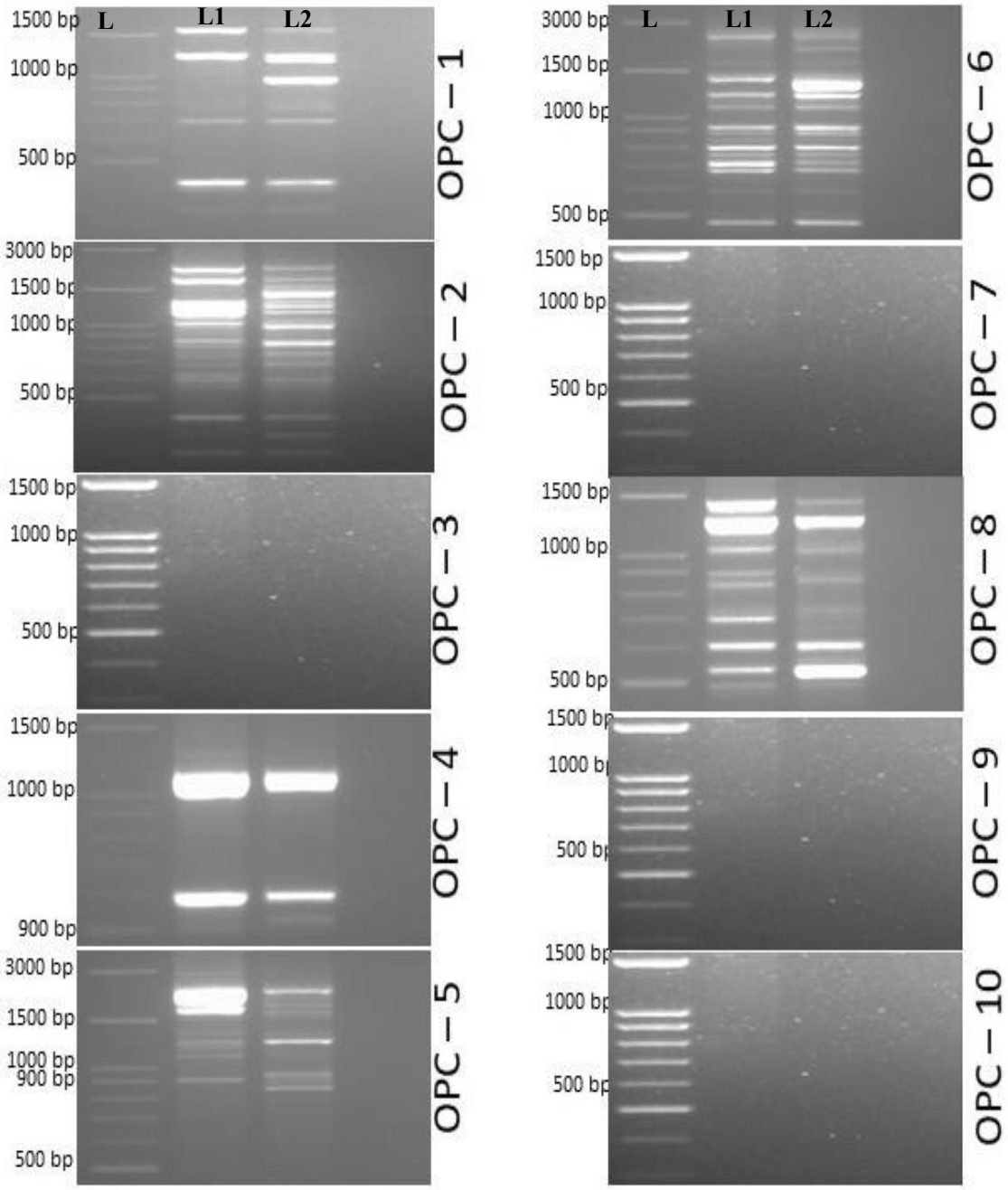


Fig 4.2.4: Amplification pattern of different RAPD Primers (OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9 and OPC10), lane L defines the 1kb DNA ladder, Lane 1 is amplified RAPD product of tissue cultured plant genome, lane 2 is amplified RAPD product of wild plant genome.

Table 4.2.2: Number of polymorphic DNA bands formed in wild and tissue cultured *T. crustacea* using RAPD primers (OPC-1 to OPC-10) in agarose gel electrophoresis

Sl no	Primer	Number of bands produced by wild plant	Number of bands produced by hardened micro propagated plants
1	OPC 01	3	4
2	OPC 02	7	7
3	OPC 03	0	0
4	OPC 04	2	2
5	OPC 05	1	2
6	OPC 06	7	7
7	OPC 07	0	0
8	OPC 08	7	4
9	OPC 09	0	0
10	OPC 10	0	0

4.2.6 *In vitro* antioxidant test of wild and tissue cultured *T. crustacea:* After successful standardization of *in vitro* propagation protocol for *T. crustacea.* Methanolic extracts were prepared form the entire plant of both wild and micropropagated *T. crustacea*. 1mg/mL of dried extracts were prepared in 70% methanol for the following tests using a UV-vis spectrophotometer and studied the total phenol content, total flavonoid content and the total antioxidant capacity in the both wild and tissue cultured *T. crustacea.*

Total Phenolic Content: FCR method was absorbed for the detection of phenolics in the plant extract of wild and tissue cultured *T. crustacea* (Fig 4.2.5a). The regression curve ($y = 276.85x + 68.485$; $R^2 = 0.9934$) was used and expressed as mg of gallic acid equivalent (GAE). From the experiment 64.7 ±5.3 mg/g GAE were found in the wild extract and 69 ±3.4 mg GAE/g powder weight in the tissue cultured *T. crustacea.*

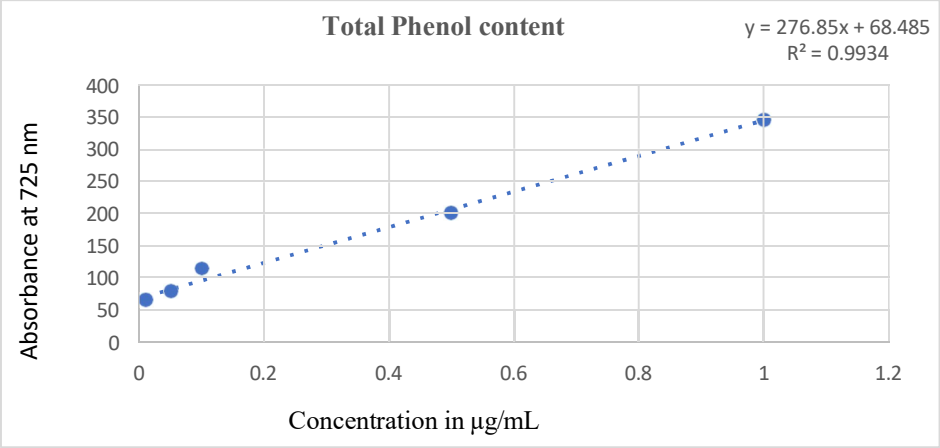


Fig 4.2.5a: Total phenolic content, standard curve of gallic acid

Total Flavonoid content: The total flavonoid content in wild and tissue cultured *T. crustacea* were determined using the regression curve (y = 404.68x + 53.647 R^2 = 0.993) and expressed as mg of quercetin equivalent (QE) per grams of the dried methanolic extract of *T. crustacea (*Fig 4.2.5b). The total flavonoid content was comparatively higher in the tissue cultured (48±3 mg QE/g) *T. crustacea* than the wild (44±3.7 mg QE/g) extract of *T. crustacea.*

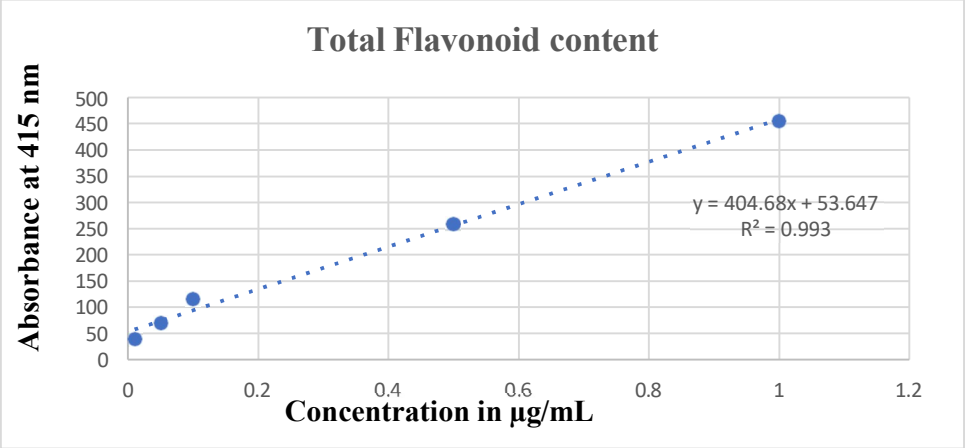


Fig 4.2.5b: Total flavonoid content, standard curve of quercetin

Total antioxidant capacity: The total antioxidant capacity of wild and tissue cultured *T. crustacea* were determined by the regression curve (y=324.98x + 45.306 R^2 = 0.9902) (Fig 4.2.5c). The antioxidant capacity of the tissue cultured methanolic

extract (143.7±4.2 mg/g AAE) was higher than the wild extract (126±4.2 mg AAE/g of dried extract) of *T. crustacea.*

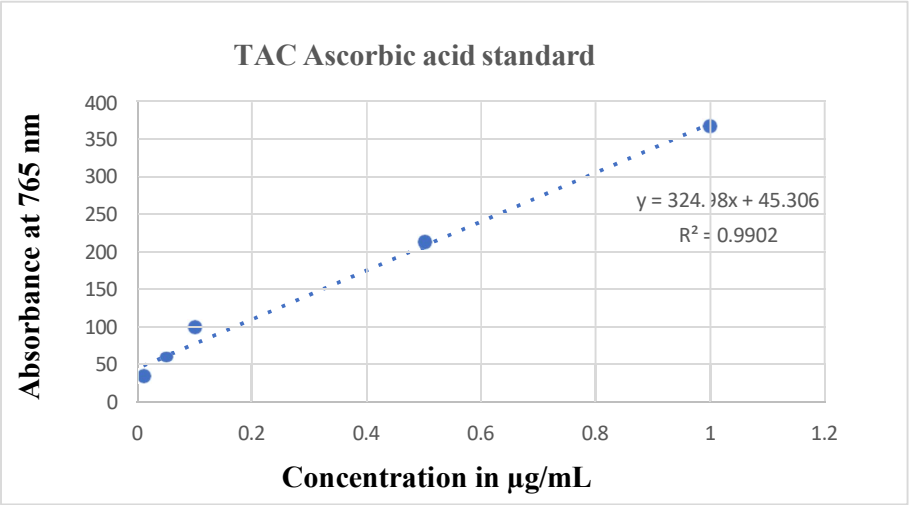


Fig 4.2.5c: Total antioxidant capacity, standard curve of ascorbic acid

DPPH scavenging Activity: The radical scavenging activity in the wild and tissue cultured *P. thyrsiformis* was determined by the regression curve (y = 16.114x + 13.25; R^2 = 0.9846) (Fig 4.2.5d). The IC_{50} value of the wild methanolic extract of *P. thyrsiformis* is 28.11±1.25 and in the tissue cultured extract was 28.677±1.13.

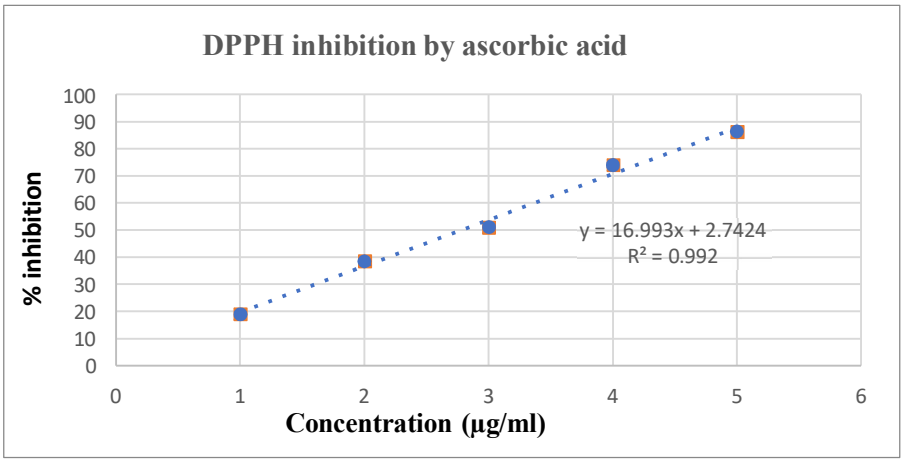


Fig 4.2.5d: DPPH inhibition by ascorbic acid standard

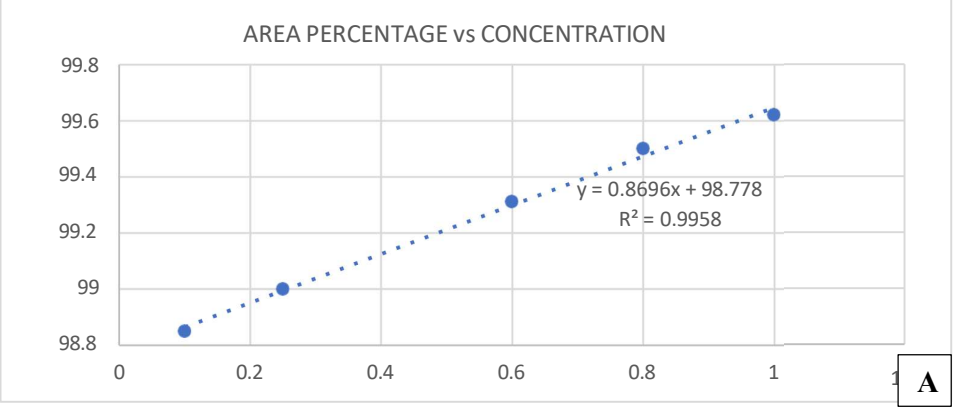
4.2.7 Quantitative detection of gallic acid and quercetin: The HPLC methods for detection of gallic acid and quercetin was validated and standardized in the Waters HPLC system. The system was stabilised and equilibrated using 70% (v/v) HPLC-grade

methanol and 30% (v/v) HPLC-grade water with a flow rate of 1 mL/min using the Waters RP-18 column. The experiment was conducted in triplicate, satisfactory results were obtained in quercetin and gallic acid in wild and micropropagated *T. crustacea*. The chromatograms and the standard curve of gallic acid and quercetin was plotted. The gallic acid content in the wild and tissue cultured *T. crustacea* was determined using the regression curve (y= 0.8696x + 98.778 R^2 = 0.9958) (Fig 4.2.6A) and the quercetin content in the wild and tissue cultured *T. crustacea* was determined using the regression curve (5.9606x + 88.794 R^2 = 0.997) (Fig 4.2.6B). The retention time of gallic acid and quercetin was 2.70 and 5.7 min, respectively (Fig 4.2.7a, 4.2.7b, 4.2.7c and 4.2.7d). From the experiment it was obtained that the gallic acid and quercetin content in tissue cultured methanolic extracts of *T. crustacea* were comparatively higher than the wild methanolic extract. The gallic acid content of wild-type and tissue-cultured extracts was 92µg/mg and 99µg/mg of dried extract, respectively. The quercetin content of wild and tissue cultured plant extracts of *T. crustacea* was 82µg/mg and 85µg/mg of dried extract, respectively (Table 4.2.3).

The chromatograms of each quercetin and gallic acid were obtained and standard curves were plotted. All the data were tabulated below in the Table. The chromatograms of each quercetin and gallic acid were obtained and standard curves were plotted. All the data were tabulated below in the Table. The gallic acid content in the tissue cultured methanolic plant extract was 99 and 92 µg/mg in the wild plant extract. The quercetin content was 85.7 µg/mg in the tissue cultured methanolic plant extract and 82.7 µg/mg in the wild methanolic extract (Table 4.2.3).

Table 4.2.3*:* Accuracy of gallic acid and quercetin content in *T. crustacea* extract using HPLC, Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Extracts	Peak name	RT	Area	% Area	Concentration in µg/mg of dried extract (Mean +SE)
Wild *Torenia crustacea*	Gallic Acid	2.70	3796799	17.77	91.66±1.24
Tissue cultured *Torenia crustacea*	Gallic Acid	2.68	3900718	28.74	99.33±1.24
Wild *Torenia crustacea*	Quercetin	5.711	1575448	11.21	82.73±1.42
Tissue cultured *Torenia crustacea*	Quercetin	5.778	5202602	14.09	86.33±0.53



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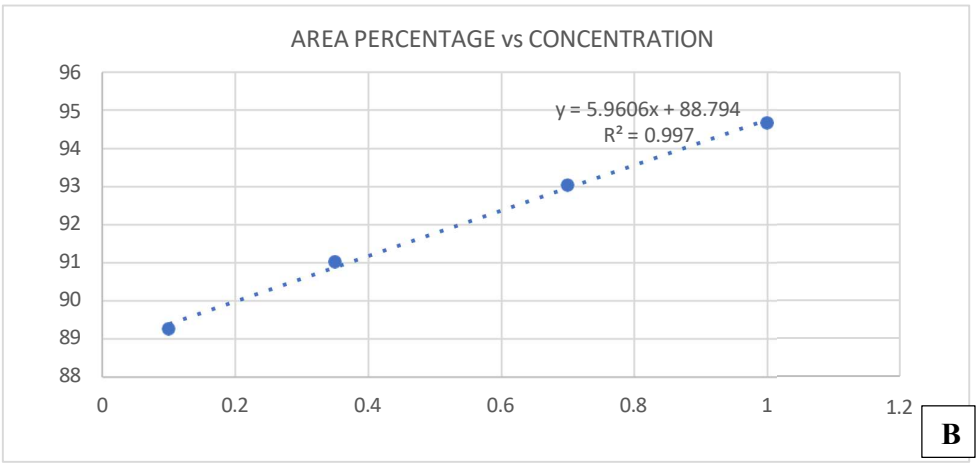


Fig 4.2.6: Linearity Graph A: Gallic ad B: Quercetin

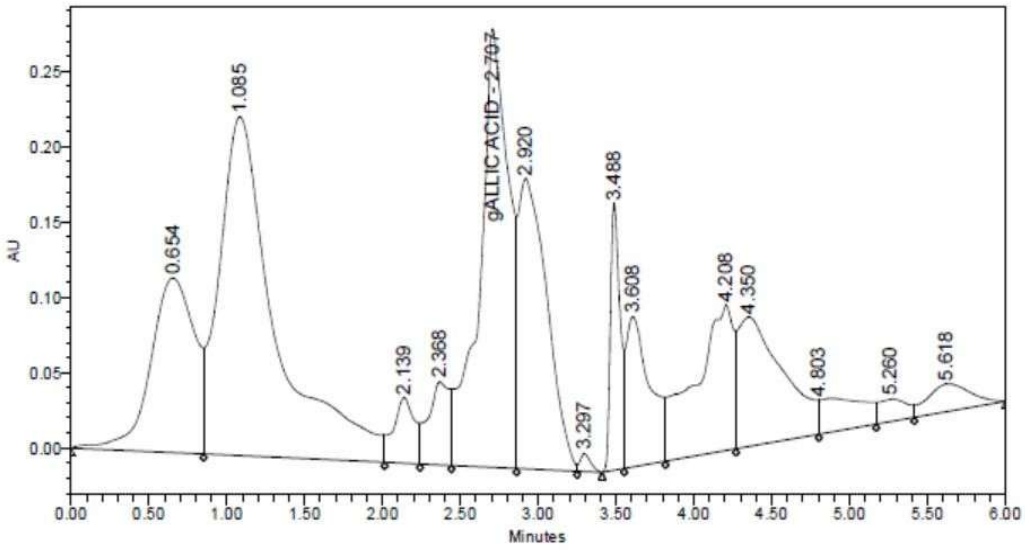


Fig 4.2.7a: HPLC Chromatogram of tissue cultured *T. crustacea* methanolic plant extract

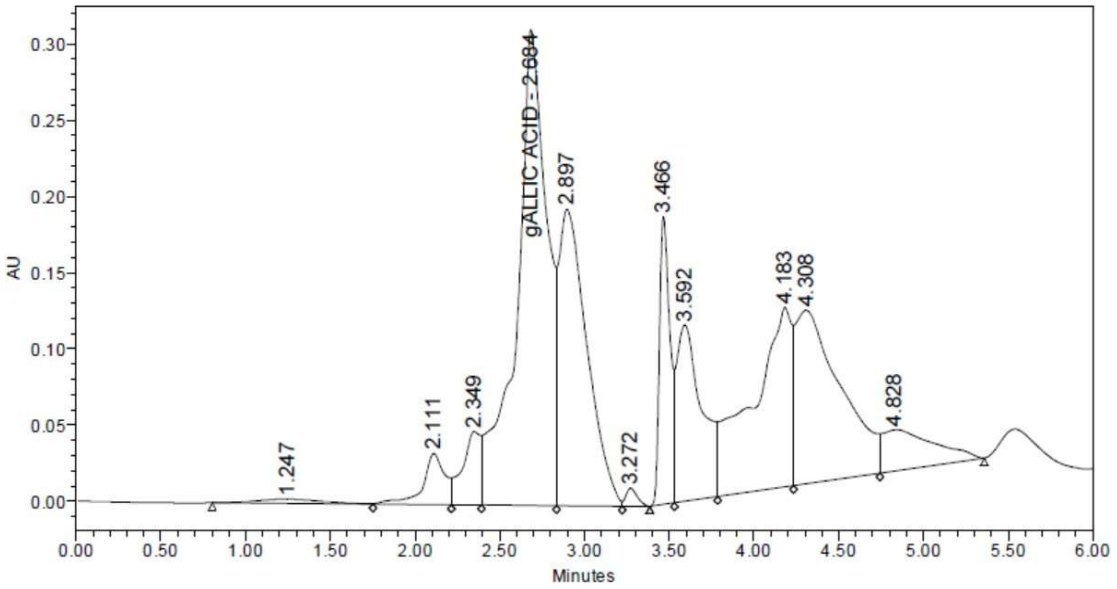


Fig 4.2.7b: HPLC Chromatogram of wild *T. crustacea* methanolic plant extract

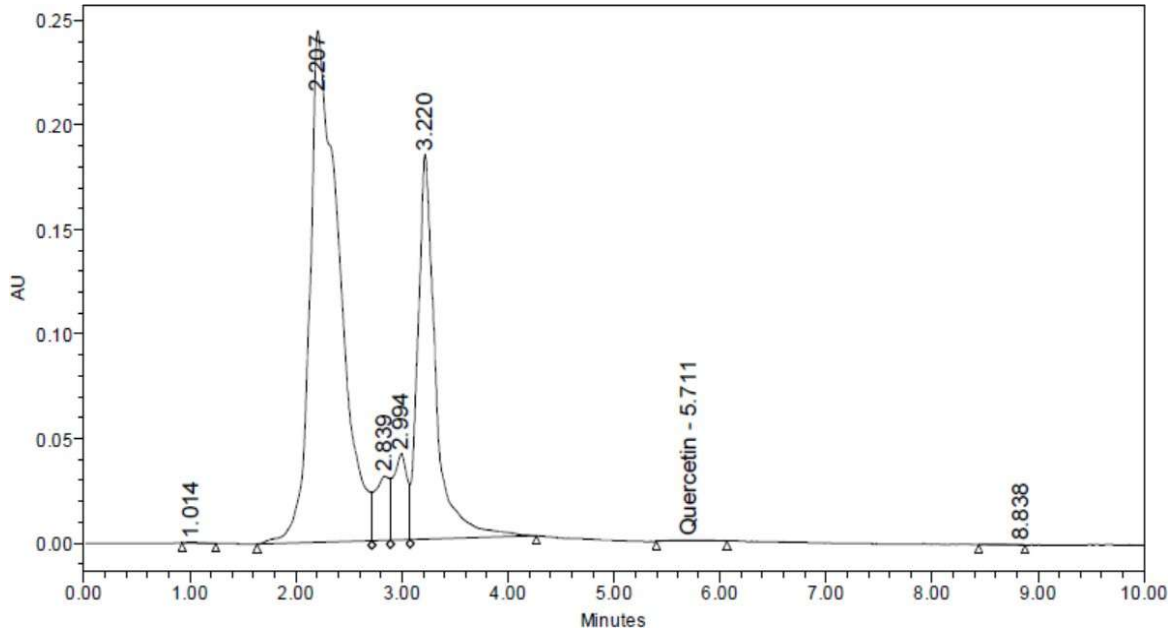


Fig 4.2.7c: HPLC Chromatogram of wild *T. crustacea* methanolic plant extract

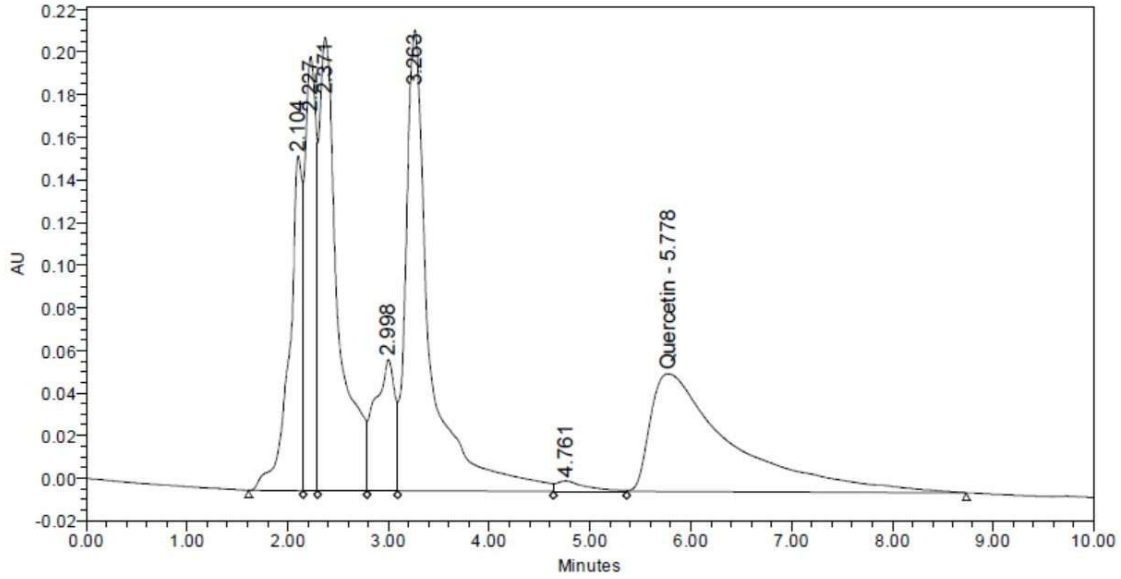


Fig 4.2.7d: HPLC Chromatogram of tissue cultured *T. crustacea* methanolic plant extract

4.2.8 GC-MS compound analysis in tissue cultured and wild extracts of *T. crustacea*:

The methanolic extracts of wild and tissue cultured *T. crustacea* plants were screened for the presence of bioactive compounds using GC-MS analysis. The GC-MS chromatogram for both wild and tissue cultured aqueous methanolic extract of *T. crustacea* were plotted (Fig. 4.2.8a & 4.2.8b). the compound search was conducted using retention time (RT), molecular weight, molecular formula, and peak area% (concentration). Ten bioactive compounds were identified in the wild extract and in the tissue cultured extract (Table 4.2.4).

The methanolic extract of wild *T. crustacea* contains the following compounds- Benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester is primarily used as a chemical derivatization agent in analytical chemistry to improve the volatility and stability of compounds for gas chromatography-mass spectrometry (GC-MS) analysis (Dionisio et al., 2018), Trimethylsilyl dimethylphosphinate serves as an effective electrolyte additive that improves the electrochemical performance in high-voltage lithium-ion batteries (Han *et al.,* 2015), Cyclohexyl propionate is primarily used as a synthetic flavouring agent (Dionisio et al., 2018), Methyl sterculate is primarily used in organic chemistry research in ring opening oxidation and metabolism studies (Pearson *et al.,* 1972), Oleic acid is a

monounsaturated omega-9 fatty acid commonly found in various animal and vegetable fats and oils. Oleic acid is widely used in the food industry for its nutritional benefits, such as lowering bad cholesterol and raising good cholesterol (Chochos & Choulis, 2011), Arsenous acid, tris(trimethylsilyl) ester primarily used as a chemical reagent in organic synthesisit is used to treat rheumatism, skin disease etc. (Manikandan *et al.,* 2019), Hexamethylcyclotrisiloxane is primarily used as a synthesis of poly siloxane, dimethoxydimethylsiloxane (Kumar & Eichinger, 1990), Octadecamethyloctasiloxane is used as dielectric fluids, damping fluids, hydraulic fluids, cosmetics and personal care additives, polishes, textile finishes, paint additives, heat-transfer oils and photocopy fuser oils (Butts M et al., 2002).

Again, in the *in vitro* methanolic extracts of *T. crustacea* the following compounds were identified using GC MS analysis- Octamethylcyclotetrasiloxane is primary function is as a conditioning agent and emollient, It may also be used as a probe liquid for nuclear magnetic resonance cryoporometry (NMRC) to analyze the pore size distribution (PSD) of oil-bearing tight sandstone (Liu et al., 2017), D-mannitol, 1,1'-O-1,16-hexadecanediylbis- is primarily used as a sweetener and stabilizer in the food and beverage industry due to its ability to preserve the texture and quality of food products during freezing and drying processes (O'Neil et al., 2013), 10-Fluorodecanoic acid is particularly useful for the fluorination of other organic compounds, greaseproofing and coating agent in furniture's, packaging, and carpets (Li et al., 2022), the chronic administration of perfluorodecanoic acid may induce weight loss and increases liver weight and lipid content in mice. Perfluorodecanoic acid reduces mRNA expression of the genes encoding IL-1β, IL-18, and cellular inhibitor of apoptosis 2 (cIAP2), as well as caspase-1, -3, and -7 in mouse liver. It has been found in marine life and as a contaminant in surface water. Formulations containing perfluorodecanoic acid have been used commercially as wetting agents and flame retardants (Li et al., 2022), 1,3-Dioxolane, 4-ethyl-5-octyl-2,2-bis(trifluoromethyl)-, trans- is primarily used as a research compound for studying chemical properties and important applications of fluorinated 1,3-dioxolane derivatives (Rollin et al., 2011).

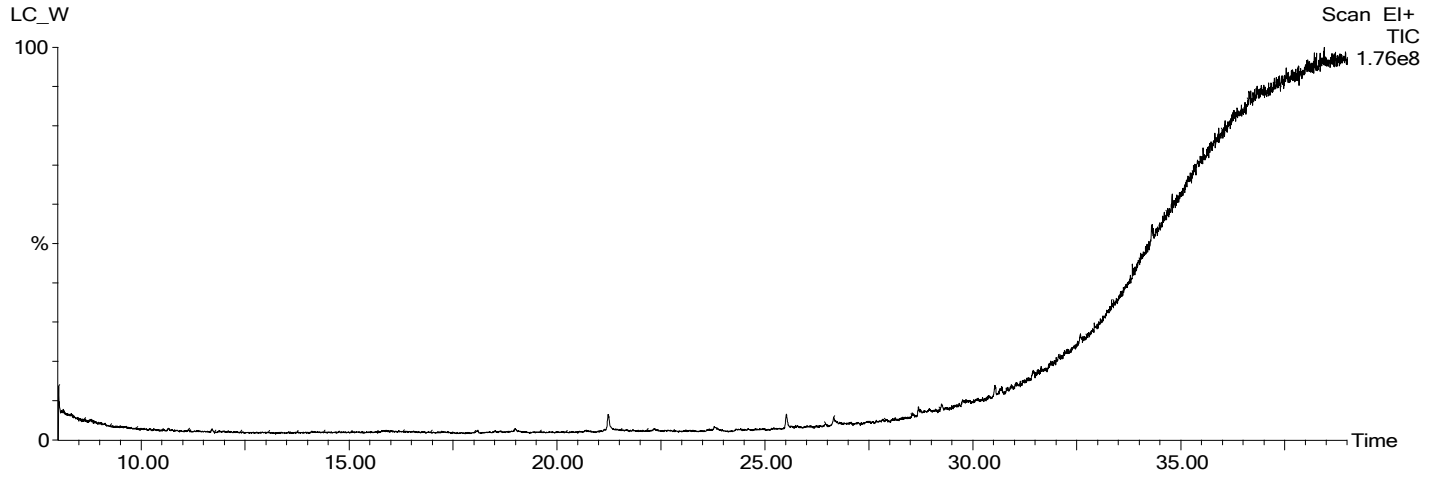


Fig 4.2.8a: GC-MS spectrum of wild *T. crustacea* plant in methanol extract.

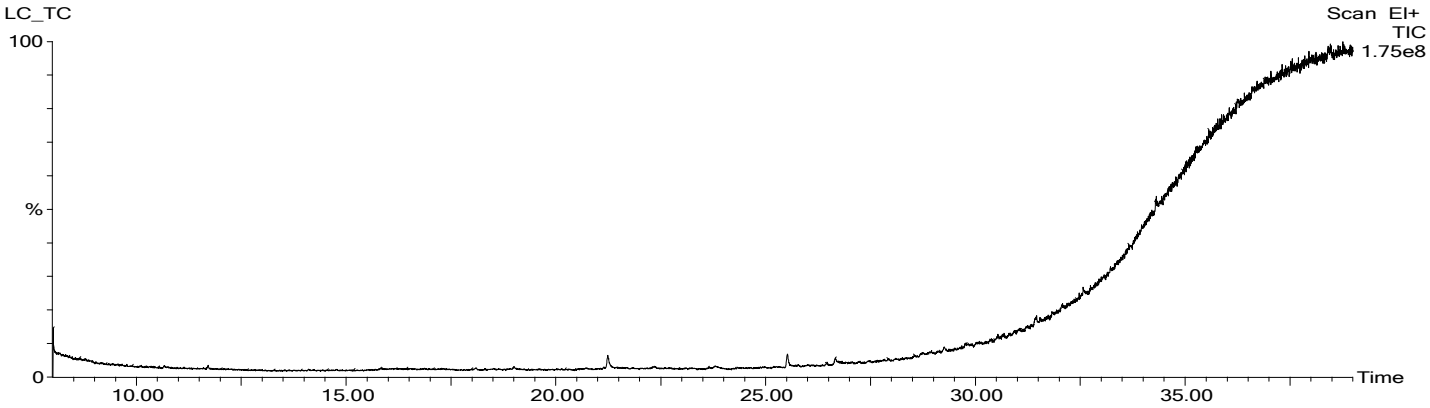
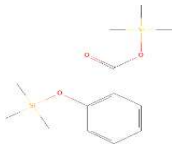
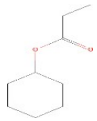
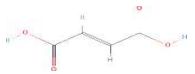


Fig 4.2.8b: GC-MS spectrum of tissue cultured *T. crustacea* plant in methanol extract

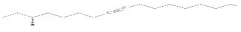
Table 4.2.4: Different compounds in the wild and tissue cultured *T. crustacea* methanolic plant extract, Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

A: Compounds in the wild *T. crustacea* methanolic plant extract

Sl no	RT	Area	Area %	Compound name	Molecular weight g/mol	Chemical formula	Structure
1	11.75	174,799.9	0.374	Benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	296.51	$C_{14}H_{24}O_3Si_2$	
2	18.097	94,824.2	0.274	Trimethylsilyl dimethylphosphinate	166.23	$C_5H_{15}O_2Psi$	
3	19.003	157,836.3	0.337	Cyclohexyl propionate	156.22	$C_9H_{16}O_2$	

4	21.239	634,978.7	1.357	Dihexadecyl phosphate Fumaric acid, 3,5-difluorophenyl tetradecyl ester	116.07	$C_4H_4O_4$	
5	25.515	395,101.7	0.844	Methyl sterculate	308.5	$C_{20}H_{36}O_2$	
6	26.651	348,751.3	0.745	Oleic acid	282.5	$C_{18}H_{34}O_2$	
7	28.692	227,807.1	0.487	Decanoic acid, 10-fluoro-, trimethylsilyl ester	262.44	$C_{13}H_{27}FO_2Si$	
8	29.247	153,925.5	0.329	Monomethyl octadecanedioate,	328.5	$C_{19}H_{36}O_4$	

9	32.588	235,562.0	0.503	Arsenous acid, tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$	
10	30.532	226,698.9	0.484	Hexamethylcyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$	
11	30.688	3,606,372		Octadecamethyloctasiloxane	607.3	C18H54O7Si8	
				B: Compounds in the Tissue cultured *T. crustacea* methanolic plant extract			
1	8.018	13,426,354.0	27.320	Octamethylcyclotetrasiloxane			
2	21.239	620,776.3	1.263	D-MANNITOL, 1,1'-O-1,16-HEXADECANEDIYLBIS-	586.8	$C_{28}H_{58}O_{12}$	

3	23.344	147,667.7	0.300	1,1,3,3,5,5,7,7,9,9,11,11,13,13-Tetradecamethylheptasiloxane	503.07	$C_{14}H_{42}O_6Si_7$	
4	23.805	266,368.1	0.542	10-Fluorodecanoic acid	190.25	$C_{10}H_{19}FO_2$	
5	25.521	414,441.7	0.843	(R)-(-)-14-METHYL-8-HEXADECYN-1-OL	252.4	$C_{17}H_{32}O$	
6	26.661	261,001.8	0.531	1,3-Dioxolane, 4-ethyl-5-octyl-2,2-bis(trifluoromethyl)-, trans-	350.34	$C_{15}H_{24}F_6O_2$	
7	29.262	165,728.5	0.337	Dodecanedioic acid, bis(trimethylsilyl) ester	374.7	$C_{18}H_{38}O_4Si_2$	

4.3 *L. pusilla*

4.3.1 Explant Surface sterilization: There are various surface sterilant are being used in tissue culture process to get rid of contamination, which includes, calcium hypochlorite, ethanol, mercuric chloride, sodium hypochlorite, hydrogen peroxide, silver nitrate, and bromine water (Teixeira da Silva, 2016); (Singh et al., 2011). Plant preservative mixture (0.1 mL/L to 1 mL/L) also used in the culture medium to reduce contamination in the culture (Plant Cell Technology 1998). The collected explants of *L. pusilla* were washed thoroughly in the running water for 30 mins. The roots, leaves were separated and dipped in tween 20 for 30 min, followed by washing in 70% alcohol for 30s after washing. The above nodal explants were treated in 0.5% Bavistin for 45min for elimination of fungus. In the experiment for surface sterilisation of the *Lindernia pusilla* explants mercuric chloride was preferred due to its effectiveness and availability. 0.1% mercuric chloride was used for surface sterilisation of the *L. pusilla* explants.

In the present experiment of explant surface sterilisation using 0.1% mercuric chloride, 2 min and 3 min of treatment showed the maximum explant survival rate after 21 days of culture initiation. 2 min of treatment with 0.1% mercuric chloride showed 76.67±4.71% explant survival and 23.33±4.71% explant contamination, and 3 min of treatment with 0.1% mercuric chloride also showed also 76.67±4.71% explant survival, but only 20±8.16% of the explants got contaminated and 10% of the explants got damaged after 21 days of culture initiation. 4 min of treatment resulted in 66.67±4.71% explant survival, and another 33.33±4.71% of the explants got damaged. 1-minute treatment showed a minimum (36.67±4.71%) explant survival rate, and explants without mercuric chloride treatment resulted in 100% explant contamination after 21 days of culture initiation (Fig. 4.3.1). Previously, it was reported that for explant surface sterilisation of *L. antipoda* (L.), 10% NaOCl treatment for 10 min and 0.15% Bavistin treatment showed the most effective results (T. et al., 2016).

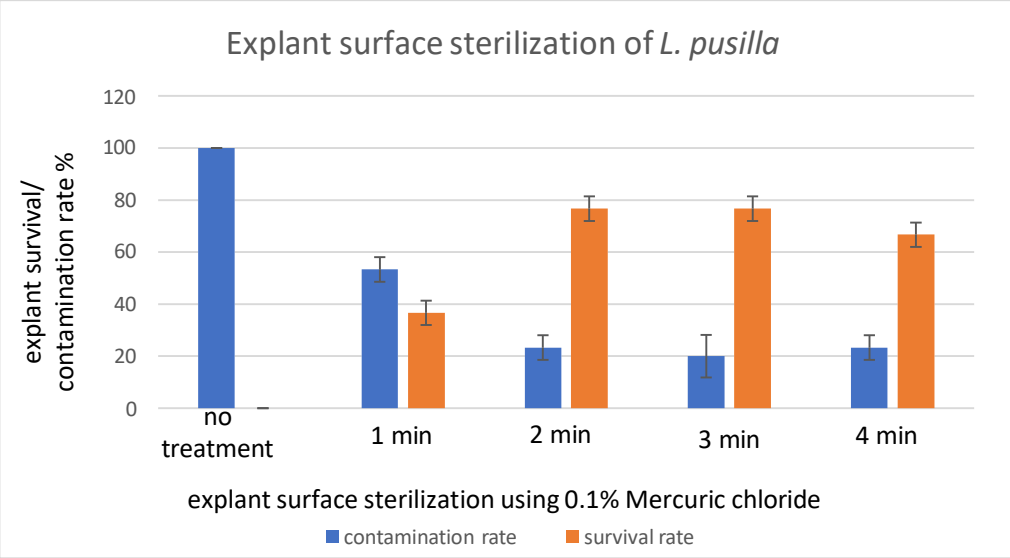


Fig 4.3.1: Explant survival rate of *in vitro* propagated *L. pusilla* after 21 days of initiation. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

4.3.2 Explant response in different Basal media: Media composition including different growth regulators may be used to grow different plant parts, the response of plant tissues varies due to in vitro conditions and growth regulators. The plant hormones auxin and cytokinin are essential for shoot regeneration, elongation and root formation (Revilla & Ryan, 2000). The callus of *Psophocarpus tetragonolobus* (L.) formed multiple shoots when it was cultured in the MS medium containing BAP along with NAA or IAA.

In the present experiment it was observed that the explants of *L. pusilla* responded differently in the MS media supplemented with different growth regulators. Highest shoot proliferation and multiplication in the explants of *L. pusilla* were observed in the BM2 media where MS medium supplemented with 1mg/mL BAP and 0.2 mg/L NAA, i.e. an average of 33 shoots per explant (Fig 4.3.2), and the lowest shoot proliferation and multiplication were observed in the BM12 media (MS + 2mg/L IAA). The control media without any growth regulator formed an average of 17±1.41 shoots per explant, 6±1.41 cm shoot length and an average of 11±1.41 number of roots. BM1 media (MS+1mg/L BAP) formed an average of 26±1.16 shoots per explant, 6±0.8cm shoot length and 11±1.41 mean number of roots per explant. An average of 26±0.74 shoots per explant, 8± 0.89 cm length, and 8±0.81 roots per explant was observed in the BM3 media

(MS+1mg/L BAP+0.4mg/L NAA). An average of 23±1.01 shoots per explant, 7±0.74 cm, and 11±1.64 roots per explant were observed in the BM5 media where MS was supplemented with 1mg/L BAP and 0.5mg/L NAA. In the BM6 media (MS+ 2.0mg/L BAP) an average of 18± 0.74 shoots per explant, 6± 1.16 cm per shoot, and 15±1.24 number of roots per explant were observed. In the BM7 media where MS was supplemented with 2mg/L BAP and 0.5mg/L NAA an average of 16± 0.81 shoots per explant, 6± .81cm shoot length per explant, and 17±1.41 roots per explant was observed. In the BM8 media (MS+ 2mg/L BAP+ 1mg/L NAA) an average of 11± 0.81 shoots per explant, 5± 1.24 cm average shoot length, and 11±1.24 number of roots per explant was observed. An average of 12± 0.94 shoots per explant, 5±0.47cm length per shoots, and 12±0.74 number of roots per explant was observed in the BM9 media (MS+3mg/L BAP). In the BM10 media (MS +0.5mg/L IAA) an average of 13± 1.63 shoots per explant, 5± 1.24 cm shoot length per explant, and 38±1.41 roots per explant was observed. In the BM11 media (MS+1mg/L IAA) an average of 11± 1.63 shoots per explant, 5± 1.41cm length per shoot, and 28±1.41 average number of roots per explant was observed (Table 4.3.1).

Both plant hormones cytokinin and auxin are essential for plant regeneration in tissue culture, cytokinin are particularly important for shoot regeneration (Revilla & Ryan, 2000). Different concentrations of BA induce shoot formation from the callus without formation of root (Rout et al., 2009).

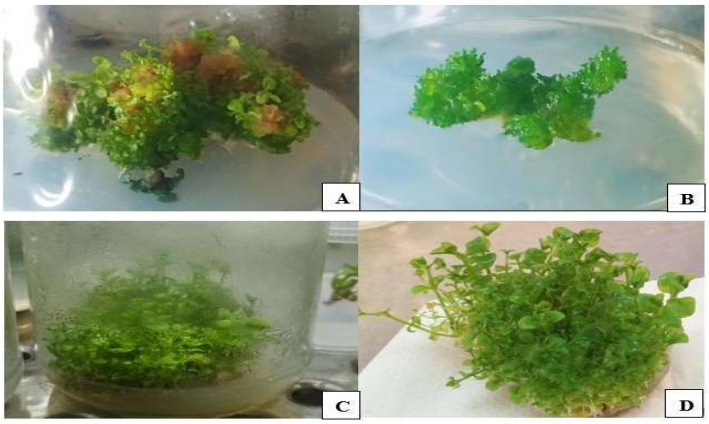


Fig 4.3.2*: in vitro* propagation of *L. pusilla*, **A**; explant initiation, **B&C**; shoot multiplication and **D**: root formation

Table 4.3.1: Effect of different growth regulators on *in vitro* propagation of *L. pusilla.* Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Sl no	Basal media	Growth regulators (mg/L)			Number of explant culture initiation	Rate (%) of explant producing shoots	Number of shoots per explant	shoot length (cm)	Number of roots
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	05	100	17±1.41	6±1.41	11±1.41
2	BM1	1.0	0.0	0.0	05	100	26±1.16	6±0.8	10±2.44
3	BM2	1.0	0.2	0.0	05	100	33±1.32	10±1.32	6±1.24
4	BM3	1.0	0.4	0.0	05	100	26±0.74	8± 0.89	8±0.81
5	BM4	1.0	0.5	0.0	05	100	23±1.01	7±0.74	11±1.64
6	BM5	1.0	1.0	00	05	100	23± 0.74	7± 0.89	12±0.74
7	BM6	2.0	0.0	0.0	05	100	18± 0.74	6± 1.16	15±1.24
8	BM7	2.0	0.5		05	100	16± 0.81	6± .81	17±1.41
9	BM8	2.0	1.0		05	100	11± 0.81	5± 1.24	11±1.24
10	BM9	3	0.0	0.0	05	100	12± 0.94	5±0.47	12±0.74
11	BM10	0.0		0.5	05	100	13± 1.63	5± 1.24	38±1.41
12	BM11	0.0	0.0	1.0	05	100	11± 1.63	5± 1.41	29±1.63
13	BM12	00	00	2.0	05	100	9± 1.24	4± 1.16	28±1.41

4.3.3 Hardening:

The primary hardening 90% of the *L. pusilla* was successfully done on the 50% vermicompost soil mixture inside the green house at a temperature of $28^0C \pm 2^0C$ and a relative humidity of 70%. After 21 days grown in the greenhouse the explants were taken out to the shed net house in natural environment with varying temperature $28^0C \pm 5^0C$. all the plantlets survived in the natural condition in this step. Finally, the plantlets were successfully grown in the natural climate condition.

4.3.4 Random Amplified Polymorphic DNA (RAPD) assay: Maximum concentration of bioactive compounds can be produced using in vitro cultivation methods but there is very high possibility for generation of new somaclones. Therefore, detection of the somaclones in the regenerated plants are important. Molecular markers may be an effective tool for the detection of somaclones in the regenerated plants. RAPD is very effective and useful tool for identification of somaclonal variation in the tissue cultured plants. In the present study, for the detection of somaclones in the in vitro derived L. pusilla plants 14 RAPD primers were used and a total of 355 polymorphic DNA bands were formed in wild and micropropagated (Hardened and non-hardened) plant. 7 RAPD primers (OPC 02, OPC 05, OPC 09, OPA 01, OPA2, OPC 03, and OPC 06) formed 177 polymorphic DNA bands out of 14 RAPD primers showing variation in the micro propagated L. pusilla plants (Fig. 4.3.3). And the 7 RAPD primers (OPC07, OPC08, OPA04, OPA13, OPA12, OPC01, and OPC04) formed 178 similar monomorphic DNA bands. the OPC 08 primer formed the highest 14 RAPD bands were formed in the micropropagated plants (hardened and non-hardened) and the lowest of 1 RAPD primer were formed by the OPC 04 primer (Table 4.3.2).

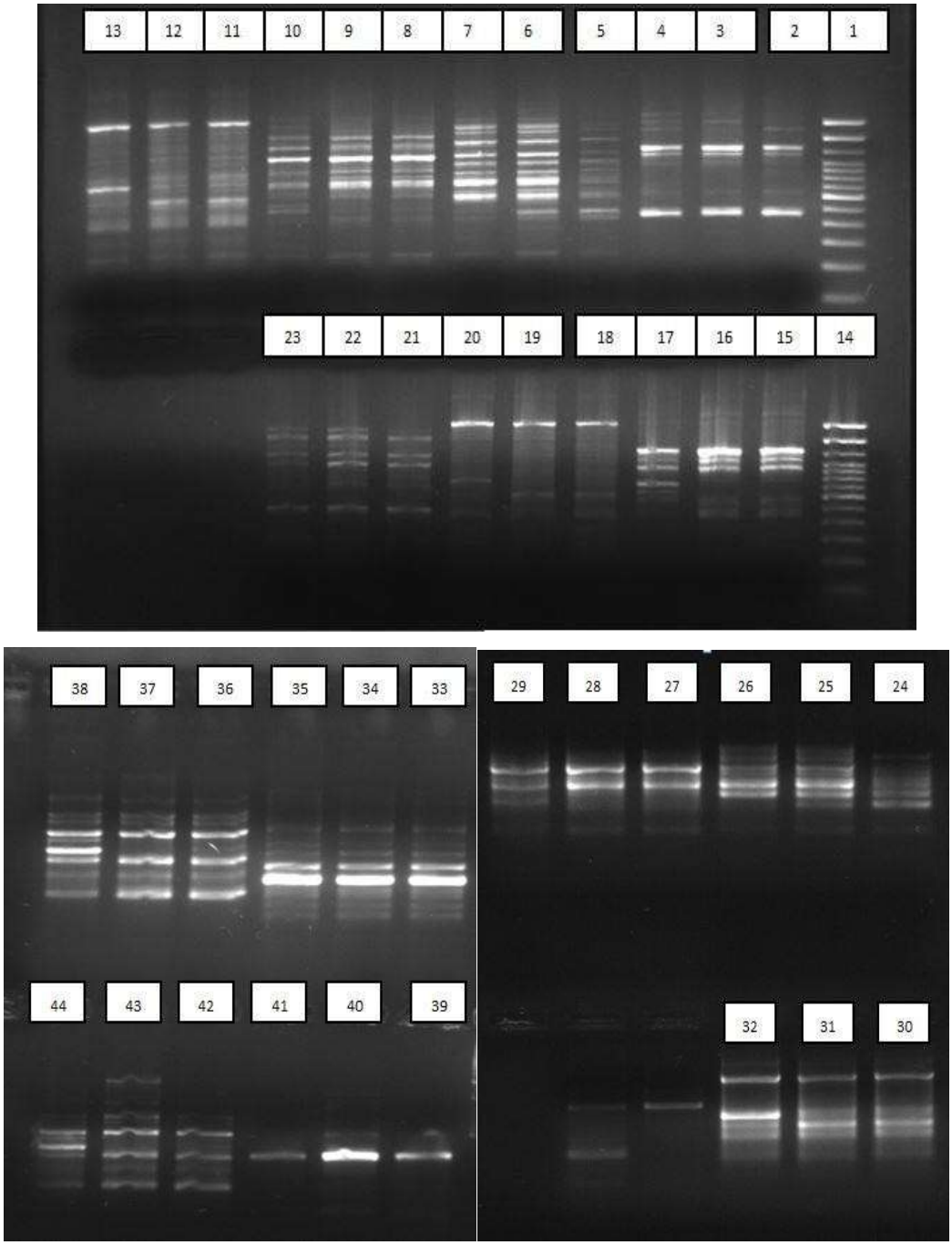


Fig 4.3.3: Lane 1: 100 bp marker; lane 3,4,5: OPC 02; lane 6,7,8: OPC 05; lane 9, 10, 11: OPC07; lane 12, 13, 14: OPC 08; lane 15, 16, 17: OPC09; lane 18, 19, 20: OPA01; lane 21, 22, 23: OPA02; lane 24, 25, 26: OPA04; lane 27, 28, 29: OPA13; lane 30, 31, 32: OPA12; lane 33, 34, 35: OPC01; lane 36, 37, 38: OPC03; lane 39, 40, 41: OPC04; lane 42, 43, 44: OPC06.

Table 4.3.2: Table showing numbers of DNA monomorphic/polymorphic bands produced by different RAPD primers in wild, micro propagated (non-hardened) and hardened micro propagated *L. pusilla*

Sl no	Primer	Number of bands produced by wild plant	Number of bands produced by micro propagated plants	Number of bands produced by hardened micro propagated plants
1	OPC 01	9	9	9
2	OPC 02	7	9	9
3	OPC 03	8	8	9
4	OPC 04	3	3	1
5	OPC 5	13	14	14
6	OPC 06	7	8	9
7	OPC 07	13	13	13
8	OPC 08	14	14	14
9	OPC 09	8	8	9
10	OPA 01	5	5	5
11	OPA 02	8	8	6
12	OPA 04	8	8	8
13	OPA 12	7	7	7
14	OPA 13	6	6	6

4.3.5 Comparative in-vitro antioxidant tests of wild and tissue-cultured extract of L. pusilla: The medicinal plants are the primary source of new drug inventions for treating various diseases. *In vitro* propagation method using cells and tissues plays a significant role in the synthesis of natural products. In a study of comparative analysis of phenolic profiles in *in vitro* propagated and wild plants of *Psophocarpus tetragonolobus* (L.) DC, significant difference in phenolic components were observed (Singh et al., 2014).

Total Phenolic Content: The total phenolic content in the dried extract of *L. pusilla* was determined using the regression curve ($y = 276.85x + 68.485$; $R^2 = 0.9934$) (Fig 4.2.5a) and expressed as mg of GAE (Gallic acid equivalent). The total phenolic content in the tissue cultured extracts was comparatively higher (61±3.4 mg GAE/g) than that of the wild (54.7 ±5.3 mg/g GAE) extracts of *L. pusilla*.

Total flavonoid content: The flavonoid content in the wild and micropropagated *L. pusilla* was determined using the regression curve (y = 404.68x + 53.647 $R^2 = 0.993$) (Fig 4.2.5b) and expressed as Quercetin equivalent mg/g of the dried extract. In the experiment the flavonoid content in the tissue cultured methanolic extract was comparatively higher (24±7.7 mg QE/g) than the wild (20±7 mg QE/g) extract of *L. pusilla*.

Total antioxidant capacity: Total antioxidant capacity in the tissue cultured and wild extract was determined using the regression curve (y=324.98x + 45.306 $R^2 = 0.9902$) (Fig 4.2.5c) and expressed as mg of ascorbic acid equivalent per grams of dried extract (AAE)**.** The total antioxidant capacity in the tissue cultured L. pusilla was 114.7±7.2 mg of AAE/g of dried extract and 94±7.2 mg AEE/g in wild extract.

DPPH scavenging Activity: The radical scavenging activity in the wild and tissue cultured *P. thyrsiformis* was determined by the regression curve (y = 16.114x + 13.25; $R^2 = 0.9846$) (Fig 4.2.5d). The IC_{50} value of the wild methanolic extract of *P. thyrsiformis* is 40.70±0.50 and in the tissue cultured extract was 45.19±0.84.

4.3.6 Quantitative Detection of gallic acid and quercetin using HPLC: The elution of the wild and the tissue cultured extract was done using the isocratic solution of 30% (v/v) HPLC-grade water and 70% (v/v) methanol. The gallic acid and quercetin content in the wild and tissue cultured methanolic extract of *L. pusilla* was satisfactory. The chromatograms and the standard curve of gallic acid and quercetin was plotted. The gallic acid content in the wild and tissue cultured *L. pusilla* was determined using the regression curve (y= 0.8696x + 98.778 $R^2 = 0.9958$) (Fig 4.2.6A) and the quercetin content in the wild and tissue cultured *L. pusilla* was determined using the regression curve (5.9606x + 88.794 $R^2 = 0.997$) (Fig 4.2.6B). The retention time of quercetin and gallic acid was 4.42min and 2.6 min respectively (Fig: 4.3.4a, 4.3.4b, 4.3.4c, 4.3.4d). From the experiment the gallic acid content and quercetin content was higher in the tissue cultured extract was than the wild extract of *L. pusilla*. The quercetin content in the micropropagated extract was 2.26± 0.032mg/g of dried extract and 2.13± 0.035mg/g of dried extract in the wild plant. Again, the gallic acid content was 10.302± 0.064mg/ g and 42.94± 0.23mg/g in the wild and tissue cultured plant extract of *L. pusilla, respectively (Table 4.3.3).*

Table 4.3.3: Accuracy of Gallic acid and Quercetin content in *L. pusilla* extract. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Extracts	Peak name	RT	Area	% Area	Height	Concentration in µg/mg of dried extract
Tissue cultured *L. pusilla*	Gallic Acid	2.549	12767793	25.40	611690	42.94± 0.23
Wild *L. pusilla*	Gallic Acid	2.602	3062882	7.43	396214	10.302± 0.064
Tissue Cultured *L. pusilla*	Quercetin	4.405	931357	1.81	53720	2.26± 0.032
Wild *L. pusilla*	Quercetin	4.427	876790	1.87	46670	2.13± 0.035

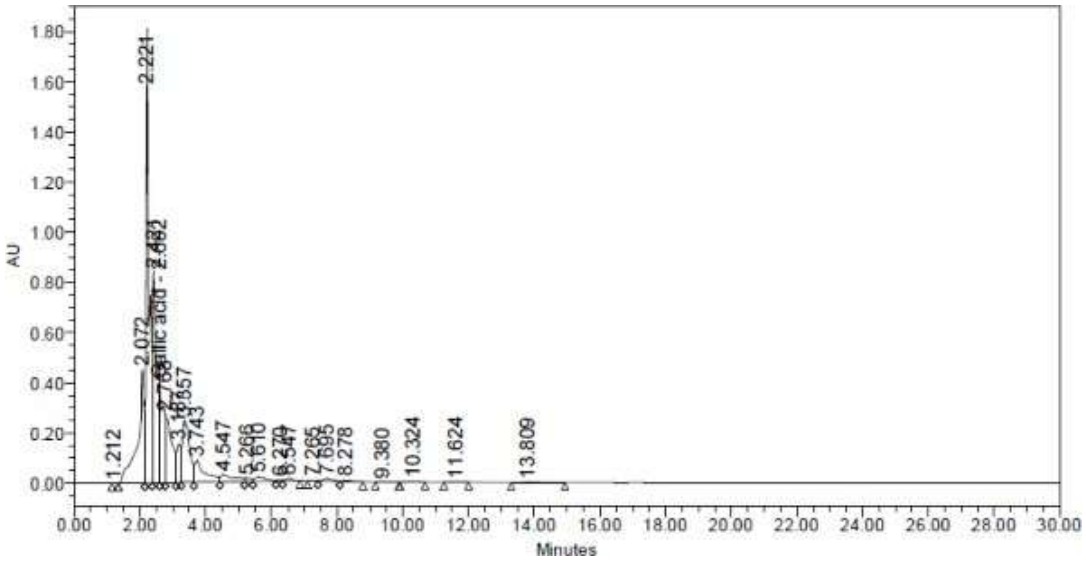


Fig 4.3.4a: Chromatograms of gallic acid in methanolic extract of tissue cultured *L. pusilla*

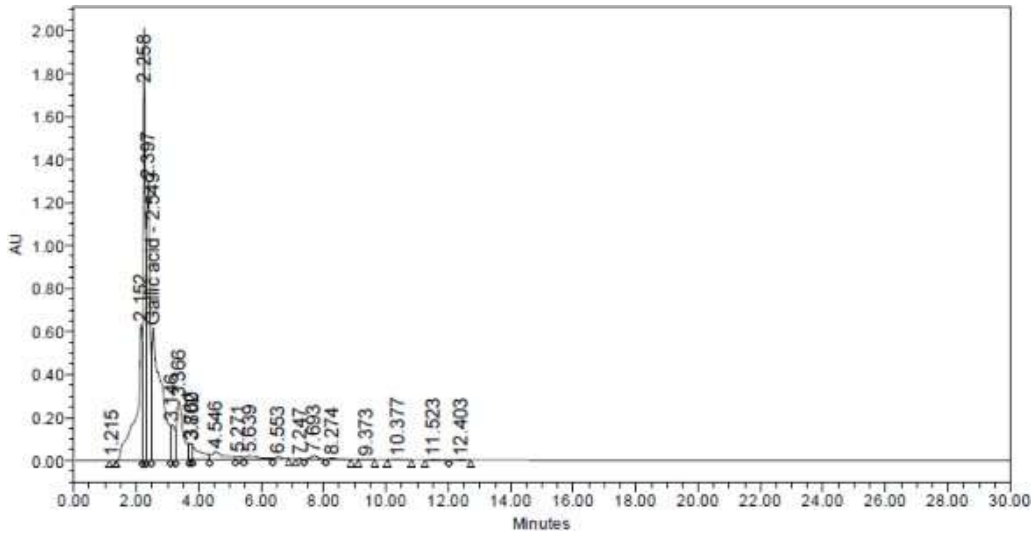


Fig 4.2.4b: Chromatograms of Gallic Acid in methanolic extract wild of *L. pusilla*

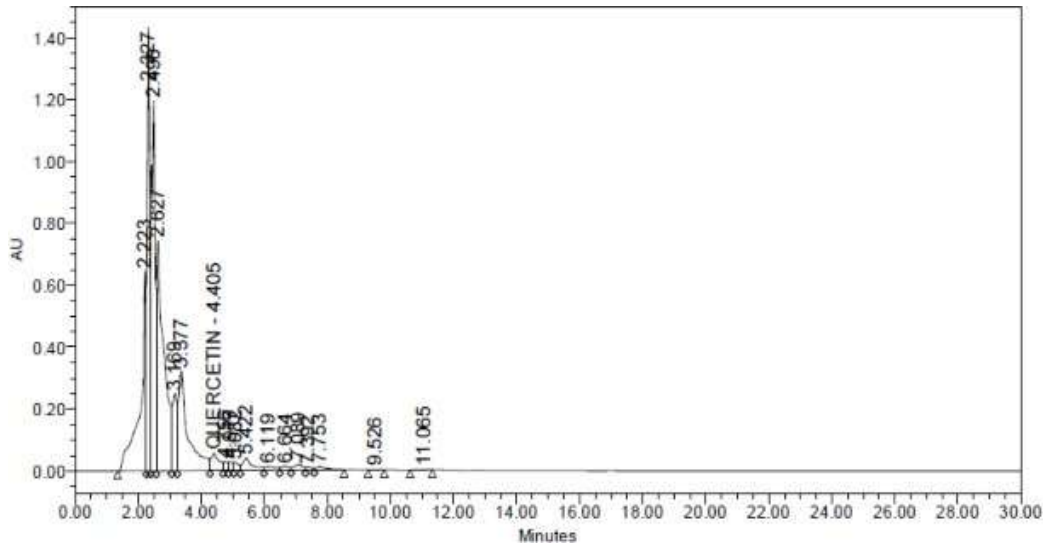


Fig 4.3.4c: Chromatograms of Quercetin in methanolic extract of tissue cultured *L. pusilla*

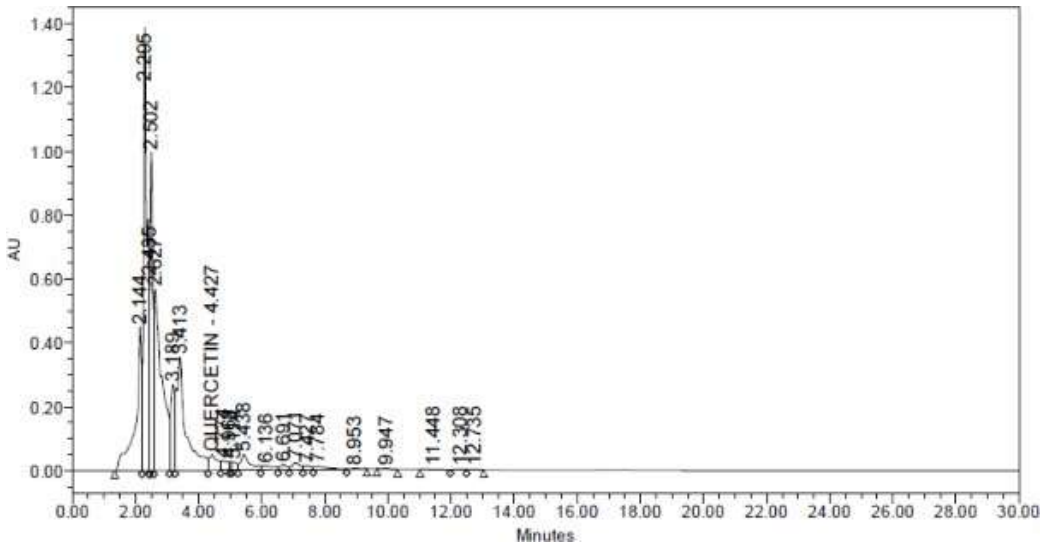


Fig 4.3.4d: Chromatogram of quercetin in methanolic extract of wild *L. pusilla*

4.3.7 Study of bioactive compounds in tissue cultured and wild extracts of L. pusilla using GC MS: 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 *L. pusilla* were studied using GC-MS analysis. The GC-MS-MS chromatogram of the aqueous methanolic extract of *L. pusilla* is shown in Figure (4.3.5a & 4.3.5b). The compound search was selected by retention time (RT), molecular formula, molecular weight, and peak area% (concentration). From the wild extract, eight chemical compounds were identified, and from the tissue-cultured extract, nine compounds were identified and presented (Table 4.3.4).

The methanolic extract of wild *L. pusilla* contains 1,2,4,5-Cyclohexanetetrol (key precursor in the biosynthesis of terpenes and steroids) (Rosatella & Afonso, 2022), 4-Aminohex-5-Enoic Acid can be used in treatment of neurological conditions, neuroscience research related to GABAergic pathways (Jung et al., 1977), Silane (2-Ethyl-3,3-Dimethyl-4-Methylene-1-Cyclopenten-1-Yl) Trimethyl which are used as Waterproofing agent in construction projects, semiconductor manufacturing (Baselt, 2014), Trisiloxane, 1,1,3,3,5,5-Hexamethyl- which are Wetting agent, emulsifier and foam builder used in cosmetics, shampoo, lotions etc. (Rayasam et al., 2022), Heptalene,

7,7'- Dihydro-6,6'-Bis (Trimethylsilyl) Methyl- is primarily used as a chemical reagent in organic synthesis, particularly for the protection of functional groups in complex organic molecules (Clegg et al., 2011), Panaxydol, Tms is an important bioactive compound that can be used as potential therapeutic for delaying liver inflammation (Kim et al., 2024), Trimethylsilyl-Di (Timethylsiloxy)-Silane is primarily used as a chemical reagent in organic synthesis, particularly for the protection of hydroxyl groups and activation of carboxylic acids (Arya et al., 1990).

The *in vitro* propagated methanolic extract of *L. pusilla* plant contains Chloroacetic Acid 2,2-Dimethylpropyl Ester, used as intermediate chemical in organic synthesis (Baselt, 2014), 4-Methylthiane S ,S-Dioxide is primarily used as intermediate chemical to synthesis of biologically active compound and pharmaceuticals, synthesis of pyrrole derivatives (Karthik et al., 2016), anthracene, 9-Ethyl-9,10-Dihydro-10-Trimethylsilyl- is used in preparation of nanostructured thin films and inhibitor in human protein kinase CK2 (López-Rojas et al., 2023), Panaxydol, Tms is an important bioactive compound that can be used as potential therapeutic for delaying liver inflammation (Kim et al., 2024), Trimethyl(5-Methyl-2-Pentylphenoxy) Silane is used as coating agents as an adhesion promoter (London et al., 2013).

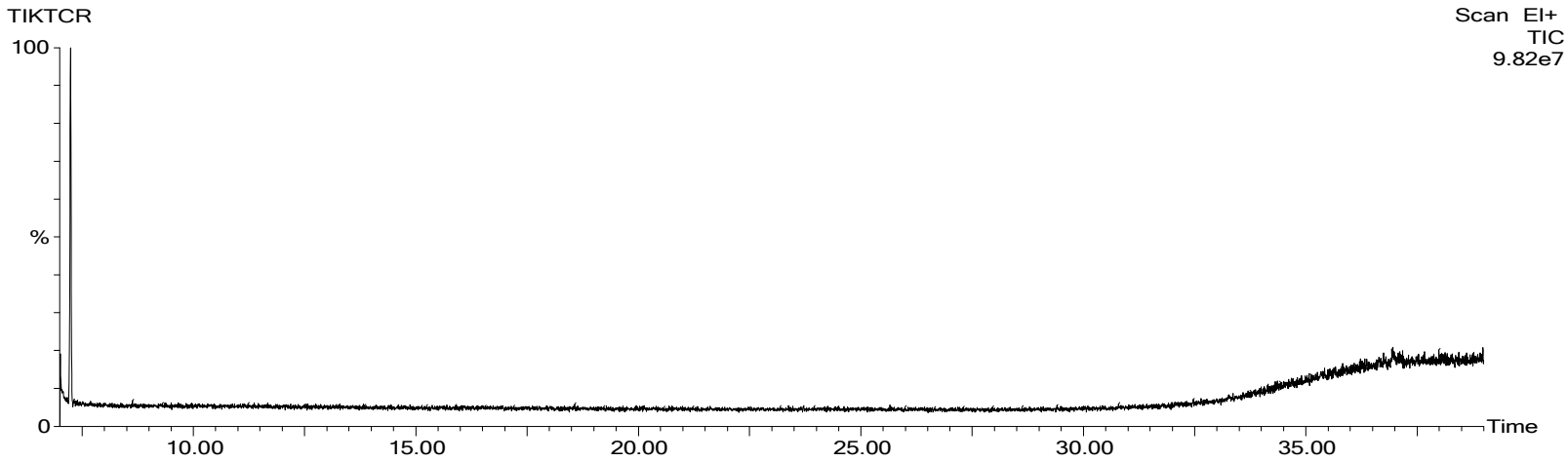


Fig 4.3.5a: GC-MS spectrum of tissue cultured *L. pusilla* plant in methanol extract

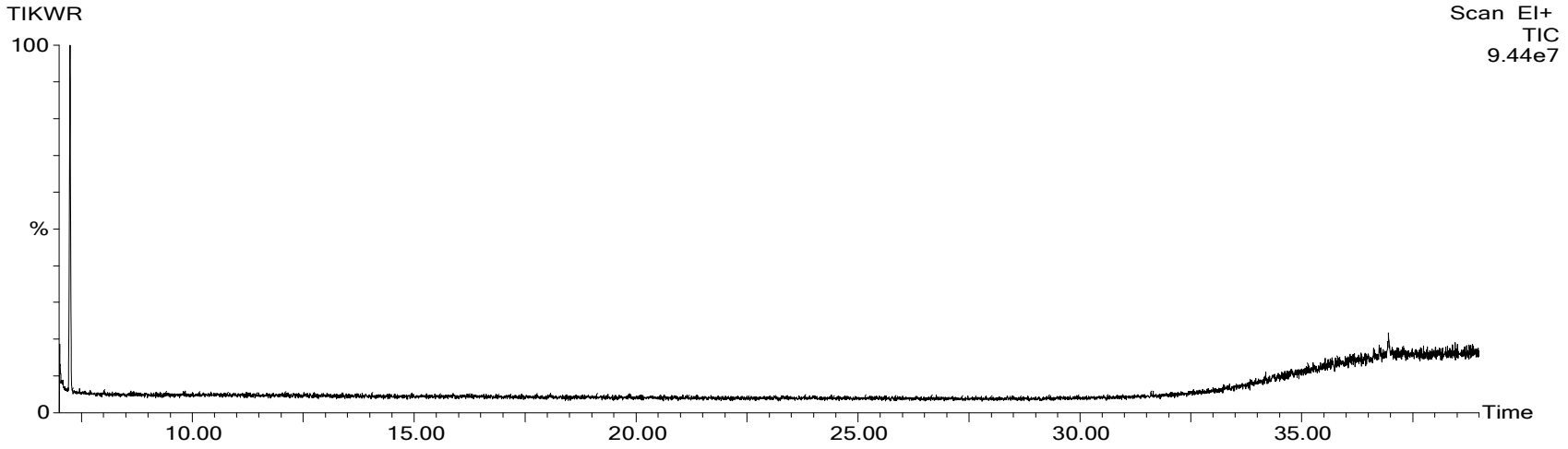
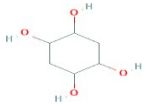
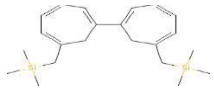
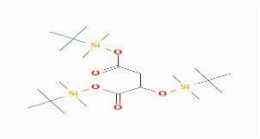


Fig 4.3.5b: GC-MS spectrum of wild *L. pusilla* plant in methanol extract

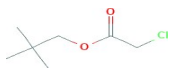
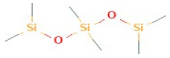
Table 4.3.4: Bioactive compounds found in aqueous methanolic extract of *L. pusilla* A: wild plant B: Tissue cultured plant.

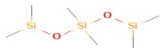
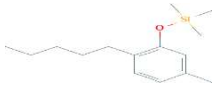
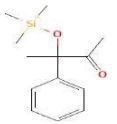
A: Bioactive compounds in wild methanolic extract of *L. pusilla*						
S. No.	**Retention time (RT in min)**	**Area (%)**	**Mol. weight (MW)**	**Formula**	**Compound name**	**Structure**
1	7.013	3.094	148	$C_6H_{12}O_4$	1,2,4,5-Cyclohexanetetrol,	
2	7.248	12.092	129	$C_6H_{11}NO_2$	4-Aminohex-5-Enoic Acid	
3	34.765	0.445	208		Silane, (2-Ethyl-3,3-Dimethyl-4-Methylene-1-Cyclopenten-1-Yl)Trimethyl	
4	35.680	0.552	208	$C_6H_{18}O_2Si_3$	Trisiloxane, 1,1,3,3,5,5-Hexamethyl-	

5	36.495	0.412	354	$C_{22}H_{34}Si_2$	Heptalene, 7,7'-Dihydro-6,6'-Bis (Trimethylsilyl) Methyl-	
6	36.740	0.560	476	$C_{22}H_{48}O_5Si_3$	Butanedioic acid	
7	36.950	1.337	332	$C_{20}H_{32}O_2Si$	Panaxydol, Tms	
8	38.451	0.184	280	$C_9H_{27}O_2Si_4$	Trimethylsilyl-Di(Timethylsiloxy)-Silane	

B: Bioactive compounds in tissue cultured methanolic extract of *L. pusilla*

S. No.	Retention time (RT, min)	Area (%)	Molecular weight (MW)	Formula	Compound name	Structure

1	7.013	4.046	164	$C_7H_{13}ClO_2$	Chloroacetic Acid, 2,2-Dimethylpropyl Ester	
2	7.244	12.732	148	$C_6H_{12}O_2S$	4-Methylthiane, S,S-Dioxide	
3	34.774	0.185	280	$C_{19}H_{24}Si$	Anthracene, 9-Ethyl-9,10-Dihydro-10-Trimethylsilyl-	
4	35.625	0.153	208	$C_6H_{18}O_2Si_3$	1,1,3,3,5,5-Hexamethyl trisiloxane	

5	36.460	0.173	280	$C_9H_{27}O_2Si_4$	Trimethylsilyl-Di (Timethylsiloxy)-Silane	
6	36.740	0.252	206	$C_6H_{18}O_2Si_3$	1,1,3,3,5,5-Hexamethyl trisiloxane	
7	36.950	0.732	332	$C_{20}H_{32}O_2Si$	Panaxydol, Tms	
8	36.975	0.545	208	$C_{15}H_{26}OSi$	Trimethyl(5-Methyl-2-Pentylphenoxy) Silane	
9	38.361		236	C13H20O2Si	3-Hydroxy-3-phenylbutan-2-one, tms derivative	

4.4 *P. thyrsiformis*

4.4.1 Explant surface sterilisation: The collected explants of *P. thyrsiformis* were sterilized using different sterilizing agents. Initially the collected explants were washed using tween 20 for 10 min, followed by 0.5% Bavistin for 45 min and 0.1% mercuric chloride(0-5min). When no treatment was used all the cultured explants got contaminated in the culture. When the explants treated with 0.1% mercuric chloride for 1 min, 36.67±4.71% of the explants survived out of 10 explants after 21 days of culture. 2min and 3 min treatment with 0.1% mercuric chloride was found best for the maximum survival rate (76.67±4.71%) of the explants after 21 days of explant culture. Though the contamination rate in the explants treated for 2 min mercuric chloride was 26.67±4.71% and with 3 min treatment the contaminated rate was 13.33±4.71%. The 4 min and 5 min treatment with 0.1% mercuric chloride showed no contamination rate but the explant survival rate was 73.33±4.71%, and 5min treatment resulted only 26.67±4.71% explant survival rate after 21 days of explant culture (Fig 4.4.1). Contaminations in the culture may also cause by the endophytes present in the explant used for the culture, it is very difficult to eliminate the endophytes from the explants. Microbes are most common and difficult problems in the *in vitro* explant culture technique which directly impacts the growth of the tissues (Webster & Mitchell, 2003). These endophytes or microbes affects adversely on the tissue culture of explants by competing for the nutrients in the culture media, most of the times presence of these microbes in the explant or culture leads to the explant mortality, tissue necrosis, and reduced growth of the tissues (Oyebanji et al., 2009).

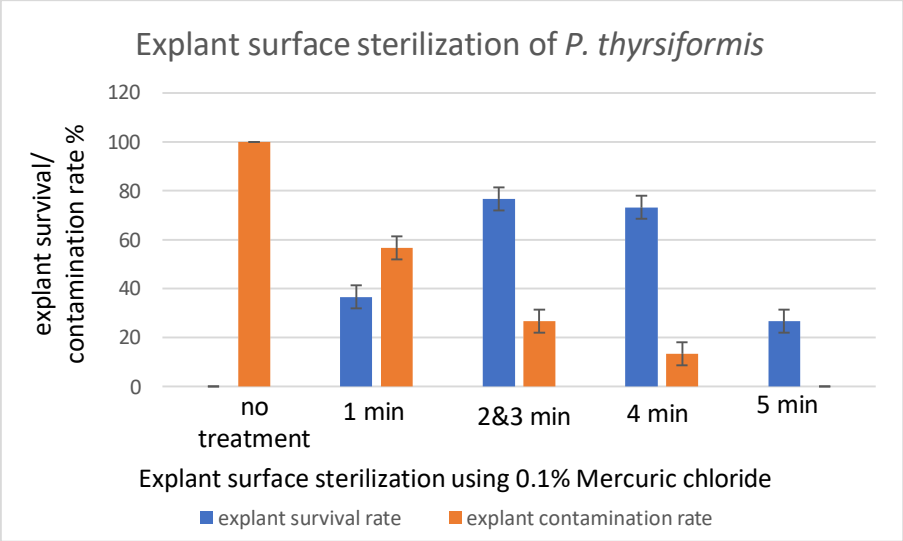


Fig 4.4.1: Graph showing explant survival rate of *in vitro* propagated *P. thyrsiformis* after 21 days of explant initiation0.1% mercuric chloride. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

4.4.2 Explant initiation and shoot multiplication and rooting: After successful surface sterilization of *P. thyrsiformis* explants using 0.1% mercuric chloride, the explants were cultured in MS media supplemented with different ratios of plant growth regulators to induce multiple shoots and rooting. The inoculated explants were monitored regularly for growth and contaminations in the culture. From the cultures it was observed that the explants cultured in the different MS media composition responded differently. The highest shoot multiplication and highest shoot length was obtained in the BM2 media where MS media was supplemented with 1mg/L BAP and 0.2mg/L NAA (Fig 4.4.2), i.e.- 9.5.4±0.8 shoots numbers per explant and 13.08±1.44 cm per explant and an average of 12.41±1.44 roots per explant. The BM12 media where MS was supplemented with 1mg/L IAA was most suitable for rooting of *P. thyrsiformis* explants. Lowest shoot numbers average shoot length and lowest rooting was observed in the BM1 where no growth regulators were added with MS media. In BM3 media (MS+1mg/L BAP +0.4mg/L NAA) formed 8.6±1.16 average number of shoots, 12.91±1.54cm average shoot length, and 14.66 ±1.54 average number of roots per explant after 21 days. BM4 media (MS+1mg/L BAP+0.5mg/L NAA) formed an average number of 7.7±1.47 shoots per explant with average shoot length of 11.7±1.24 cm and 14.66±1.24 root numbers. In BM5(MS+1mg/L

BAP+1mg/L NAA) media formed an average of 7.45±0.98 shoots per explant with an average of 9.08±1.32 shoot length and an average of 19.08±1.32 roots per explant. In the BM6 media (MS+2mg/L BAP) an average of 7.83±1.34 shoots per explant with an average of 11±0.70 shoot length and 11±0.70 root numbers per explant. In BM7 media (MS+2mg/L BAP+0.5mg/L NAA) formed an average of 7.5±1.44 shoots per explant with 8.6±1.25cm shoot length and 13.08±1.25 root numbers. In BM8 media (MS+2mg/L BAP+ 1mg/L NAA) an average of 7.66±1.17 shoot per explant with 7.8±1.49cm shoot length and 12.91±1.50 root numbers. BM9 media (MS+2mg/L BAP+ 1mg/L NAA) formed an average of 7.25±1.08 shoots per explant with 5±0.70cm shoot length and 8.91±1.80 average number of roots per explant. The BM10 media (MS+0.5mg/L IAA) formed an average of 6.25±1.01 shoots per explant with 4.9±11.11cm average shoot length and 15.08±1.18 average root numbers per explant. The BM11 media (MS+1mg/L IAA) formed an average of 4.41±0.86 shoots per explant with 4.08±0.86 cm shoot length and 22.25±2.06 root numbers. Finally, BM12 media (MS+2mg/L IAA) formed an average of 3.83±0.79 shoots per explant with an average of 3.69±0.94 shoot length and 18±1.22 number of roots (Table 4.4.1).

The *in vitro* multiplied shoots were taken out of the media after every 4 weeks, excised into single shoots, and subcultured into a new fresh medium. After complete shooting and rooting the explants were taken out for hardening grown in the green house.

Fig 4.4.2: *in vitro* propagation of *P. thyrsiformis*, A; explant initiation, B&C; shoot multiplication and root formation, D; Hardening

Table 4.4.1: Effect of different growth regulators on *in vitro* propagation of *P. thyrsiformis*. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Sl no	**Basal media**	**Growth regulators (mg/L)**			**Number of explant culture initiation**	**Rate (%) of explant producing shoots**	**number of shoots per explant**	**Shoot length (cm)**	**No of roots**
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	05	100	3.5±1.04	2.66±0.74	8.58±1.60
2	BM1	1.0	0.0	0.0	05	100	3.41±1.18	5±1.22	11.75±1.73
3	BM2	1.0	0.2	0.0	05	100	9.5.4±0.8	13.08±1.44	12.41±1.44
4	BM3	1.0	0.4	0.0	05	100	8.6±1.16	12.91±1.54	14.66 ±1.54
5	BM4	1.0	0.5	0.0	05	100	7.7±1.47	11.7±1.24	14.66±1.24
6	BM5	1.0	1.0	00	05	100	7.45±0.98	9.08±1.32	19.08±1.32
7	BM6	2.0	0.0	0.0	05	100	7.83±1.34	11±0.70	11±0.70
8	BM7	2.0	0.5		05	100	7.5±1.44	8.6±1.25	13.08±1.25
9	BM8	2.0	1.0		05	100	7.66±1.17	7.8±1.49	12.91±1.50
10	BM9	3	00	00	05	100	7.25±1.08	5±0.70	8.91±1.80
11	BM10	00	00	0.5	05	100	6.25±1.01	4.9±11.11	15.08±1.18
12	BM11	0.0	0.0	1.0	05	100	4.41±0.86	4.08±0.86	22.25±2.06
13	BM12	00	00	2.0	05	100	3.83±0.79	3.69±0.94	18±1.22

4.4.3 Hardening:

The primary hardening 90% of the *P. thyrsiformis* was successfully done on the 50% vermicompost soil mixture inside the green house at a temperature of $28^0C \pm 2^0C$ and a relative humidity of 70%. After 21 days grown in the greenhouse the explants were taken out to the shed net house in natural environment with varying temperature $28^0C \pm 5^0C$. all the plantlets survived in the natural condition in this step. Finally, the plantlets were successfully grown in the natural climate condition (Fig 4.4.3).

Fig 4.4.3: A: primary hardening in coco pit and soil mixture, **B**: Secondary hardening of *P. thyrsiformis*

4.4.4 Genomic DNA extraction: The whole genome of both wild and tissue cultured *P. thyrsiformis* was extracted using Qiagen DNeasy Plant Mini kit followed by steps mentioned in the kit (Fig:4.3.4). Lane 1 and lane 2 is the genomic DNA extracted from leaf explant of tissue cultured *P. thyrsiformis,* lane L is 1kb DNA ladder, Lane 3 and lane 4 is genomic DNA extracted from leaf explant of wild *P. thyrsiformis* (Fig 4.4.4).

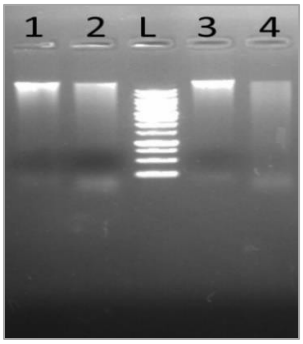


Fig 4.4.4: Isolated Genomic DNA of *P. thyrsiformis*, where **1&2** – Tissue cultured (using leaf explant), **3&4** – Wild Plant (using leaf explant),

4.4.5 RAPD assay

After the extraction of the whole genome from wild and micropropagated *P. thyrsifromis* using Qiagen DNeasy Plant Mini kit, RAPD assay was conducted using 8 RAPD primers OPC 1 to OPC 10(OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, and OPC-8). A total of 52 distinct DNA bands were observed in the 1.5% agarose gel electrophoresis in both the genome samples (Fig 4.4.5) (wild and micropropagated *P. thyrsiformis)*. Out of the total ten RAPD primers four primers (OPC 2, OPC-8, OPC-9, OPC-10) did not bind any targets, out of the six RAPD primers five primers formed polymorphic DNA bands in wild and tissue cultured *P. thyrsiformis*, only one RAPD primer (OPC-6) formed monomorphic bands. The highest polymorphic DNA bands were formed by the OPC-4 RAPD primer in the wild plant, and the OPC-7 in wild plant of *P. thyrsiformis*. The OPC-1 primer in the wild plant formed a total of five polymorphic band while the micropropagated plant formed six polymorphic amplified DNA bands, OPC-3 formed a total of 3 polymorphic DNA bands in wild plant and only one band in the tissue cultured plant, OPC-4 plant formed a total eight polymorphic DNA bands and three polymorphic DNA bands in wild plant, OPC-6 formed a total of three DNA bands in both wild and tissue cultured plant and the OPC-7 formed a total of seven polymorphic DNA bands in wild and only five polymorphic DNA bands in tissue cultured plant (Table 4.4.2).

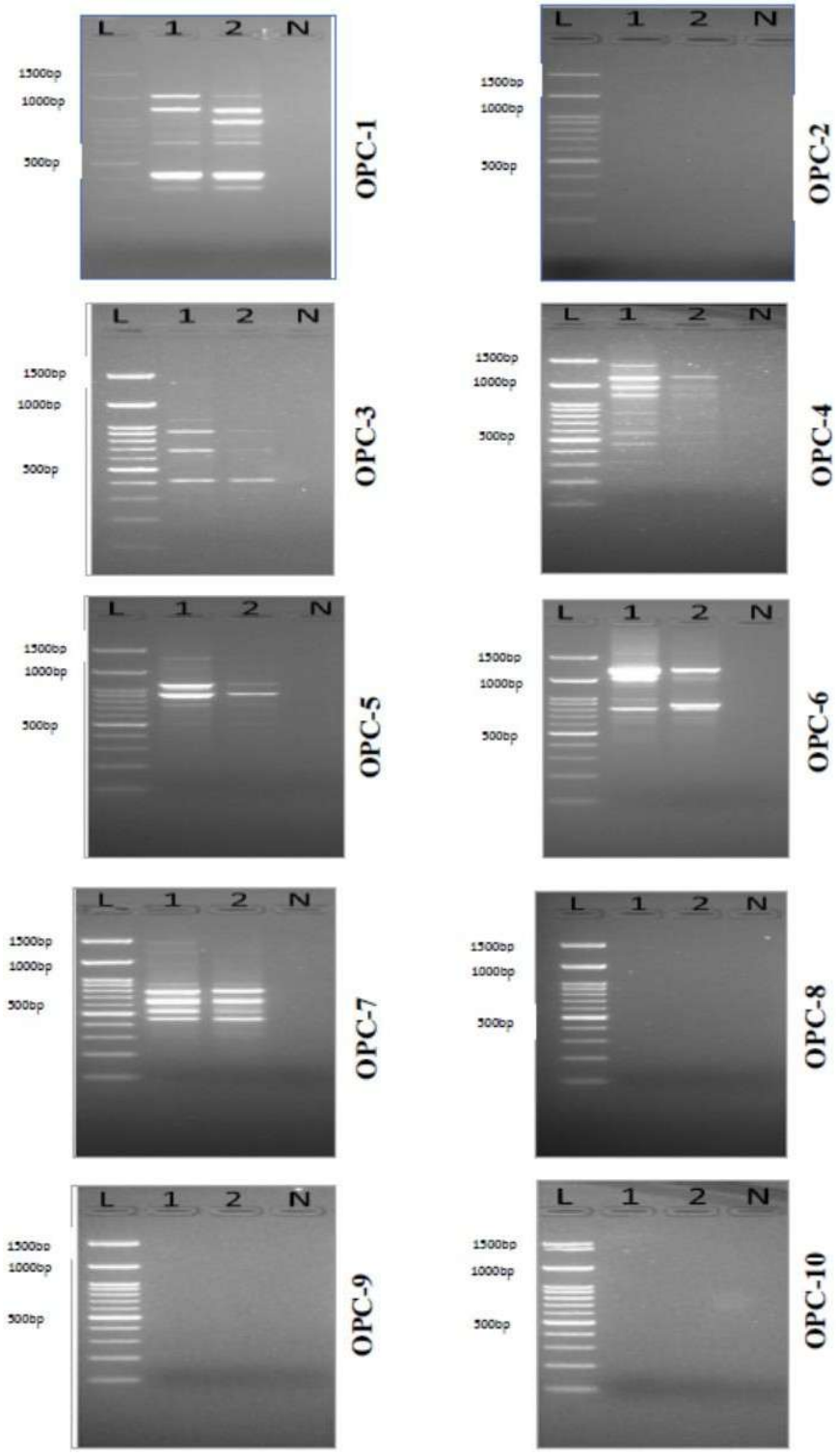


Fig 4.4.5: Amplification pattern of different RAPD Primers (OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9 and OPC10), lane L defines the 1kb DNA ladder,

Lane 1 is amplified RAPD product of tissue cultured plant genome, lane 2 is amplified RAPD product of wild plant genome.

Table 4.4.2: Number of polymorphic DNA bands formed in wild and tissue cultured *P. thyrsiformis* using RAPD primers (OPC-1 to OPC-10) in agarose gel electrophoresis.

Sl no	Primer	Number of Polymorphic DNA bands of wild plant produced by random primer	Number of polymorphic DNA bands of micro-propagated plants produced by random primers
1	OPC-1	5	6
2	OPC-2	0	0
3	OPC-3	3	1
4	OPC-4	8	3
5	OPC-5	5	3
6	OPC-6	3	3
7	OPC-7	7	5
8	OPC-8	0	0
9	OPC-9	0	0
10	OPC-10	0	0

RAPD is most widely used technique for detection of variation in the plant genomes due to its simplicity and rapidity, also this technique does not require any genetic information of the species. The RAPD fingerprinting outcomes are very consistent and target specific for any species of any age group (Micheli et al., 1994). In some species *in vitro* mutation may be induced for improvement of plant product yield or plant improvement. In sugarcane, banana, and potato genome *in vitro* mutation was induced for improving specific characteristics such as disease resistant and some important characters for upgradation of commercial values (Pérez et al., 2000). Somaclonal variation in the plant genome also may induce some significant characteristics in plants such as, drought, biotic or abiotic stress, high or low soil pH, high salinity, and disease resistance (Srivastava & Singh, 2013).

4.4.6 *In vitro* antioxidant assay in wild and tissue cultured extracts of *Phlogacanthus thyrsiformis*: *In vitro* antioxidant assays were conducted in wild and tissue cultured *P. thyrsiformis conducted after successful* standardisation of the *in vitro* propagation method. 1 mg/mL extracts were prepared in 70% methanol for the following tests using a UV-vis spectrophotometer.

Total Phenolic Content: For the detection of phenolic on the plant extract of wild and tissue cultured *P. thyrsiformis,* FCR method was used. The regression curve ($y = 276.85x + 68.485$; $R^2 = 0.9934$) (Fig 4.2.5a) was used and expressed as mg of gallic acid equivalent (GAE). From the experiment using spectrophotometer 237.66±3.4 mg/g GAE was found in the wild extract and 206.6667±4.92 mg GAE/g powder weight in the tissue cultured *P. thyrsiformis.*

Total Flavonoid content: The total flavonoid content in wild and tissue cultured *P. thyrsiformis* were determined using the regression curve ($y = 404.68x + 53.647$ $R^2 = 0.993$) (Fig 4.2.5b) and expressed as mg of quercetin equivalent (QE) per grams of the dried methanolic extract of *P. thyrsiformis.* The total flavonoid content was comparatively higher in the tissue cultured (125.66±1.69 mg QE/g) *P. thyrsiformis* than the wild (106±4.54 mg/g QE) extract of *P. thyrsiformis.*

Total antioxidant capacity: The total antioxidant capacity of wild and tissue cultured *P. thyrsiformis* were determined by the regression curve ($y=324.98x + 45.306$ $R^2 = 0.9902$) (Fig 4.2.5c). The antioxidant capacity of the tissue cultured methanolic extract (228.33±3.39 mg/g AAE) was higher than the wild extract (182.33±4.1 mg AAE/g of dried extract) of *P. thyrsiformis.*

DPPH scavenging Activity: The radical scavenging activity in the wild and tissue cultured *P. thyrsiformis* was determined by the regression curve ($y = 16.114x + 13.25$; $R^2 = 0.9846$) (Fig 4.2.5d). The results were confirmed to bear potent nutraceutical potential. The IC_{50} value of the wild methanolic extract of *P. thyrsiformis* is 49.33±1.24 and in the tissue cultured extract was 56.33±1.24.

4.4.7 Quantitative detection of gallic acid and quercetin: The HPLC method for detection of gallic acid and quercetin were successfully validated and standardised for P. thyrsiformis. The system was stabilised and equilibrated using 70% (v/v) HPLC-grade

methanol and 30% (v/v) HPLC-grade water with a flow rate of 1 mL/min using the Waters RP-18 column. The chromatograms and the standard curve of gallic acid and quercetin was plotted. The gallic acid content in the wild and tissue cultured *P. thyrsiformis* was determined using the regression curve (y= 0.8696x + 98.778 R^2 = 0.9958) (Fig 4.2.6A) and the quercetin content in the wild and tissue cultured *P.thyrsiformis* was determined using the regression curve (5.9606x + 88.794 R^2 = 0.997) (Fig 4.2.6B). The experiment was conducted in triplicate, satisfactory results were obtained in quercetin and gallic acid in wild and micropropagated P. thyrsiformis (Fig. 4.4.6a, 4.4.6b, 4.4.6c and 4.4.6d). The chromatograms of each quercetin and gallic acid were obtained and standard curves were plotted. All the data were tabulated below in the Table 5.14. The gallic acid content in the tissue cultured methanolic plant extract was 129.24±4.06 and 117.31±2.06 in the wild plant extract. The quercetin content was 85.66±1.82 in the tissue cultured methanolic plant extract and 85.67667±1.81 in the wild methanolic extract (Table 4.4.3).

Table 4.4.3: Showing concentration of gallic acid and quercetin (µg/ml) in wild and tissue cultured *P. thyrsiformis* using HPLC. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Compound	RT	Concentration (µg/mg)
Gallic acid content in tissue cultured plant extract	2.7	129.24±4.06
Gallic acid content wild plant extract	2.67	117.31±2.06
Quercetin content in tissue cultured plant extract	5.78	85.67±1.82
Quercetin content in wild plant extract	5.8	85.67±1.81

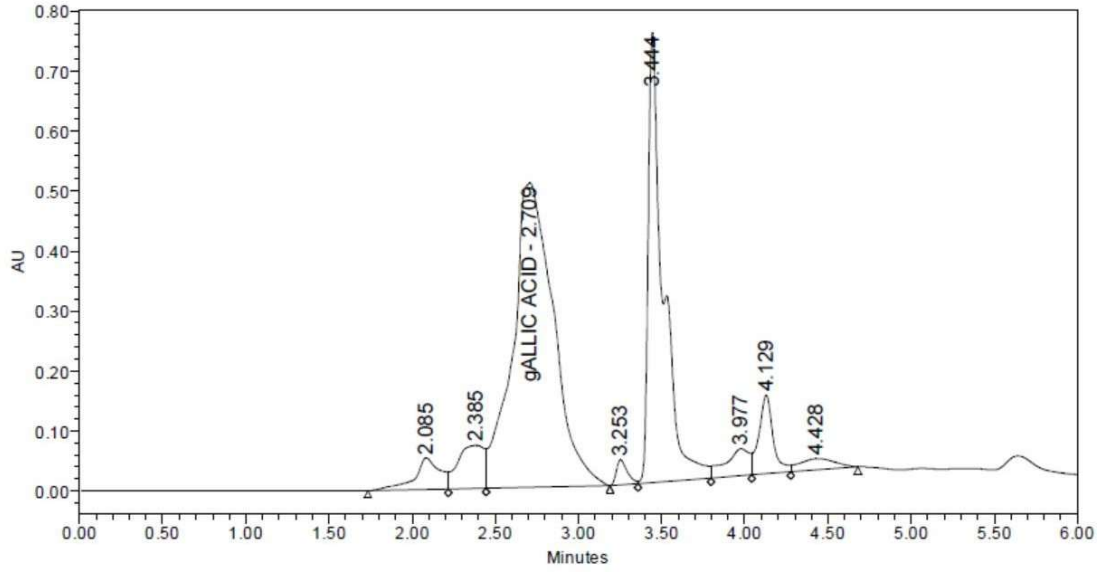


Fig 4.4.6a: HPLC Chromatogram; Gallic acid peak of tissue cultured *P. thyrsiformis* plant extract

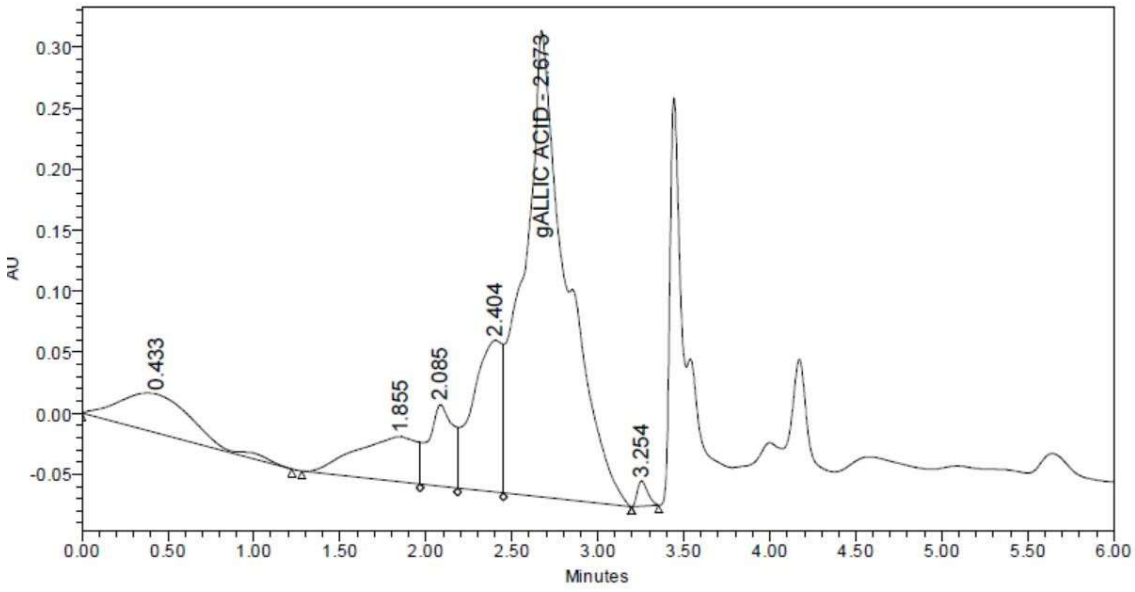


Fig 4.4.6b: HPLC Chromatogram; Gallic acid peak of wild *P. thyrsiformis* plant extract.

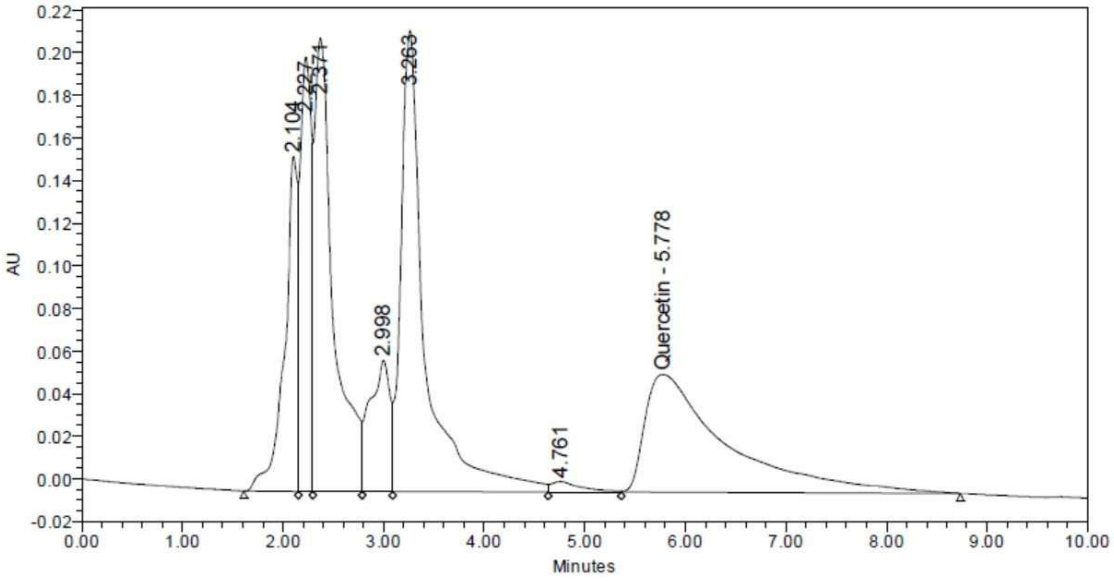


Fig 4.4.6c: HPLC Chromatogram; quercetin peak of tissue cultured *P. thyrsiformis* plant extract.

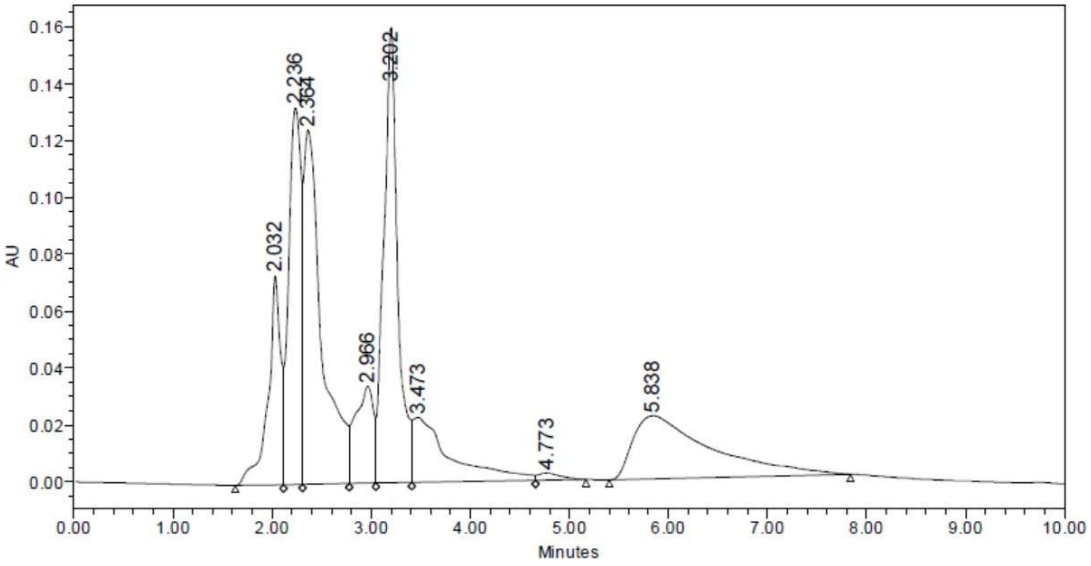


Fig 4.4.6d: HPLC Chromatogram; quercetin peak of wild *P. thyrsiformis* plant extract.

4.4.8 Identification of bioactive compounds in tissue cultured and wild extracts of *P. thyrsiformis*: The methanolic extracts of wild and tissue cultured *P. thyrsiformis* plants were screened for the presence of bioactive compounds using GC-MS analysis. The GC-MS chromatogram for both wild and tissue cultured aqueous methanolic extract of *P. thyrsiformis* were plotted (Figure 4.4.7a & 4.4.7b). the compound search was conducted using retention time (RT), molecular weight, molecular formula, and peak area% (concentration). Ten bioactive compounds were identified in the wild extract and in the tissue cultured extract (Table 4.3.4). In the wild plant extract 7,7,9,9,11,11-Hexamethyl-3,6,8,10,12,15-hexaoxa-7,9,11-Trisilaheptadecane; 1,1,1,3,5,5,7,7,7-nonamethyl-3-(trimethylsiloxy)tetrasiloxane; methanol, oxo-, benzoate which is used in the naturally occurring oils of clove, tuberose, used in perfumes, also as flavouring agents, disinfectant additives, dye carrier, and solvents (resin, cellulose ether and esters, rubber) (Lewis, 2016), 2-hydroxyoctanoic acid, TMS derivative; 2-methylenebornane is used as a precursor molecule in the petrochemical industry (e.g. synthesis of isooctane) (Wilkerson, 2017); Cis-dihydrocarvone is primarily used in flavour (aroma, food and beverages, cosmetics and personal care) fragrances, synthesis of various compounds (Bora et al., 2022); trisiloxane 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]- bears antioxidant properties (Momin & Thomas, 2020); 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane; Asarone, is an important bioactive compound which possesses anti-anxiety, anti-depressant, anti-Parkinson's, anti-Alzheimer's, anti-cancer, anti-epileptic, anti-thrombic, anti-hyperlipidemic, anti-cholestatic, and radioprotective activities (Sandhir et al., 2021); Isoelemicin is commonly used as pharmaceutical adjuvant, nutritional, and cosmetics etc. (Maduabuchi, & Awucha, 2020).

On the other end in the tissue cultured methanolic extract of *P. thyrsiformis* extract cyclotetrasiloxane, octamethyl- contains antimicrobial properties (Olaoye et al., 2024); Formic acid is used as antibacterial agent, preservative and in manufacturing (Okoye-Chine et al., 2022), 2-methylhex-3-yl ester; Lauryl glycidyl ether; 2-Nonadecanone 2,4-dinitrophenylhydrazine is used for the detection of presence and absence of aldehyde and ketones (Mohrig, 1998); Maltosuronic acid, 1',6'-anhydro-penta-O-benzyl-; Silandrone is used as anabolic androgenic steroid (Elks, 2014); Chloroacetic acid dodec-9-ynyl ester is used as a building block and in chemical researches (Tareq et al., 2023); 2-Iodocinnamic acid is important precursor in medicinal chemistry (Assaleh et al., 2022);

d-Mannitol, 1-O-(22-hydroxydocosyl)- is used as an internal standard in NMR analysis, and it is a component of test sugar solution (Rundlöf et al., 2021); and Arsenous acid, tris(trimethylsilyl) has an important role in nematostatic effect, and bears anticancer and antioxidant properties (Wiraswati et al., 2023) (Table 4.4.4).

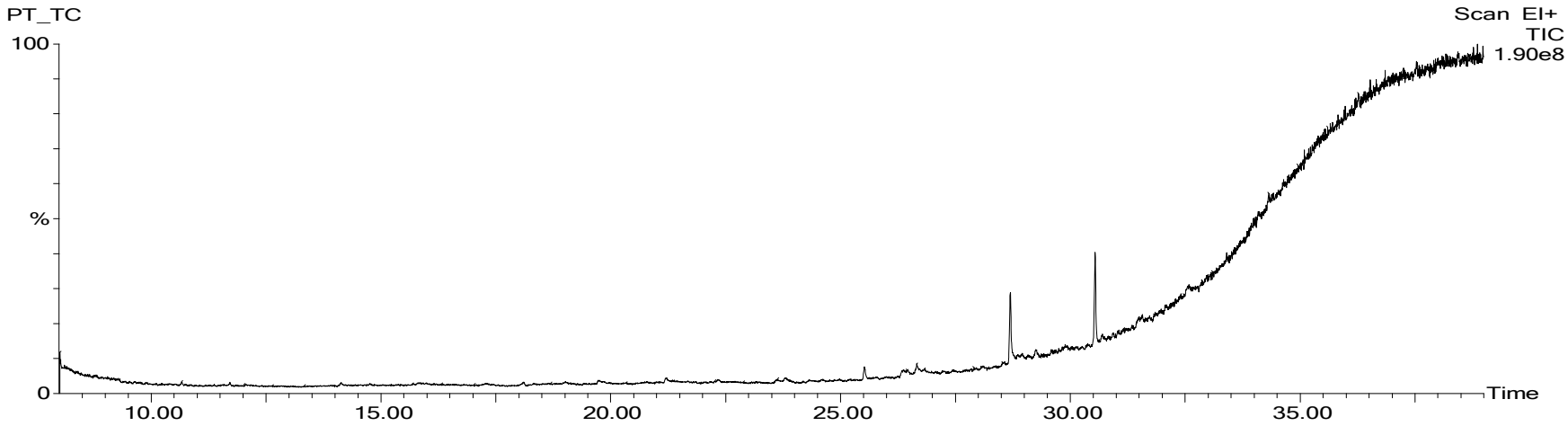


Fig 4.4.7a: GC-MS spectrum of tissue cultured *P. thyrsiformis* plant in methanol extract

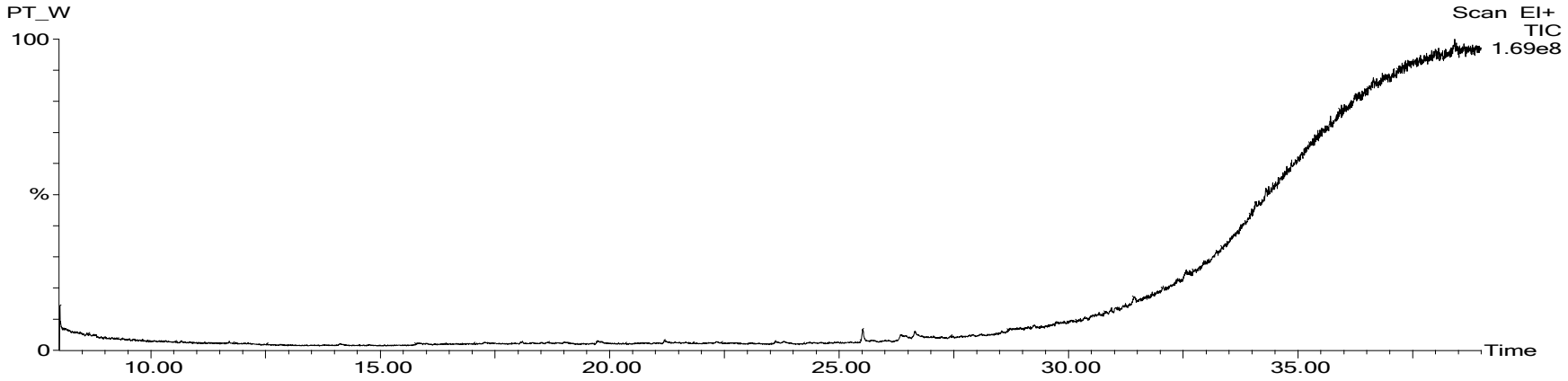
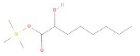
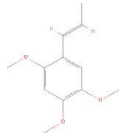
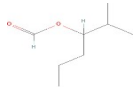
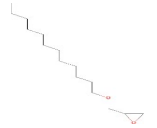


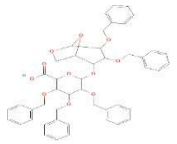
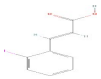
Fig 4.4.7b: GC-MS spectrum of tissue cultured *P. thyrsiformis* plant in methanol extract

Table 4.4.4*:* Compounds found in aqueous methanolic extract of *P. thyrsiformis* **A:** wild plant **B**: Tissue cultured plant.

A: Compounds in the wild *P. thyrsiformis* methanolic plant extract						
Sl no	**RT**	**Area**	**Compound name**	**Molecular weight g/mol**	**Chemical formula**	**Structure**
1	10.664	276,045.1	7,7,9,9,11,11-HEXAMETHYL-3,6,8,10,12,15-HEXAOXA-7,9,11-TRISILAHEPTADECANE	384	$C_{14}H_{36}O_6Si_3$	
2	11.715	410,915.1	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384.84	$C_{12}H_{36}O_4Si_5$	
3	19.763	2,541,330	METHANOL, OXO-, BENZOATE	150.13	$C_8H_6O_3$	
4	21.224	261,901.0	2-Hydroxyoctanoic acid, TMS derivative	232.39	$C_{11}H_{24}O_3Si$	

5	23.785	211,329.0	2-Methylenebornane	70.13	C_5H_{10}	
6	25.520	7,199,134	Cis-Dihydrocarvone	152.23	$C_{10}H_{16}O$	
7	26.466	3,388,776	TRISILOXANE, 1,1,1,5,5,5-HEXAMETHYL-3,3-BIS[(TRIMETHYLSILYL)OXY]-	384.84	$C_{12}H_{36}O_4Si_5$	
8	26.656	3,892,045	2-METHYL-3-(3-METHYL-BUT-2-ENYL)-2-(4-METHYL-PENT-3-ENYL)-OXETANE	222.37	$C_{15}H_{26}O$	
9	28.697	2,019,462	ASARONE	208.25	$C_{12}H_{16}O_3$	

10	30.537	2,307,857	Isoelemicin	208.25	$C_{12}H_{16}O_3$	
B: Compounds in the Tissue cultured *P. thyrsiformis* methanolic plant extract						
1	8.108	7,803,004.5	CYCLOTETRASILOXANE, OCTAMETHYL-	296.61	$C_8H_{24}O_4Si_4$	
2	14.136	211,526.5	FORMIC ACID, 2-METHYLHEX-3-YL ESTER	144.21	$C_8H_{16}O_2$	
3	15.847	173,879.1	Lauryl glycidyl ether	242.40	$C_{15}H_{30}O_2$	
4	18.102	185,860.2	2-Nonadecanone 2,4-dinitrophenylhydrazine	462.6	$C_{25}H_{42}N_4O_4$	

5	19.738	227,709.1	Maltosuronic acid, 1',6'-anhydro-penta-O-benzyl-	788.9	$C_{47}H_{48}O_{11}$	
6	21.189	367,527.8	Silandrone	360.6	$C_{22}H_{36}O_2Si$	
7	25.510	424,721.6	CHLOROACETIC ACID, DODEC-9-YNYL ESTER	258.78	$C_{14}H_{23}ClO_2$	
8	26.351	470,491.4	2-Iodocinnamic acid	274.05	$C_9H_7IO_2$	
9	26.651	698,921.4	d-Mannitol, 1-O-(22-hydroxydocosyl)-	506.8	$C_{28}H_{58}O_7$	
10	31.443	311,431.8	Arsenous acid, tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$	

4.5 *Enydra fluctuans*

4.5.1 Explant surface sterilization: The technique *in vitro* propagation is an essential tool for producing disease free and uniform plants and production of secondary metabolites including plant germplasm conservation. Various methods including organ, cell, and tissue culture can be possible for plant improvement and production of clonal copies of mother plants. Successful *in vitro* propagation techniques for *E. fluctuans* have been standardised. The rapidly growing disease-free epical meristem parts of the plant were found most suitable for the successful establishment of explant culture. The explant surface sterilization step was most crucial step in the entire process. Contaminations were observed in the cultures after 7–20 days of culture initiation. Therefore, selection of the suitable surface sterilizing agent is the most important. After treatment with 0.5% Bavistin for 30 min for all the explants, they are introduced in to the 0.1% mercuric chloride for different time intervals. Treatment with 0.1% mercuric chloride for 3 min and 4 min showed highest explant survival rate (76.67±4.71%) after 28 days of the culture initiation, 1 min treatment with 0.1% mercuric chloride showed 23.33±4.71% explant survival, 2 min treatment showed 36.67±4.71% explant survival rate and 5 min showed 56.67±4.71% explant survival rate after 28 days of culture initiation. On the other view while treating the explants using 0.1% mercuric chloride for longer duration the explants are damaged, i.e. while treating for 4 min 13.33±4.71% of the explants got damaged and while treating for 5 min 40±4.71% of the *E. fluctuans* explants got damaged (Fig 4.5.1).

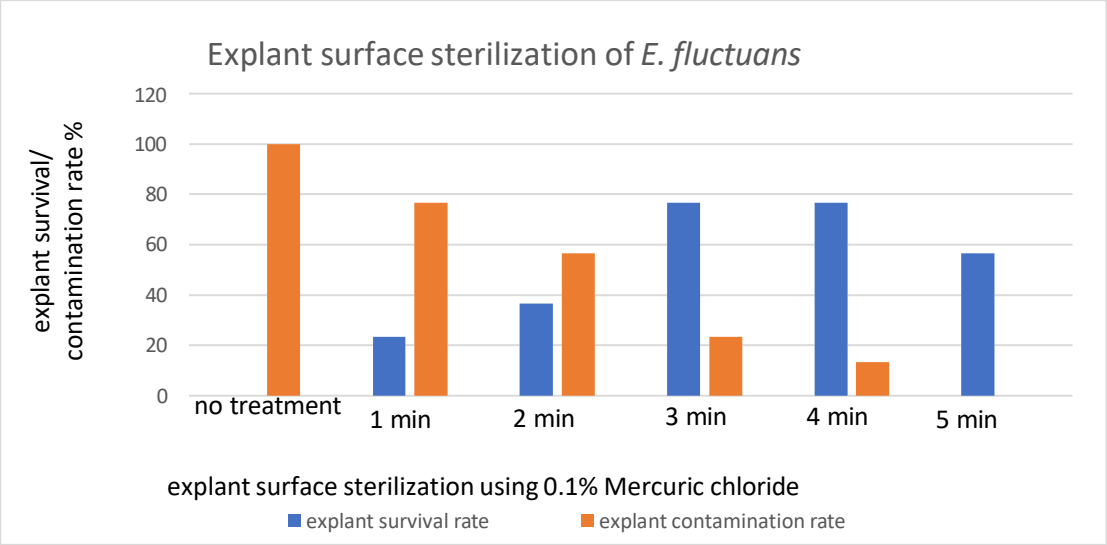


Fig 4.5.1: Graph showing explant survival rate of *in vitro* propagated *E. fluctuans* after 21 days of explant initiation0.1% mercuric chloride. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

4.5.2 Explant initiation and shoot multiplication and rooting: After the successful surface sterilisation of explants, the explants were induced for multiple shoots and rooting *in vitro* in new culture media. The explants were monitored regularly to observe growth and contamination. The explants were successfully developed into multiple shoots and roots, but the explants responded differently to different compositions of hormones. The *in vitro* multiplied shoots were taken out of the media after every 4 weeks, excised into single shoots, and sub cultured into a new fresh medium. The highest numbers of shoot multiplication of *E. fluctuans* were observed in the MS medium with 2 mg/L BAP (BM6), which is an average of 21±1.30 shoots and an average shoot length of 15.4±1.35 cm. The highest average shoot length was obtained in the media MS with 2 mg/L BAP (BM6), also, the most effective shoot multiplication and the highest average shoot length were observed (Fig. 4.5.2), with an average of 21±1.30 shoots and an average of 15.4±1.35 cm of shoot length. The lowest average shoot lengths were observed in control medium where no growth regulator was used, i.e.-an average of 8±1.5 shoots per explant, an average of 13.2±0.74cm shoot length and 12.6±1.62 roots per explant (Table 5.13). BM1 media formed an average of 8±1.4 shoots per explant, 13±0.89cm shoot length, and an average

of 14.8±0.74 roots per explant. BM2 media formed an average of 15±1.51 shoots per explant, 13±0.63 cm average shoot length, and 14.6±2.05 root numbers per explant. An average of 11±0.83 shoots per explant, 14.2±0.74cm shoot length, and 15.4±1.01 average root numbers were formed when the explants were cultured in the BM4 media. In the BM5 media an average of 17±1.14 shoots per explant, 13.8±0.74cm shoot length, and 15±0.63 root numbers were formed. An average of 21±1.30 shoots per explant, 13.8±0.74 cm average shoot length, and 15.8±1.46 number of roots were observed in the basal media BM6. In the BM8 media an average of 11±1.40 shoots per explant, 15±0.89cm average shoot length, and 15.6±1.01 roots per explant were observed. An average of 8±1.14 shoots per explant, 13.4±1.01cm average shoot length, and 21.6±1.49 number of average roots were observed in the BM10 media. An average of 9±1.51 number of shoots per explant, 12.4±0.48cm average shoot length, and 24.8±1.85 average number of roots were observed. Finally, in the BM12 media an average of 8±1.30 shoots per explant, 12.2±0.74cm average shoot length, and 21±1.16 average root numbers were observed (Table 4.5.1).

Fig 4.5.2: *in-vitro* propagation of *Enydra fluctuans*

Table 4.5.1: Effect of different concentrations of PGRs on callus induction, shoot proliferation, elongation and rooting from nodal explants of *Enydra fluctuans in vitro.* Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Sl no	Basal media	Growth regulators (mg/L)			Explant culture initiation (nos)	Rate (%) of explant producing shoots	Number of shoots per explant	Shoot length (cm)	Number of roots
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	05	100	8±1.5	13.2±0.74	12.6±1.62
2	BM1	1.0	0.0	0.0	05	100	8±1.4	13±0.89	14.8±0.74
3	BM2	1.0	0.2	0.0	05	100	15±1.51	13±0.63	14.6±2.05
4	BM3	1.0	0.4	0.0	05	100	11±0.83	14.2±0.74	15.4±1.01
5	BM4	1.0	0.5	0.0	05	100	10±1.30	14.6±1.01	14.8±1.16
6	BM5	1.0	1.0	0.0	05	100	17±1.14	13.8±0.74	15±0.63
7	BM6	2.0	0.0	0.0	05	100	21±1.30	15.4±1.35	15.8±1.46
8	BM7	2.0	0.5	0.0	05	100	10±1.14	15±0.63	16.6±1.35
9	BM8	2.0	1.0	0.0	05	100	11±1.40	15±0.89	15.6±1.01
10	BM9	3	0.0	0.0	05	100	12±1.51	14.2±1.72	15.2±1.16
11	BM10	0.0	0.0	0.5	05	100	8±1.14	13.4±1.01	21.6±1.49
12	BM11	0.0	0.0	1.0	05	100	9±1.51	12.4±0.48	24.8±1.85
13	BM12	0.0	00	2.0	05	100	8±1.80	12.2±0.74	21±1.16

***In vitro* rooting and acclimatization:** complete developed multiple shoots were sub cultured in the rooting media containing various concentration of IAA. Maximum rooting was obtained in the MS media supplemented with 1mg/L IAA (24.8± 1.85 roots per explant). The above plantlets were grown in the vermicompost coco pit mixture and kept inside green house at 28±2ºC temperature and 70% relative humidity for hardening.

4.5.3 Hardening: The primary hardening 80% of the *E. fluctuans* was successfully survived on the 50% vermicompost soil mixture inside the green house at a temperature of 28^0C±2^0C and a relative humidity of 70%. After 21 days grown in the greenhouse the explants were taken out to the shed net house in natural environment with varying temperature 28^0C±5^0C. all the plantlets survived in the natural condition in this step. Finally, the plantlets were successfully grown in the natural climate condition.

4.5.4 Genomic DNA extraction: The whole genome of both wild and tissue cultured *E. fluctuans* was extracted using Qiagen DNeasy Plant Mini kit followed by steps mentioned in the kit (Fig 4.5.3). Lane 1 and lane 2 is the genomic DNA extracted from leaf explant of tissue cultured *E. fluctuans,* lane L is 1kb DNA ladder, Lane 3 and lane 4 is genomic DNA extracted from leaf explant of wild *E. fluctuans.*

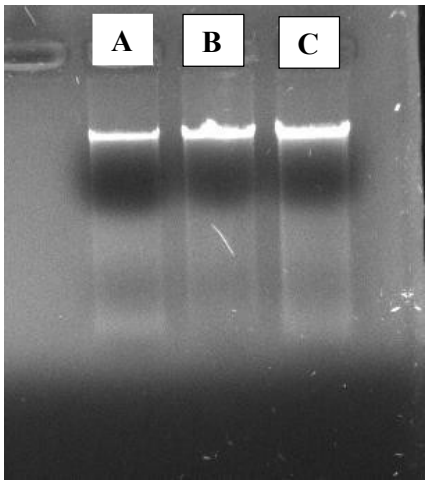


Fig 4.5.3: Isolated Genomic DNA of *E. fluctuans* in 0.8% agarose gel, where **A**–Tissue cultured (using leaf explant), **B&C** – Wild Plant (using leaf explant),

4.5.5 RAPD assay: When compared to the mother plant, the phenotypic and genetic makeup of the in vitro produced plants may undergo a variety of alterations. Once these variations have spread, it is crucial to find them. Using molecular markers to identify somaclones can be a helpful method.

The RAPD assay is an effective method for analysing and identifying DNA alterations and repairs, which may have a direct effect on the survivility and devel opment of certain species or organisms. This method can also detect the mutation in the genome induced by chemicals (Atienzar & Jha 2004). For the detection of somaclonal variation in the micropropagated *E. fluctuans* nine distinct RAPD primers were used (OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OPC-8, OPC-9 and OPC-10). Out of the ten RAPD primers four primers (OPC-3, OPC7, OPC-9, OPC-10) did not bind any target sequence on the template forming no DNA bands in the gel electrophoresis. Out of six RAPD primers the five RAPD primers formed polymorphic DNA bands in wild and tissue cultured *E. fluctuans,* (Fig 4.5.4). The OPC-1 primer in the wild plant formed a total of three polymorphic band while the micropropagated plant formed one polymorphic amplified DNA bands, OPC-2 formed a total of six polymorphic DNA bands in wild plant and seven polymorphic DNA band in the tissue cultured plant, OPC-4 formed a total four polymorphic DNA bands and one polymorphic DNA bands in wild plant, OPC-5 formed a total of two DNA band and one in tissue cultured plant, OPC-6 formed a total of 3 DNA bands in micropropagated plant and one in wild plant, and the OPC-8 formed a total of 5 polymorphic DNA bands in both wild and tissue cultured plant. From the result it can be concluded that the *in vitro* cultured plant undergoes somaclonal variation in the process of *in vitro* propagation (Table 4.4.2).

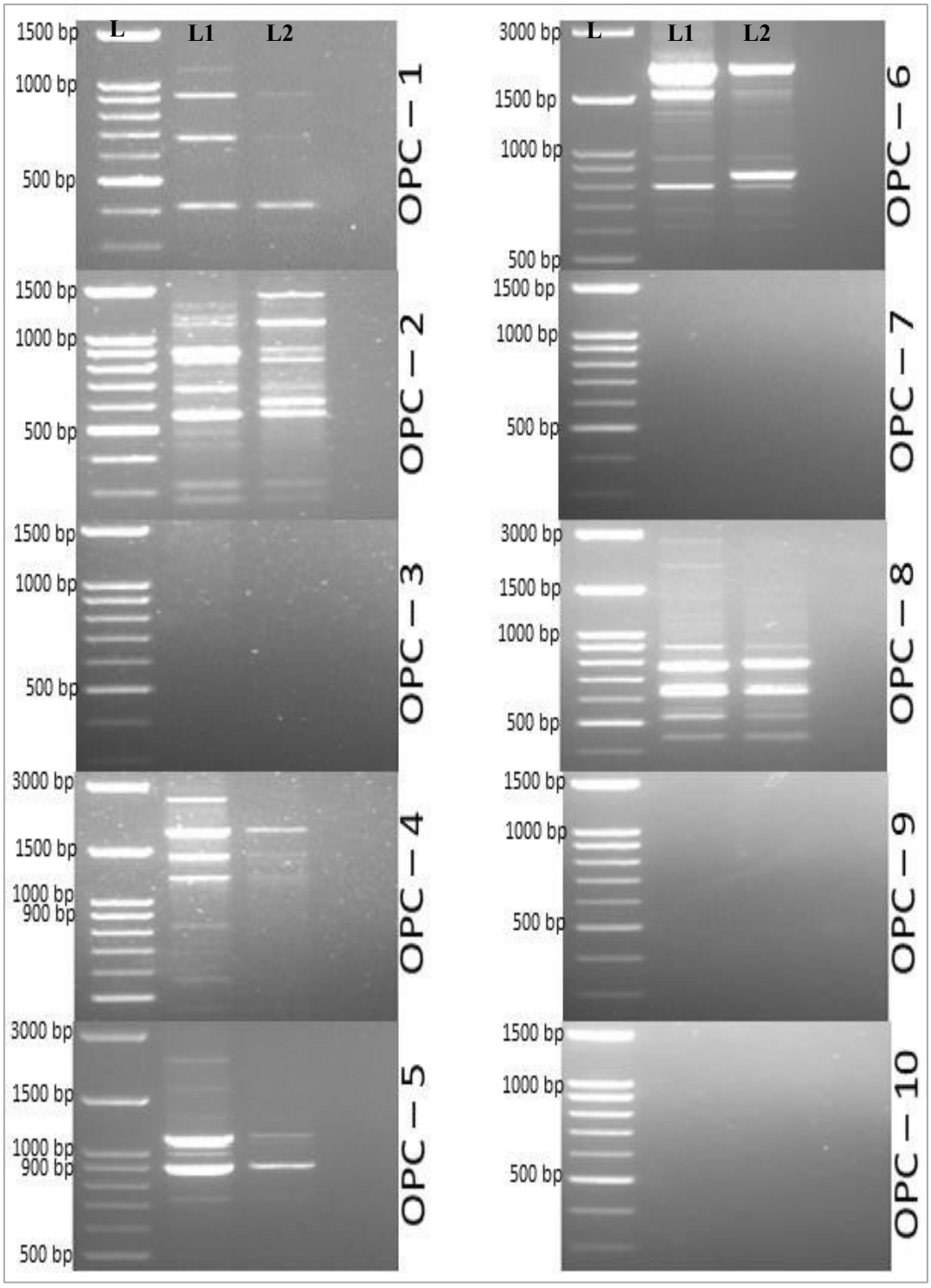


Fig 4.5.4: Amplification pattern of different RAPD Primers (OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9 and OPC10). Lane L defines the 1kb DNA ladder, Lane 1 is amplified RAPD product of tissue cultured plant genome, lane 2 is amplified RAPD product of wild plant genome.

Table 4.5.2: Table showing number of amplified bands using RAPD assay in wild and micropropagated *E. fluctuans*

Sl no	Primer	Number of bands produced by wild plant	Number of bands produced by hardened micro propagated plants
1	OPC 01	3	1
2	OPC 02	6	7
3	OPC 03	0	0
4	OPC 04	4	1
5	OPC 05	2	1
6	OPC 06	3	2
7	OPC 07	0	0
8	OPC 08	5	5
9	OPC 09	0	0
10	OPC-10	0	0

4.5.6 *In-vitro* antioxidant assay in wild and tissue cultured extract of *E. fluctuans:* *In vitro* antioxidant assay in wild and tissue cultured *E. fluctuans were conducted after successful* standardisation of the *in vitro* propagation method. 1 mg/mL extracts were prepared in 70% methanol for the following tests using a UV-vis spectrophotometer.

Total Phenolic Content: FCR method was absorbed for the detection of phenolics in the plant extract of wild and tissue cultured *E. fluctuans.* The regression curve ($y = 276.85x + 68.485$; $R^2 = 0.9934$) (Fig 4.2.5a) was used and expressed as mg of gallic acid equivalent (GAE). From the experiment 54.7 ±5.3 mg/g GAE were found in the wild extract and 61 ±3.4 mg GAE/g powder weight in the tissue cultured *E. fluctuans.*

Total Flavonoid content: The total flavonoid content in wild and tissue cultured *E. fluctuans* were determined using the regression curve ($y = 404.68x + 53.647$ $R^2 = 0.993$) (Fig 4.2.5b) and expressed as mg of quercetin equivalent (QE) per grams of the dried methanolic extract of *E. fluctuans.* The total flavonoid content was

comparatively higher in the tissue cultured (24±7.7 mg QE/g) *E. fluctuans* than the wild (20±7 mg/g QE) extract of *E. fluctuans.*

Total antioxidant capacity: The total antioxidant capacity of wild and tissue cultured *E. fluctuans* were determined by the regression curve (y=324.98x + 45.306 R^2 = 0.9902) (Fig 4.2.5c). The antioxidant capacity of the tissue cultured methanolic extract (114.7±7.2 mg/g AAE) was higher than the wild extract (94±7.2 mg AAE/g of dried extract) of *E. fluctuans.*

DPPH scavenging Activity: The radical scavenging activity in the wild and tissue cultured *P. thyrsiformis* was determined by the regression curve (y = 16.114x + 13.25; R^2 = 0.9846) (Fig 4.2.5d). The IC_{50} value of the wild methanolic extract of *P. thyrsiformis* is 37.70±2.33 and in the tissue cultured extract was 45.19±2.84.

4.5.7 Quantitative detection of gallic acid and quercetin: The HPLC method for detection of gallic acid and quercetin were successfully validated and standardised for ***E. fluctuans***. The system was stabilised and equilibrated using 70% (v/v) HPLC-grade methanol and 30% (v/v) HPLC-grade water with a flow rate of 1 mL/min using the Waters RP-18 column. The experiment was conducted in triplicate, satisfactory results were obtained in quercetin and gallic acid in wild and micropropagated *E. fluctuans*. The chromatograms and the standard curve of gallic acid and quercetin was plotted. The gallic acid content in the wild and tissue cultured *E. fluctuans* was determined using the regression curve (y= 0.8696x + 98.778 R^2 = 0.9958) (Fig 4.2.6A) and the quercetin content in the wild and tissue cultured *E. fluctuans* was determined using the regression curve (5.9606x + 88.794 R^2 = 0.997) (Fig 4.2.6B). The chromatograms of each quercetin and gallic acid were obtained and standard curves were plotted. All the data were tabulated below in the Table. The retention time of gallic acid and quercetin was 2.4 and 4.42 min, respectively (Fig: 4.4.5a, 4.4.5b, 4.4.5c and 4.4.5d). From the experiment it was obtained that the gallic acid and quercetin content in tissue cultured methanolic extracts of *E. fluctuans* were comparatively higher than the wild methanolic extract. The gallic acid content of wild-type and tissue-cultured extracts was 8.059µg/mg and 10.1934µg/mg of dried extract, respectively. The quercetin content of wild and tissue cultured plant extracts of *E. fluctuans* was 2.0199µg/mg and 5.1292µg/mg of dried extract, respectively (Table 4.5.3).

Table 4.5.3: Accuracy of Gallic acid and Quercetin content in *E. fluctuans* extract using HPLC. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Extracts	Peak name	RT	Area	% Area	Height	Concentration in µg/mg of dried extract (Mean +SE)
Tissue cultured *E. fluctuans*	Gallic Acid	2.463	21375374	67.41	2936952	10.1934±1.18
Wild *E. fluctuans*	Gallic Acid	2.389	16900835	69.58	2334686	8.059±0.65
Tissue cultured *E. fluctuans*	Quercetin	4.498	1754045	1.47	80402	5.1292±1.08
Wild *E. fluctuans*	Quercetin	4.491	690760	0.91	52157	2.0199±1.09

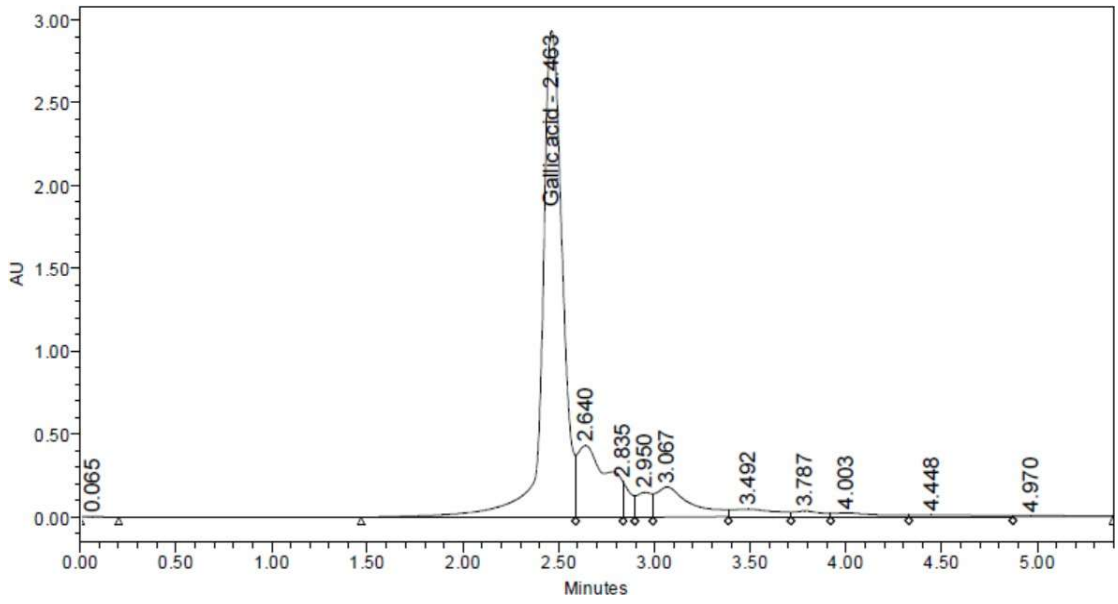


Fig 4.5.5a: HPLC Chromatogram; Gallic acid peak of tissue cultured *E. fluctuans* extract

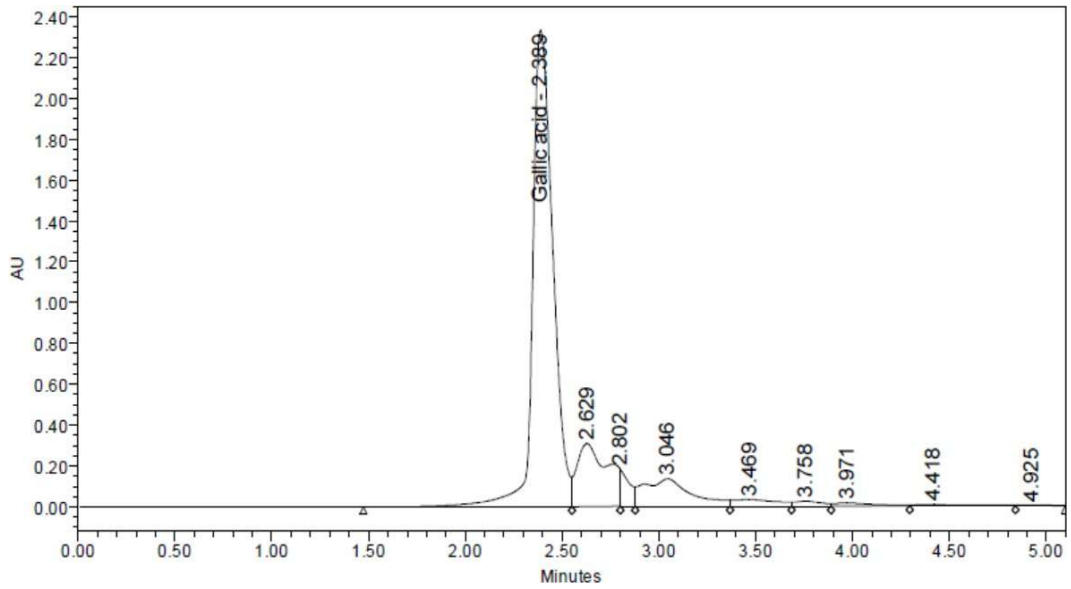


Fig 4.5.5b: HPLC Chromatogram; Gallic acid peak of wild *E. fluctuans* extract

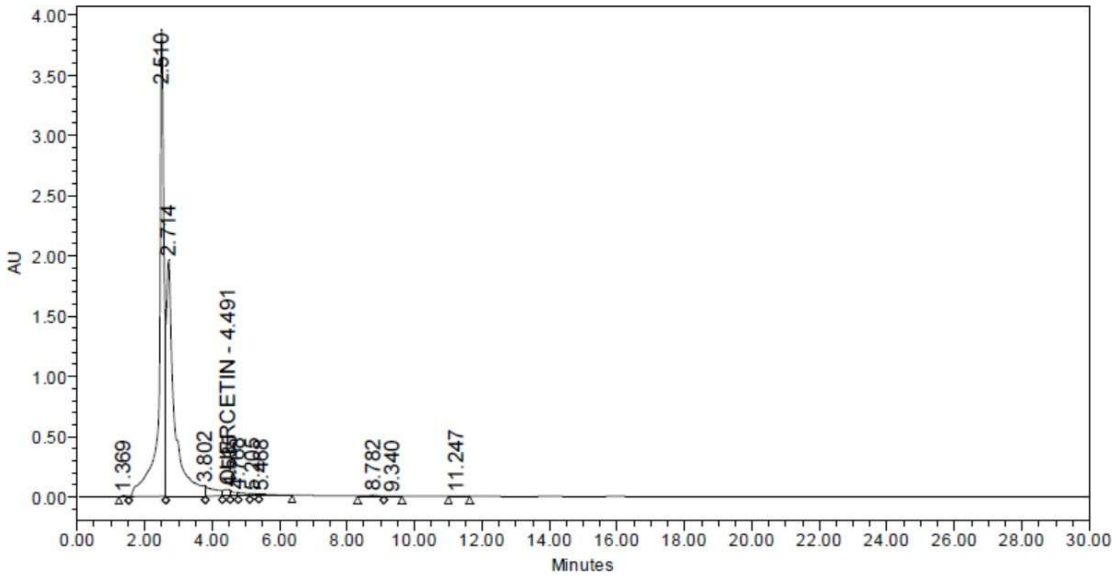


Fig 4.5.5c: HPLC Chromatogram; Quercetin peak of tissue cultured *E. fluctuans* extract

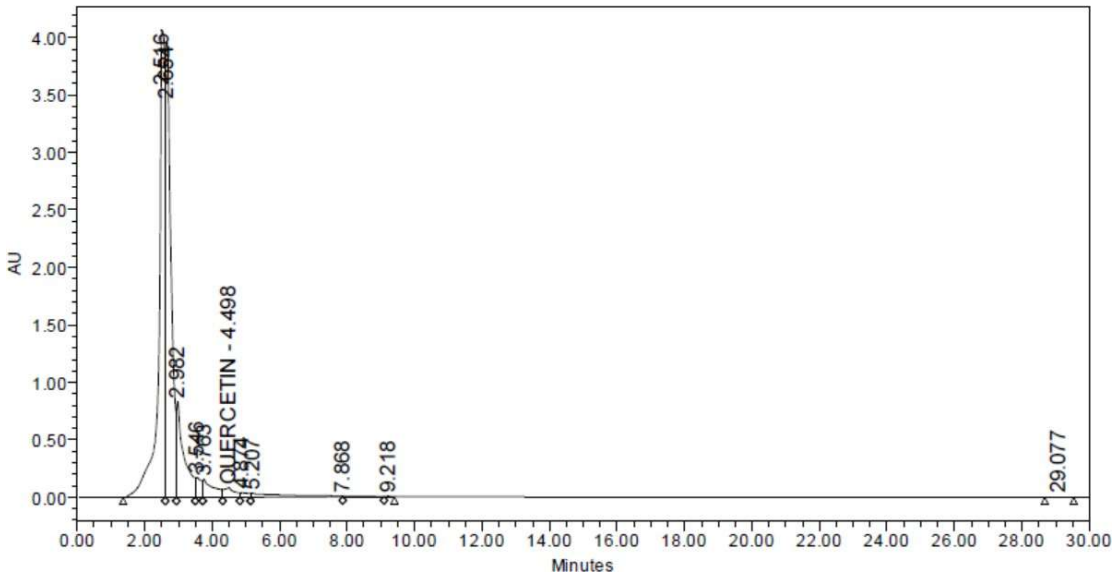


Fig 4.5.5d: HPLC Chromatogram; Quercetin peak of wild *E. fluctuans* extract

4.5.8 Study of bioactive compounds in tissue cultured and wild extracts of E. fluctuans: The methanolic extracts of wild and tissue cultured *E. fluctuans* plants were screened for the presence of bioactive compounds using GC-MS analysis. The GC-MS chromatogram for both wild and tissue cultured aqueous methanolic extract of *E. fluctuans* were plotted (Figure: 4.5.6a & 4.5.6b). the compound search was conducted using retention time (RT), molecular weight, molecular formula, and peak area% (concentration). Nine bioactive compounds were identified in the wild extract and eight active chemical compounds were identified in the tissue cultured extract (Table: 4.5.4). The following compounds were identified in the methanolic wild extract of *E. fluctuans*, L-Norvaline has a role of bacterial metabolite, a hypoglycaemic agent and a neuro protective agent (De et al., 2016), Metoprolol is used to treat high blood pressure, chest pain due to poor blood flow to the heart, and a number of conditions involving an abnormally fast heart rate (Koch-Weser, 1979), 2-Propenoic acid is used in production of coatings, polymers, plastics, adhesives, and other applications like water treatment and detergents (Huang et al., 2014), ethyl nipecotate is an enantiopure cholinergic drug that has been shown to have antinociceptive and anti-inflammatory properties (Rustam 1995), ritalinic Acid is the drug commonly prescribed to children for treatment of attention-

deficit hyperactivity disorder is the ester dl-methylphenidate (Ritalin) (Lockridge et al., 2018), L-Glutamic acid help treat epilepsy and muscular dystrophy (Mok *et al.,* 2006), treat low blood sugar (hypoglycemia) in people with diabetes adenine, 9-(2,3-dideoxy-beta. -D-glycero-pent-2-enofuranosyl) can be used anti-HIV activity (Jafari, 2020).

The *in vitro* propagated methanolic extract of *E. fluctuans* contains Hentriacontane bears anti-microbial, anti-tumoral, and anti-inflammatory activities (Ouellette & Rawn 2015). Z,z-6,28-heptatriactontadien-2-one is used as vasodilator, larvicidal effects on A. aegypti and A. stephensi (Mallikadevi et al., 2012), 2-Azetidinecarboxylic acid is used as teratogenic agent, causes the production of abnormal proteins with impaired biological activity (Rubenstein et al., 2006), L-Norvaline has a role of bacterial metabolite, a hypoglycemic agent and a neuro protective agent (De et al., 2016), Ritalinic acid is the drug commonly prescribed to children for treatment of attention-deficit hyperactivity disorder is the ester dl-methylphenidate (Ritalin) (Lockridge et al., 2018).

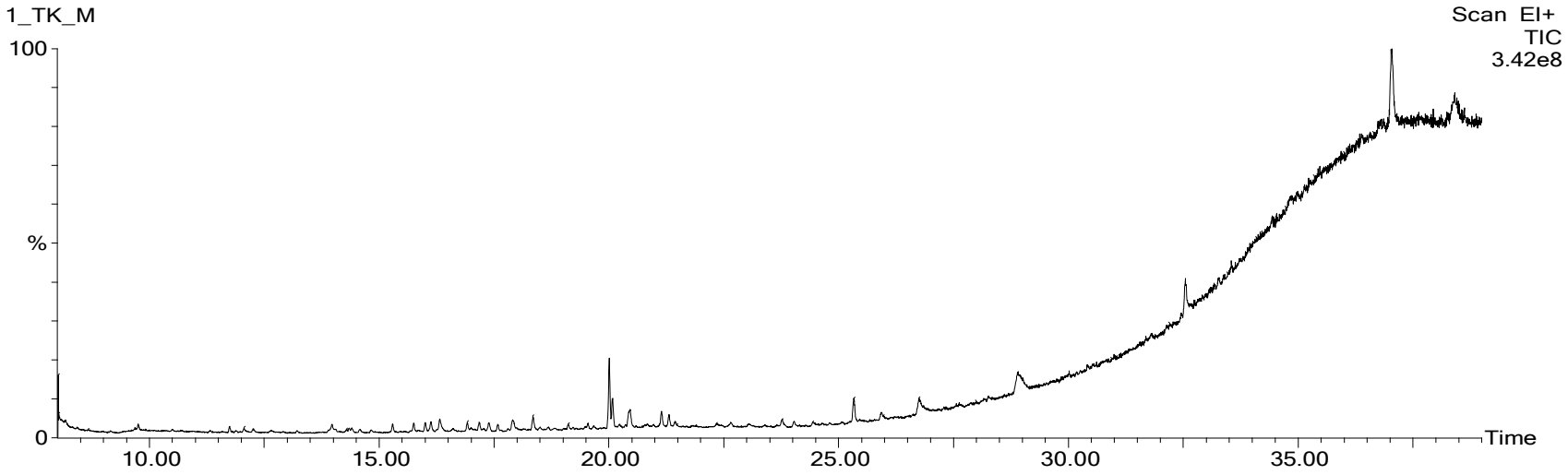


Fig 4.5.6a: GC-MS spectrum of tissue cultured *E. fluctuans* plant in methanol extract

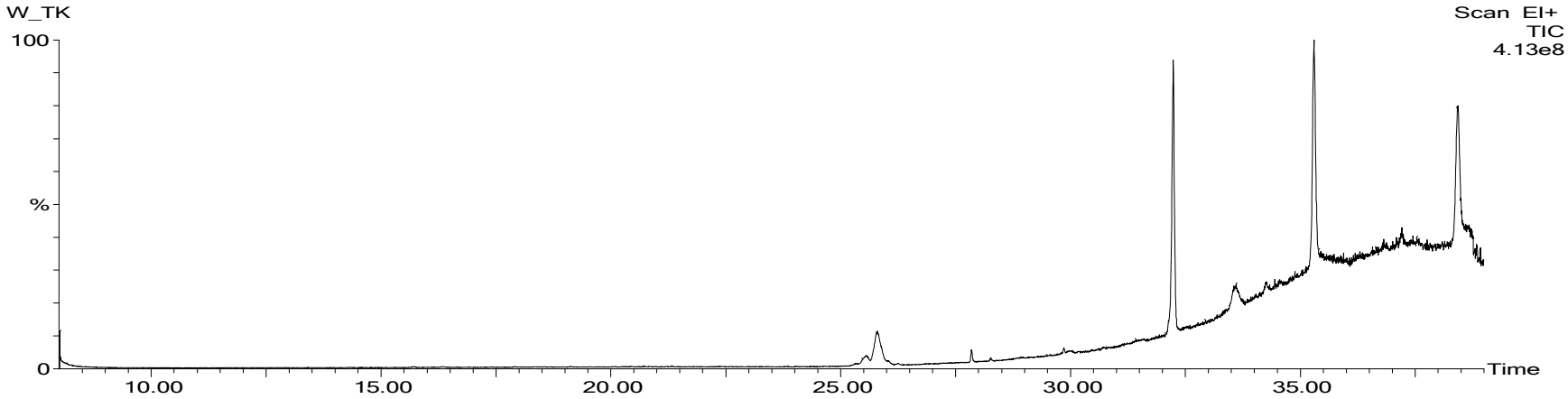
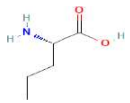
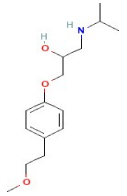
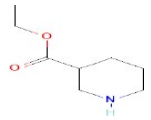
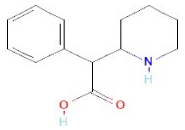
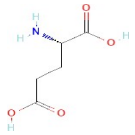
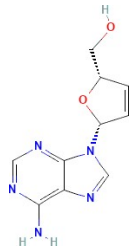


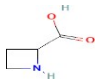
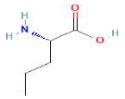
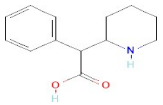
Fig 4.5.6b: GC-MS spectrum of wild *E. fluctuans* species in methanol extract.

Table 4.5.4*:* Bioactive compounds found in aqueous methanolic extract of *E. fluctuans* **A:** wild plant **B**: Tissue cultured plant

A: Compounds in the Tissue cultured *E. fluctuans*						
Sl no	**Compound name**	**Retention time (RT)**	**Area (%)**	**Molecular Weight**	**Mol Formula**	**2D structure**
1	L-Norvaline	20.008	1.379	245	$C_5H_{11}NO_2$	
2	Metoprolol	20.008	1.379	411	$C15H_{25}NO_3$	
3	2-Propenoic Acid	21.149	0.400	282	$C_3H_4O_2$	

4	Ethyl Nipecotate	21.149	0.400	157	$C8H_{15}NO_2$	
5	Ritalinic Acid	25.330	0.754	291	$C_{13}H_{17}NO_2$	
6	L-Glutamic Acid	25.330	0.754	147	$C_5H_9NO_4$	
7	Adenine, 9-(2,3-dideoxy-beta.-D-glycero-pent-2-enofuranosyl)	32.543	1.215	233	$C_{10}H_{11}N_5O_2$	

A: Compounds in wild *E. fluctuans*

Sl no	Compound name	Retention time (RT)	Area (%)	Mol. weight	Molecular Formula	2D structure
1	Hentriacontane	32.233	12.363	436	C32H64	
2	Z,z-6,28-heptatriactontadien-2-one	29.857	0.221	530	$C_{37}H_{70}O$	
3	2-Azetidinecarboxylic Acid	16.322	0.483	101.104	$C_4H_7NO_2$	
4	L-Norvaline	20.008	1.379	245	$C_5H_{11}NO_2$	
5	Ritalinic Acid	25.330	0.754	291	$C_{13}H_{17}NO_2$	

4.6 *Hygrophila auriculata*

4.6.1 Explant surface sterilization: The collected explants of *H. auriculata* were sterilized using different sterilizing agents. Initially the collected explants were washed using tween 20 for 10 min, followed by 0.5% Bavistin for 45min and 0.1% mercuric chloride(0-5min). When no treatment was used all the cultured explants got contaminated in the culture. When the explants treated with 0.1% mercuric chloride for 1 min, 13.33±4.71% of the explants survived out of 10 explants after 21 days of culture. 2 min treatment with 0.1% mercuric chloride resulted 23.33±4.71% explant survival rate after 21 days of explant culture. 3min treatment resulted 46.67±4.71% explant survival rate, 4 min showed 56.67±4.71% explant survival rate and 5 min treatment with 0.1% mercuric chloride 46.46±4.71% explant survival rate after 21 days of explant culture. Though the contamination rate in the explants treated for 4 min mercuric chloride was 30% and with 5 min treatment the contaminated rate was 26.67±4.71% rest 20% explants got damaged (Fig 4.6.1).

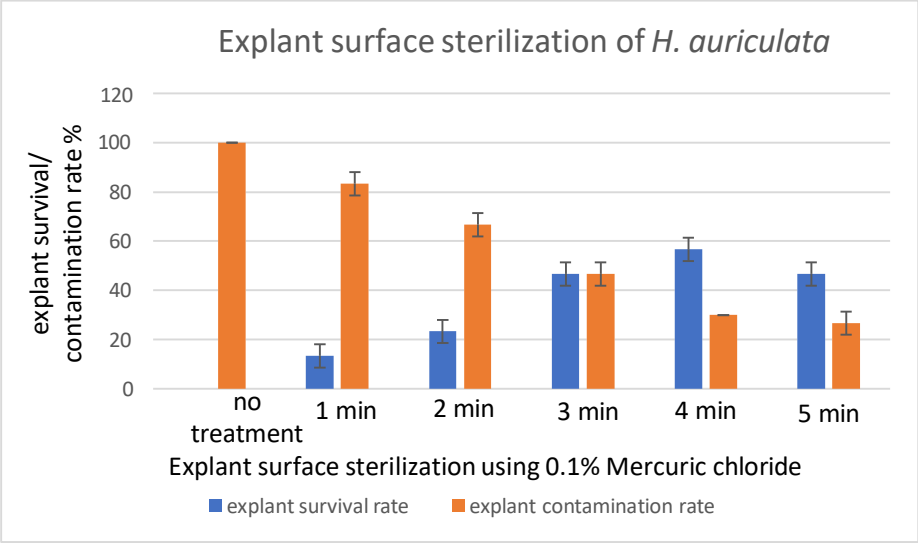


Fig 4.6.1: Graph showing explant survival rate of *in vitro* propagated *P. thyrsiformis* after 21 days of explant initiation0.1% mercuric chloride. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

4.6.2 Explant initiation and shoot multiplication and rooting: After the successful surface sterilisation of explants, the explants were induced for multiple shoots and rooting *in vitro* in new culture media. The explants were monitored regularly to observe growth and contamination. The explants were successfully developed into multiple shoots and roots, but the explants responded differently to different compositions of hormones. The *in vitro* multiplied shoots were taken out of the media after every 4 weeks, excised into single shoots, and subcultured into a new fresh medium. The highest numbers of shoot multiplication of *H. auriculata* were observed in the MS medium with 1 mg/L BAP+0.5mg/L NAA (BM4), which is an average of 12.28±1.08 shoots and an average shoot length of 15.57±1.1cm and an average of 15±1.22 roots per explant (Fig: 4.6.2). The lowest average shoot lengths were observed in control medium where no growth regulator was used, i.e.-an average of 3.57±0.84 shoots per explant, an average of 6±1cm shoot length and 10.71±1.71 roots per explant (Table 4.5.1). BM1(MS+1mg/L BAP) media formed an average of 6.14±0.59 shoots per explant, 7.57±1.1cm shoot length, and an average of 13.85±1.53 roots per explant. BM2(MS+1mg/L BAP+0.2mg/L NAA) media formed an average of 8.42±1.10 shoots per explant, 11.42±0.84 cm average shoot length, and 11.42±0.84 root numbers per explant. In the BM3 medium (MS+1mg/L BAP+0.4mg/L NAA) formed an average of 10.71±0.96 shoots per explant, 13.14±0.59 cm average shoot length, and an average of 15.42±1.48 roots per explant. In the BM5(MS+1mg/L BAP+1mg/L NAA) media an average of 9.85±0.77 shoots per explant, 11.85±0.59 cm shoot length, and 13.57±0.46 root numbers were formed. An average of 6.71±0.65 shoots per explant, 10.28±0.96 cm average shoot length, and 13±1 number of roots were observed in the basal media BM6(MS+2mg/L BAP). In the BM7(2mg/L BAP+0.5mg/L NAA) media an average of 9.14±0.92 shoots per explant, 9.14±0.92 cm average shoot length, and 12.28±0.96 roots per explant were observed. An average of 9.42±0.84 shoots per explant, 14±0.70 cm average shoot length, and 14.57±1.10 number of average roots were observed in the BM8(2mg/L BAP+1mg/L NAA) media. An average of 7.57±0.84 number of shoots per explant, 8.71±0.65 cm average shoot length, and 12.14±0.59average number of roots were observed in the BM9(MS+3mg/L BAP) medium. In the BM10 (MS+0.5mg/L IAA) media an average of 4.71±0.65 shoots per explant, 8.42±0.84 cm average shoot length, and 23.14±1.16 average root numbers were observed. An average of 6±1 shoot per explant, with an average of 9.28±0.82 cm shoot

length and 28.71±2.04 average root numbers were observed in the BM11 medium (MS+1mg/L IAA). Finally, in the BM12(MS+2mg/L IAA) medium an average of 7.28±1.19 shoots per explant, with an average shoot length of 8.85±0.5, and 23.14±1.16 number of average roots per explant were observed.

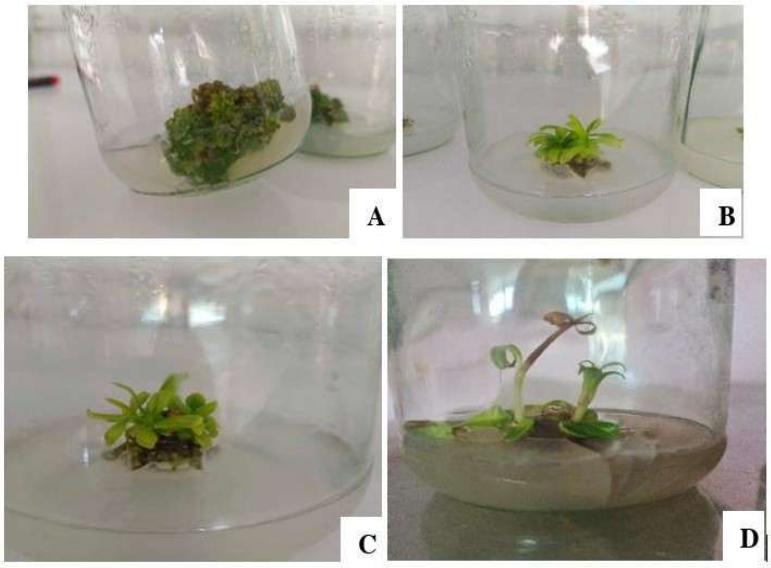


Fig 4.6.2*:* *in vitro* propagation of *H. auriculata*, A; explant initiation, B, C&D; shoot multiplication

Table 4.6.1: Showing effect of different basal medium (BM) on the *in vitro* propagation of *H. auriculata.* Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Sl no	**Basal media**	**Growth regulators (mg/L)**			**Number of explant culture initiation**	**Rate (%) of explant producing shoots**	**Mean number of shoots per explant**	**Mean shoot length (cm)**	**Mean no of roots**
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	05	100	3.57±0.84	6±1	10.71±1.71
2	BM1	1.0	0.0	0.0	05	100	6.14±0.59	7.57±1.1	13.85±1.53
3	BM2	1.0	0.2	0.0	05	100	8.42±1.10	11.42±0.84	11.42±0.84
4	BM3	1.0	0.4	0.0	05	100	10.71±0.96	13.14±0.59	15.42±1.48
5	BM4	1.0	0.5	0.0	05	100	12.28±1.08	15.57±1.1	15±1.22
6	BM5	1.0	1.0	00	05	100	9.85±0.77	11.85±0.59	13.57±0.46
7	BM6	2.0	0.0	0.0	05	100	6.71±0.65	10.28±0.96	13±1
8	BM7	2.0	0.5		05	100	9.14±0.92	9.14±0.92	12.28±0.96
9	BM8	2.0	1.0		05	100	9.42±0.84	14±0.70	14.57±1.10
10	BM9	3	00	00	05	100	7.57±0.84	8.71±0.65	12.14±0.59
11	BM10	00	00	0.5	05	100	4.71±0.65	8.42±0.84	23.14±1.16
12	BM11	0.0	0.0	1.0	05	100	6±1	9.28±0.82	28.71±2.04
13	BM12	00	00	2.0	05	100	7.28±1.19	8.85±0.59	23.14±1.16

4.6.3 Hardening: The primary hardening 80% of the *H. auriculata* was successfully survived on the 50% vermicompost soil mixture inside the green house at a temperature of $28^0C \pm 2^0C$ and a relative humidity of 70%. After 21 days grown in the greenhouse the explants were taken out to the shed net house in natural environment with varying temperature $28^0C \pm 5^0C$. all the plantlets survived in the natural condition in this step. Finally, the plantlets were successfully grown in the natural climate condition.

4.6.4 Genomic DNA extraction: The whole genome of both wild and tissue cultured *H. auriculata* was extracted using Qiagen DNeasy Plant Mini kit followed by steps mentioned in the kit (Fig 4.6.3). Lane A is the genomic DNA extracted from leaf explant of tissue cultured *H. auriculata,* Lane B is genomic DNA extracted from leaf explant of wild *H. auriculata.*

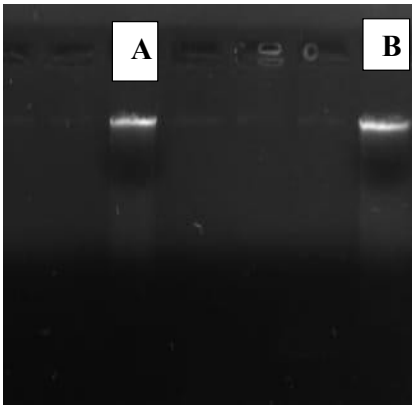


Fig 4.6.3: Isolated Genomic DNA of *H. auriculata*, where **A**– Tissue cultured (using leaf explant), **B**– Wild Plant (using leaf explant)

4.6.5 RAPD assay: When compared to the mother plant, the phenotypic and genetic makeup of the in vitro produced plants may undergo a variety of alterations. Once these variations have spread, it is crucial to find them. Using molecular markers to identify somaclones can be a helpful method. For the detection of somaclonal variation in the micropropagated *H. auriculata* nine distinct RAPD primers were used (OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OPC-8, OPC-9 and OPC-10). Out of the ten RAPD primers four primers (OPC-3, OPC7, OPC-9, OPC-10) did not bind any target sequence on the template forming no DNA bands in the gel electrophoresis. Out of six RAPD primers the four RAPD primers formed polymorphic DNA bands in wild and tissue cultured *H. auriculata,* (Fig 4.6.4). The OPC-1 primer in the wild plant formed a total of three polymorphic band while the micropropagated plant formed one polymorphic

amplified DNA bands, OPC-2 formed a total of nine polymorphic DNA bands in wild plant and eight polymorphic DNA band in the tissue cultured plant, OPC-4 formed a total six polymorphic DNA bands and five polymorphic DNA bands in wild plant, OPC-5 formed a total of three DNA band and both wild tissue cultured plant, OPC-6 formed a total of three DNA bands in micropropagated plant and two in wild plant, and the OPC-8 formed a total of 5 polymorphic DNA bands in both wild and tissue cultured plant (Table 4.6.2). From the result it can be concluded that the *in vitro* cultured plant undergoes somaclonal variation in the process of *in vitro* propagation.

Table 4.6.2: Number of polymorphic DNA bands formed in wild and tissue cultured *H. auriculata* using RAPD primers (OPC-1 to OPC-10) in agarose gel electrophoresis

Sl no	Primer	Number of bands produced by wild plant	Number of bands produced by hardened micro propagated plants
1	OPC 01	3	1
2	OPC 02	9	8
3	OPC 03	0	0
4	OPC 04	6	5
5	OPC 05	3	3
6	OPC 06	3	2
7	OPC 07	0	0
8	OPC 08	5	5
9	OPC 09	0	0
10	OPC 10	0	0

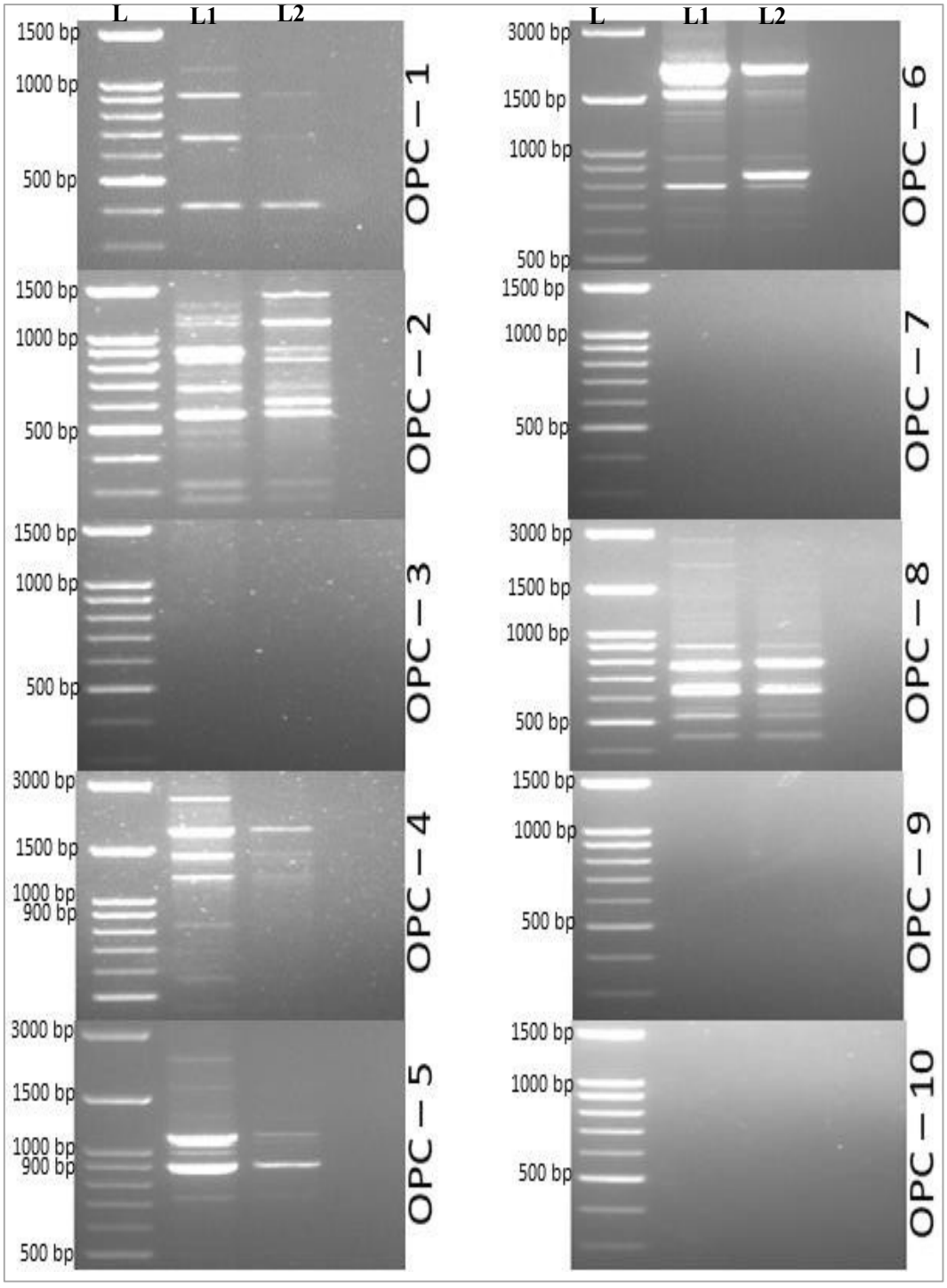


Fig 4.6.4: Amplification pattern of different RAPD Primers (OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9 and OPC10), lane L defines the 1kb DNA ladder, Lane 1 is amplified RAPD product of tissue cultured plant genome, lane 2 is amplified RAPD product of wild plant genome.

4.6.6 *In-vitro* antioxidant assay in wild and tissue cultured extract of *H. auriculata:* *In vitro* antioxidant assay in wild and tissue cultured *H. auriculata were conducted after successful* standardisation of the *in vitro* propagation method. 1 mg/mL extracts were prepared in 70% methanol for the following tests using a UV-vis spectrophotometer.

Total Phenolic Content: The total phenolic content in the dried extract of *H. auriculata* was determined using the regression curve ($y = 276.85x + 68.485$; $R^2 = 0.9934$) (Fig 4.2.5a) and expressed as mg of GAE (Gallic acid equivalent). The total phenolic content in the tissue cultured extracts was comparatively higher (184±3.4 mg GAE/g) than that of the wild (154.7 ±5.3 mg/g GAE) extracts of *H. auriculata.*

Total flavonoid content: The flavonoid content in the wild and micropropagated *H. auriculata* was determined using the regression curve ($y = 404.68x + 53.647$ $R^2 = 0.993$) (Fig 4.2.5b) and expressed as Quercetin equivalent mg/g of the dried extract. In the experiment the flavonoid content in the tissue cultured methanolic extract was comparatively higher (124±2.6 mg QE/g) than the wild (112±3 mg QE/g) extract of *H. auriculata.*

Total antioxidant capacity: Total antioxidant capacity in the tissue cultured and wild extract was determined using the regression curve ($y=324.98x + 45.306$ $R^2 = 0.9902$) (Fig 4.2.5c) and expressed as mg of ascorbic acid equivalent per grams of dried extract (AAE)**.** The total antioxidant capacity in the tissue cultured H. auriculata was 114.7±7.2 mg of AAE/g of dried extract and 94±7.2 mg AEE/g in wild extract.

DPPH scavenging Activity: The radical scavenging activity in the wild and tissue cultured *P. thyrsiformis* was determined by the regression curve ($y = 16.114x + 13.25$; $R^2 = 0.9846$) (Fig 4.2.5d). The IC_{50} value of the wild methanolic extract of *P. thyrsiformis* is 67.70±0.50 and in the tissue cultured extract was 74.19±0.84

4.6.7 Quantitative Detection of gallic acid and quercetin using HPLC: The elution of the wild and the tissue cultured extract was done using the isocratic solution of 30% (v/v) HPLC-grade water and 70% (v/v) methanol. The gallic acid and quercetin content in the wild and tissue cultured methanolic extract of *H. auriculata* was satisfactory.

The chromatograms and the standard curve of gallic acid and quercetin was plotted. The gallic acid content in the wild and tissue cultured *T. crustacea* was determined using the regression curve (y= 0.8696x + 98.778; R^2 = 0.9958) (Fig 4.2.6A) and the quercetin content in the wild and tissue cultured *T. crustacea* was determined using the regression curve (5.9606x + 88.794 R^2 = 0.997) (Fig 4.2.6B). The retention time of quercetin and gallic acid was 2.7 min and 8.7 min respectively (Fig. 4.6.5a, 4.6.5b, 4.6.5c, & 4.6.5d). From the experiment the gallic acid content and quercetin content was higher in the tissue cultured extract was than the wild extract of *H. auriculata.* The quercetin content in the micropropagated extract was 42.7±1.28 mg/g of dried extract and 42.7±1.28 mg/g of dried extract in the wild plant. Again, the gallic acid content was 43.1±1.33mg/ g and 56.26667± 0.78 mg/g in the wild and tissue cultured plant extract of *H. auriculata respectively (Table 4.6.3).*

Table 4.6.3: Showing concentration of gallic acid and quercetin (µg/ml) in wild and tissue cultured *H. auriculata* using HPLC. Values are mean ±SE (n=3). Statistical analysis was done by one-way ANOVA between groups, and the values were considered significant at $p<0.05$.

Compound	**RT**	**Concentration mg/g**
Gallic acid content in wild extract	2.68	43.1±1.33
Gallic acid content in tissue cultured extract	2.89	56.266± 0.78
Quercetin content in tissue cultured extract	8.78	42.7±1.28
Quercetin content in wild extract	8.788	41.56667±0.96

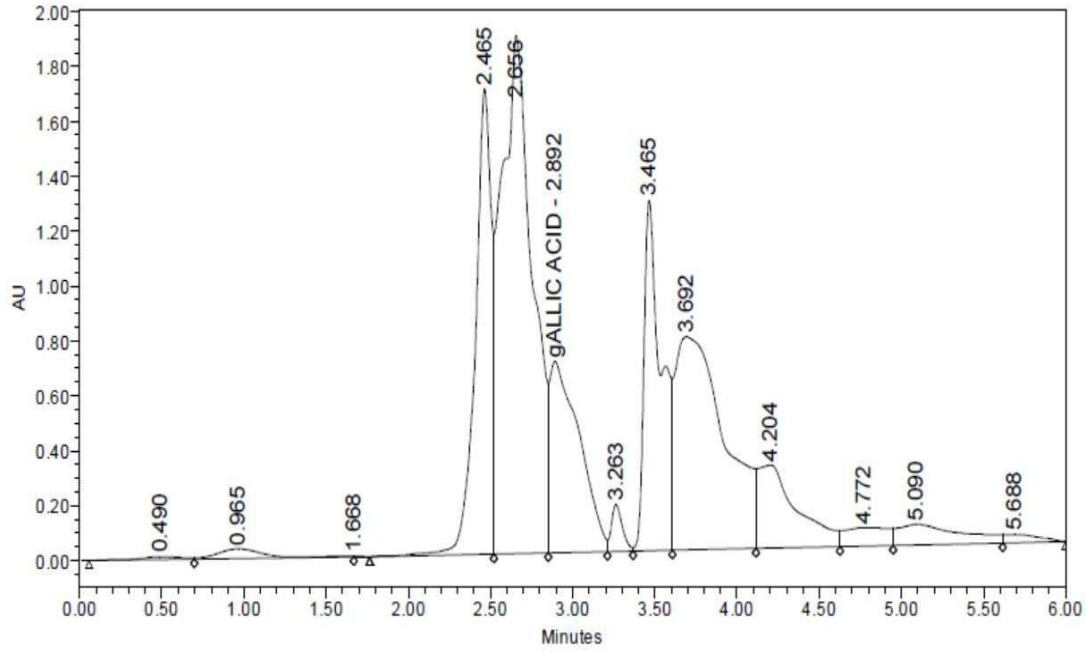


Fig 4.6.5a: HPLC Chromatograms of gallic acid peak in Tissue cultured *H. auriculata* methanolic extract

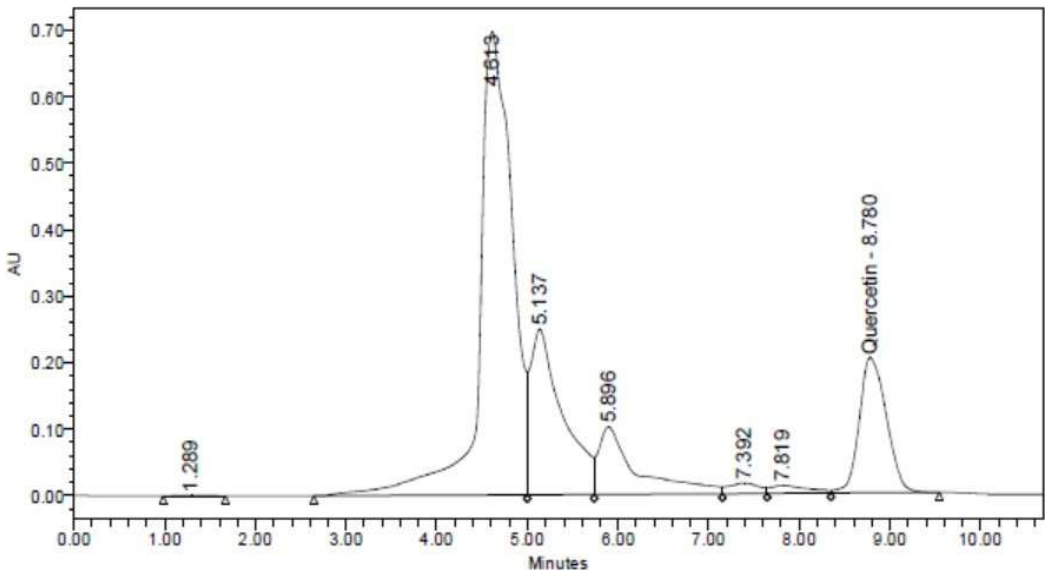


Fig 4.6.5b: HPLC Chromatograms of gallic acid peak in wild *H. auriculata* methanolic extract

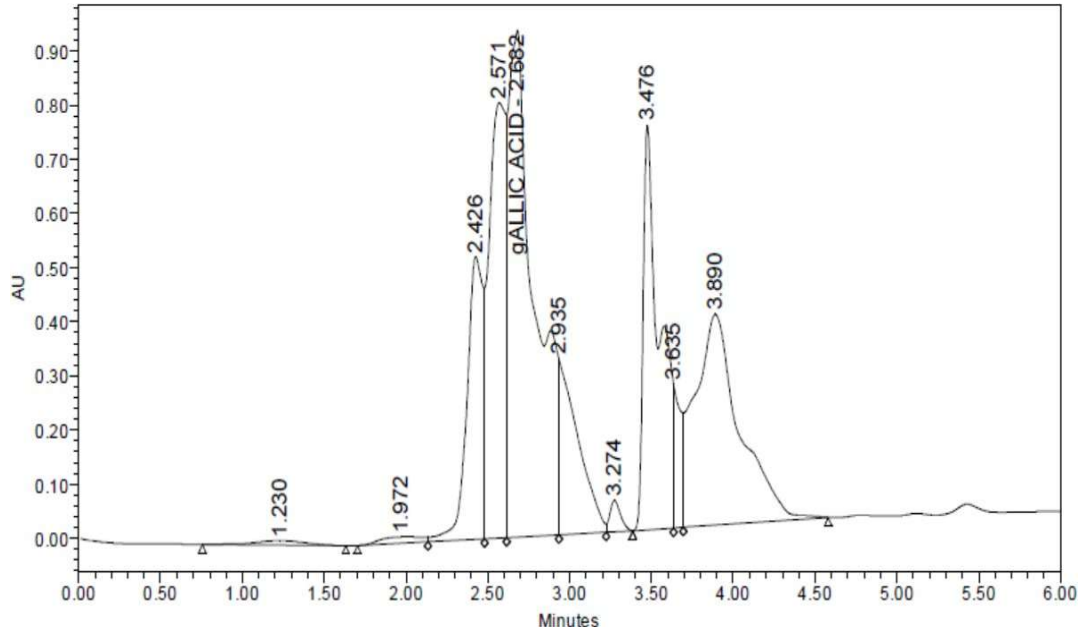


Fig 4.6.5c: HPLC Chromatograms of quercetin peak in tissue cultured methanolic extract of *H. auriculata*

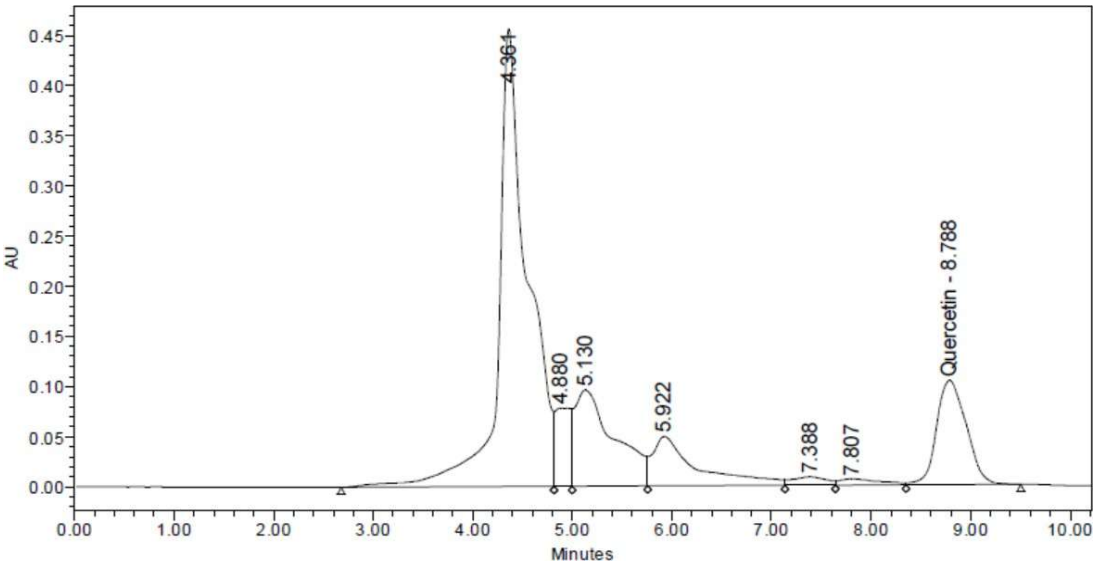


Fig 4.6.5d: HPLC Chromatograms of quercetin peak in wild methanolic extract of *H. auriculata*

4.6.8 Study of bioactive compounds in tissue cultured and wild extracts of H. auriculata: The methanolic extracts of wild and tissue cultured *H. auriculata* plants were screened for the presence of bioactive compounds using GC-MS analysis. The GC-MS chromatogram for both wild and tissue cultured aqueous methanolic extract of *H. auriculata* were plotted (Fig: 4.6.6 a & b). the compound search was conducted using retention time (RT), molecular weight, molecular formula, and peak area% (concentration). Nine bioactive compounds were identified in the wild extract and eight active chemical compounds were identified in the tissue cultured extract (Table: 4.6.4). The following compounds were identified in the methanolic wild extract of *H. auriculata*- Methyl 9-eicosenoate, Methyl 9-tetradecenoate, Methyl 13-eicosenoate, Heptacosanoic acid, 25-methyl-, methyl ester, 1-methylbicyclo [3.2.1] octane, 1,2,3-benzotriazin-4(3h)-one, 3-[(8-quinolinyloxy) methyl]-, heptacosanoic acid, 25-methyl-, methyl ester, methyl 8-heptadecenoate,

And the following compounds were identified in the methanolic extract of tissue cultured *H. auriculata*- 1,1,1,3,5,5,7,7,7-nonamethyl-3-(trimethylsiloxy) tetrasiloxane that can be used in the biological activity research compound and phytochemical analysis (Gideon & Ladan, 2023), 3-methylsalicylic acid, 2tms derivative used in analytical chemistry for compound analysis using GC-MS specifically in metabolomic studies (Abadie et al., 2022), 1-heptene, 1,3-diphenyl-1-(trimethylsilyloxy)-, 1,2,3-benzotriazin-4(3h)-one, 3-[(8-quinolinyloxy)methyl]-, p-cyanobenzyl alcohol, 3-(phenoxymethyl)-1,2,3-benzotriazin-4(3h)-one, cyclotrisiloxane, hexamethyl can be used in emollient, hair conditioning, skin conditioning (Dionisio et al., 2018), decamethyltetrasiloxane used in the deodorant, general moisturizing, hair styling and care hair styling and care, fragrance, (Dionisio et al., 2018), hexamethylcyclotrisiloxane can be used in emollient, hair conditioning, skin conditioning (Dionisio et al., 2018), 1,1,1,3,5,5,5-heptamethyltrisiloxane can be used in Surfactant (surface active agent).

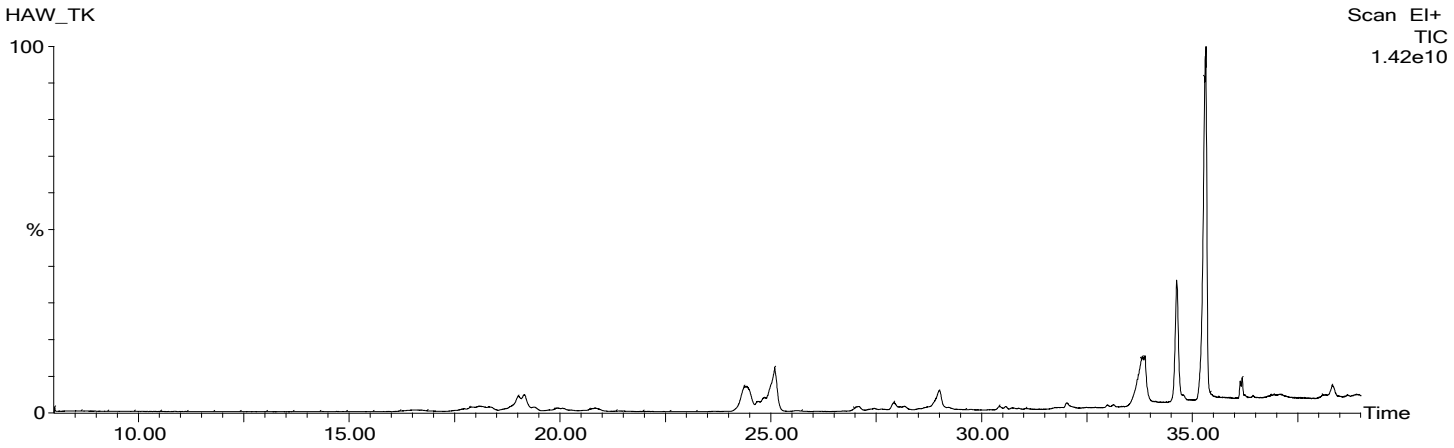


Fig 4.6.6a: GC-MS spectrum of wild *P. thyrsiformis* plant in methanol extract

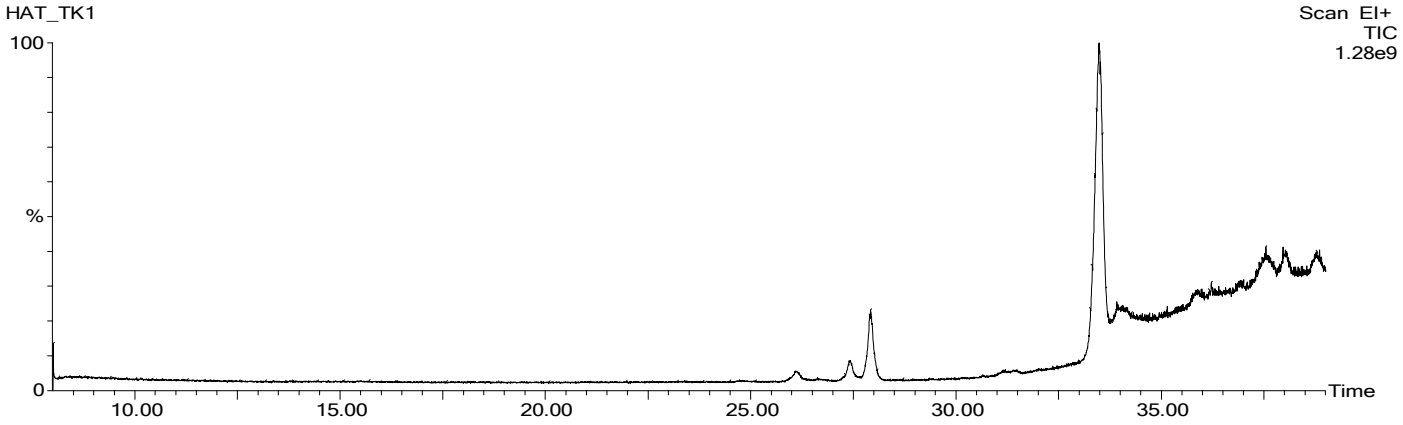
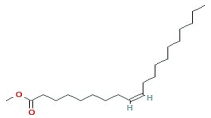
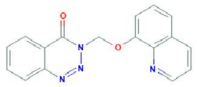
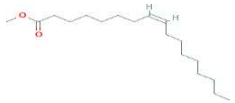


Fig 4.6.6b: GC-MS spectrum of tissue cultured *P. thyrsiformis* plant in methanol extract

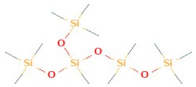
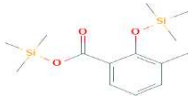
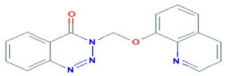
Table 4.6.4*:* Compounds found in aqueous methanolic extract of *H. auriculata* **A:** wild plant **B**: Tissue cultured plant

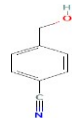
Compounds identified in the methanolic extract of wild *H. auriculata*

Sl no	RT	Height	area	Area %	Compound	Molecular formula	Molecular weight	Structure
1	8.509	74,508,728	49,902,868.0	3.53	METHYL 9-EICOSENOATE	$C_{21}H_{40}O_2$	324.5	
2	18.087	186,375,904	43,094,732.0	3.05	METHYL 9-TETRADECENOATE	$C_{15}H_{28}O_2$	240.38	
4	19.148	607,467,200	87,376,912.0	6.18	METHYL 13-EICOSENOATE	$C_{21}H_{40}O_2$	324.5	
5	24.435	789,125,184	188,800,848.0	13.36	HEPTACOSANOIC ACID, 25-METHYL-, METHYL ESTER	$C_{29}H_{58}O_2$	438.8	

7	27.916	284,072,992	44,495,068.0	3.15	1-methylbicyclo [3.2.1] octane	C_9H_{16}	124.22	
8	29.002	761,084,544	159,535,968.0	11.29	1,2,3-benzotriazin-4(3h)-one, 3-[(8-quinolinyloxy) methyl]-	$C_{17}H_{12}N_4O_2$	304.3	
9	33.874	1,845,016,320	431,668,192.0	30.55	heptacosanoic acid, 25-methyl-, methyl ester	$C_{29}H_{58}O_2$	438.8	
10	34.629	4,719,517,184	462,019,008.0	32.7	methyl 8-heptadecenoate	$C_{18}H_{34}O_2$	282.5	
11	35.319	13,698,238,464	1,413,024,896.0	100	HEPTACOSANOIC ACID, 25-METHYL-, METHYL ESTER	$C_{29}H_{58}O_2$	438.8	

B: Compounds identified in the methanolic extract of tissue cultured *H. auriculata*

Sl no	RT	Height	Area	Area%	Compound			Structure
1	8.659	48,646,768	16,085,132.	2.552	1,1,1,3,5,5,7,7,7-nonamethyl-3-(trimethylsiloxy)tetrasiloxane	$C_{12}H_{36}O_4Si_5$	384.84	
2	8.874	45,311,912	21,028,834.0	3.337	3-methylsalicylic acid, 2tms derivative	$C_{14}H_{24}O_3Si_2$	296.51	
3	9.464	37,175,696	6,509,299.5	1.033	1-heptene, 1,3-diphenyl-1-(trimethylsilyloxy)-	$C_{22}H_{30}OSi$	338.6	
5	26.121	38,790,108	8,324,550.	1.321	1,2,3-benzotriazin-4(3h)-one, 3-[(8-quinolinyloxy) methyl]-	$C_{17}H_{12}N_4O_2$	304.3	

6	27.426	70,351,064	11,269,521.0	1.788	p-cyanobenzyl alcohol	C_8H_7NO	133.15	
7	27.916	255,186,976	42,118,560.0	6.683	3-(phenoxymethyl)-1,2,3-benzotriazin-4(3h)-one	$C_{14}H_{11}N_3O_2$	253.26	
9	33.914	76,094,736	6,965,018.0	1.105	hexamethylcyclotrisiloxane	$C_6H_{18}O_3Si_3$	222.46	
10	37.450	104,389,496	6,815,113.5	1.081	decamethyltetrasiloxane	$C_{10}H_{30}O_3Si_4$	310.68	
12	37.960	125,988,640	11,200,743.0	1.777	1,1,1,3,5,5,5-heptamethyltrisiloxane