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# *In vitro* propagation and comparison of bioactive compounds in wild and micropropagated medicinal herb *Enydrafluctuans*

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**Abstract**

*Enydrafluctuans* Lour is a medicinal flowering plant found in the Asian and African subtropics and tropics. Plant tissue culture is an important technique through which plant regeneration, mass propagation, and conservation are possible. The present study aims to standardise *in vitro* mass propagation technique, study of comparative antioxidant properties, and bioactive compound analysis in wild and tissue-cultured plant extracts of *E.fluctuans*. The explant surface sterilisation for tissue culture was best with 0.1% mercuric chloride for a 3-minute treatment, resulting in an 80% explant survival rate. In the RAPD assay, the tissue-cultured plants showed polymorphism in DNA bands by two RAPD primers. Antioxidant tests for total phenol, flavonoid contents, and total antioxidant capacity tests showed the tissue-cultured extracts carried a higher concentration of GAE (gallic acid equivalent) and QE (quercetin equivalent) comparatively than those of the wild plant. The HPLC analysis revealed that the tissue cultured extracts carried higher concentrations of gallic acid (10.1934µg/mg) than in the wild plant extract (8.059µg/mg) and higher concentration of quercetin ( 5.1292µg/mg of dried extract) than the wild plant extracts (2.0199µg/mg). The bioactive compounds in the extracts of wild and tissue-cultured plants were studied using GC-MS analysis.

**Keywords**: bioactive compound; explant culture; HPLC; micropropagation; RAPD.

## Introduction

Plant extracts are being used as primary healthcare to treat many diseases. Also, the medicinal plants play a vital role in pharmaceuticals and drug discoveries (Sharma, M., & Das, B. 2018). Medicinal plants are used to cure variety of diseases directly or indirectly due to abundance of their important bioactive components (Das, D., & Das, S. 2022). Traditional medicines play a crucial role in the health care and treatment of diseases in most developing countries (Naveen Kasagana, V., & Sree Karumuri, S.). *Enydrafluctuans* Lour is a semi-aquatic herb belonging to the family Asteraceae that contains medicinal properties. The plant

including leaves are consumed as a vegetable and spread throughout the country (Shah, N. A.,*et al,* 2013a). The herb is commonly known for its anticancer, analgesic, anti-diarrheal, antioxidant, hepatoprotective, and neuropharmacological properties (Sarma, U.,*et al,* 2014). The stem is used to treat gastric, ulcers, and the whole plant is used to treat constipation by the people of Assam. It is also used in ascites, dropsy, anasarcha, and snakebite (Ghani, A., 2003). The herb has significant antimicrobial and cytotoxic activities (Ruhul Amin, *et al,* 2012). The herb is rich in β-carotene and a high source of protein. Isoflavone glycoside 4',5,6,7-tetrahydroxy-8-methoxyisoflavone-7-O-β-D galactopyranosyl-(1→3)-O-β-Dxylopyranosyl-(1→4) O-α-l-rhamnopyranoside, a novel compound, has been reported in the methanolic extract of *E. fluctuans* Lour (Yadava, R. N., & Singh, S. K., 2007).

With time, the plant resources in the biodiversity are declining continuously due to the continuous consumption and higher demands of the species. Deforestation, increasing population, and other factors also cause decline of the species. Numerous important plant species got extinct in nature; most of the species have been extinct without being explored or identified. Therefore, preservation and mass propagation of desired plant species are prior needs of the time to meet the demands of the pharmacological industries. Plant tissue culture is an efficient and useful tool for mass propagation of uniform and disease-free plants. Most of the time, it is used for preservation and conservation of important species. This technique is also used for genome transformation and production of bioactive compounds and secondary metabolites (Debnath, M., *et al,* 2006; Altpeter, F, *et al,* 2016).

This technique is very profitable as it offers a remarkable dominance over the conventional propagation method since it can provide round-year propagation and the production of uniform, disease-free plants (Debnath, M.,*et al,* 2006). Sometimes, the resulting plant product through the tissue culture process may undergo genetic or epigenetic variation. From the study, it was found that all the tissue-cultured plantlets are not always clonal copies of the mother plant (Bhattacharyya, P., *et al,* 2017). Therefore, detection of the genetic stability in the propagated plants is most important. The somaclonal variation in the plants may be detected using some molecular markers, i.e., RAPD, SSCP, AFLP, RFLP, etc. The effect of somaclonal variation may have a large impact on the production of secondary metabolites (Debnath, M.,*et al,* 2006). In the tissue culture process, the collected explants undergo several treatments, the use of different growth promoters, and environmental conditions that may induce alterations in the genetic compositions of the *in vitro* plants, which is also known as somaclonal variation. It is thought that treatments with different sterilants and growth conditions can also alter the genetic composition of tissue-cultured plants (Koduru, *et al,* 1981;Stanišić, M., *et al,* 2015; Govindaraju, S., & Indra Arulselvi, P. 2018; Gyulai, G., *et al*; &Bairu, M. W *et al,* 2011).

One of the major challenges in the entire tissue culture process is contamination in the cultured explants; hence, surface sterilisation is the most crucial step for the initiation of contamination-free explant culture. Different chemical sterilants are being used for the surface sterilisation process, including ethanol, sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, mercuric chloride, silver nitrate, and bromine water (Teixeira da Silva, J. A., 2016&Singh, V., 2011). However, to achieve a contamination-free culture, the required concentrations and treatment of the sterilant vary from species to species, also depends on plant parts which are used for the culture. Plant preservative mixture(0.1 mL/L to 1 mL/L) can be used along with the media to treat airborne contaminants in the culture. 0.5 mL/L to 1 mL/L PPM in the culture media can be used to reduce airborne contamination in the culture (Plant Cell Technology, 1998). Plant growth regulators play a remarkable role in plant growth and development; proper combinations and ratios are required for tissue development. There is no report published yet on *in vitro* propagation of *Enydrafluctuans*. Therefore, the objective of the present study are to standardise *in vitro* propagation protocol for *Enhydra*

*fluctuans*from the nodal explants and to study the effects of different plant growth regulators on plant propagation, shoot proliferation, and root formation.

**Materials and Methodology**

**Media preparation:** For plant tissue culture, full- and half-strength MS media were prepared supplemented with different concentrations of plant growth regulators. Stock solutions of different reagents were prepared and kept inside the refrigerator at 8$^{o}$ C. MS media supplemented with different concentrations of PGR were prepared and sterilised at 121C temperature and 15 psi for 15 min (Baro, T., & Das, S., 2022a). For the preparation of 500 mL of MS media, initially 300 mL of sterile distilled water was taken in a conical flask, all the required components of the media were added, and the volume was adjusted to 500mL. The pH was adjusted to 5.6 and 0.8% agar (w/v) was added, and 3% (w/v) sucrose was added. The media were sterilised at 121$^{o}$C at 15 psi for 15 minutes and brought to the LAF (laminar air flow hood). The plant growth regulators and PPM were sterilised with the help of a 0.22µM filter syringe and added to the media when the temperature of the media was below 60$^{o}$ C and poured into the culture jars and kept under UV light inside the LAF.

**Plant material collection and authentication:** Disease-free, and healthy nodal explants of *Enhydra fluctuans*were collected from Baksa District, Assam. The voucher specimen was then submitted Bodoland University Botanical Herbarium, Department of Botany, Bodoland University, Assam for identification.

**Explant preparation and surface sterilization:** The collected explants were washed in running water, the leaves and roots were removed. The explants were washed in 300 mL of distilled water containing 1-2 drops of tween-20 for 20 minutes, and washed in distilled water. The explants were then treated with 70% alcohol for 30 seconds, and washed in sterile distilled water three times. After that, the explants were treated into 100 mL of 0.5% Bavistin for 40 minutes and washed in sterile distilled water three times. The surface sterilisation was performed using 0.1% mercuric chloride (0–5 min treatment in 0.1% mercuric chloride inside the laminar air flow cabinet) and immediately washed in sterile distilled water three times. After washing in sterile distilled water, the explants were trimmed to get a 1cm nodal explant in length.

**Explant culture initiation:** The above explants were inoculated in already prepared MS media supplemented with different concentrations of plant growth regulator. For initiation of explant culture, BAP (0–5 mg/L), NAA (0–1 mg/L), IBA (0–1 mg/L), and different combinations of the growth regulators were used. After inoculation, the explants were shifted to the culture racks inside the growth room at 28$^{o}$ C and 16 hours of photoperiod.

**Shoot inducing and multiplication:** After the initiation of the explant culture, the explants were monitored in every weekly basis for their responses, and the data were recorded. After 6 weeks, the explants were subcultured into a new, fresh medium. The multiple shoots that emerged were taken out, excised, and introduced into different fresh medium.

**Rooting, hardening and acclimatization:** After successful shoot multiplication, the shoots were induced for rooting *in vitro. In vitro* rooting experiments were conducted in MS media supplemented with different concentrations and combinations of BAP and NAA, as well as BAP and IAA. After successful rooting, the plantlets were taken out and grew in pots containing coco pits supplemented with vermicompost fertiliser. They were kept in a controlled environment at 28$^{o}$ C and 60% relative humidity, and 12 hours of photoperiod for 10 days. After 10 days, the plantlets were shifted to the agro-net house for another 7 days and finally, the explants were taken out and grew in the natural environment.

**Genome extraction and RAPD assay**: The whole genome of the was extracted from the dried leaves of both wild and micro-propagated plants using the DNeasy Plant Mini kit (Qiagen), following the steps mentioned in the kit manual. RAPD amplification was carried out as mentioned in our previous work (Baro, T., & Das, S., 2022b) using RAPD primers

(OPC-01 to OPC 09). The PCR cycle consisted of initial denaturation at 94 °C for 5 minutes, the denaturation step at 94 °C for 30 seconds, annealing at 36 °C for 2 minutes, extension at 72 °C for 2 minutes, final extension at 72 °C for 7 minutes, and hold at 4 °C. After completion of the PCR cycle, the PCR product were separated in 1.5% ultrapure agarose gel under gel electrophoresis. Ethidium bromide was added to the agarose gel to stain the DNA. The separated products were visualised under the Gel-Doc E-Gel Imager camera hood (Life Technologies).

**Comparative *in-vitro* antioxidant tests of wild and tissue cultured extract of *E. fluctuans:***

**Extract preparation:**The collected wild and tissue cultured *E. fluctuans* plants (after hardening) were washed and dried at 45 °C for 7 days inside a hot air oven. The dried leaves were crushed up, and ground to get a homogeneous fine powder using a grinder, and stored in a container. 5g powder of the both tissue-cultured and wild plants was extracted in 100 ml of 70% methanol and water for 72 hours and filtered using Whatmann's filter paper number 42. The filtrate was concentrated by freeze-drying in a lyophilizer until complete drying. The antioxidant activity of both wild and tissue-cultured extracts of *E. fluctuans*was studied using the following methods.

**Total phenolic Content**: For the determination of total phenolic content, the Folin-Ciocalteau method (Maheshwari, D. T.,*et al,* 2011) was used. In this method, 100 µL (1 mg/ml) methanolic extract of *E. fluctuans* was taken in a test tube, and 1600 µL of distilled water was added to it. Again, 100 µL of FCR reagent was added and mixed thoroughly. After 3 minutes, 150 µL of the 1N sodium carbonate was added to the above mixture and incubated for 2 hours. Finally, the absorbance was measured at 725nm using a spectrophotometer using gallic acid as a standard and expressed in milligrammes of gallic acid equivalent (GAE/g) of dried extract.

**Total flavonoid content:**Total flavonoid content was determined using the aluminium chloride (Zhishen, J, *et al* 1999). In this method, 100 µL of 1 mg/mL extract was reacted with 1.5 mL of 95% methanol, followed by the addition of 0.1 mL of aluminium chloride hexahydrate (10%) to the mixture. To the above mixture, 0.1 mL of potassium acetate (1M) was added, and finally, 2.8 mL of double-distilled water was added and kept in room temperature for 40 minutes. Finally, the absorbance was measured at 415 nm, taking quercetin as the standard.

**Total antioxidant capacity:**The phosphomolybdate method was used for the determination of the antioxidant capacity of *E. fluctuans*(Shah, N. A, *et al*, 2013a). In this method, 0.3 ml of extract was added to 3 ml of phosphomolybdate reagent and incubated at 95 °C in a water bath for 90 minutes. After cooling, the absorbance was measured at 765 nm. Results were expressed in mg of ascorbic acid equivalent (AEE)/g of dried extract.

**Quantitative analysis of gallic acid and quercetin using HPLC:** The following working procedure were captivated for the quantitative analysis of gallic acid and quercetin.

**Extract Preparation**: The micropropagated explants and wild *E. fluctuans*were collected and dried in a hot air oven at 45 °C overnight. The dried explants were crushed to get a homogenised, fine powder. 5g of both micropropagated and wild powder of *E. fluctuans*were dissolved in 70% methanol separately and extracted inside the ultrasonicator. After 24 hours, the extracts were filtered through Whatman 41 filter paper. The second extraction was done with the remaining sample. And the filtrate was lyophilized until complete drying, and a 1 mg/mL concentration was prepared with 70% methanol for analysis.

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

**Precision:** The method was examined for its accuracy by administering a 20-mg/mL standard solution of gallic acid and quercetin. The peak regions were identified and contrasted. Six replicas were performed, the repetition and reproduction were done by injecting a 1 mg/mL solution. Inter- and intra-day variation was done. Peak areas were measured and compared for the repeatability and reproducibility of the HPLC method.

**Calibration plots:** Standard solutions of gallic acid and quercetin were prepared in various concentrations (10 to 1000 µg/mL). A linear regression was fitted to the triplicate values obtained to plot a standard curve of gallic acid and quercetin at each of the five concentrations. A volume of 20 µL was administered in the column for chromatography and ran for 10 minutes each. The peak areas were calculated and recorded. The linear range of gallic acid and quercetin was then determined by plotting peak areas for each concentration.

**Method validation:** The accuracy, precision, and specificity were validated by spiking the standards of gallic acid and quercetin. The intra-day and inter-day variation of the peak area and retention time was used to gauge precision. All experiments were conducted in triplicate. In the intra-day experiment, all the experiments for gallic acid and quercetin were conducted in triplicate on the same day at intervals of 2 hours; in the inter-day experiment, they were conducted on three different days. Injecting standard solutions allowed for the evaluation of the method's specificity and selectivity.

**HPLC Analysis:** The experiment was carried out on a Waters 1525 binary HPLC pump with a Waters 2998 PDA detector. Empower software was used for data analysis. 20 µL samples were loaded in the HPLC system, and watered spherisorb 5.0 µm The ODS2 column was used with a dimension of 4.6 mm å 250 mm. The absorbance was measured at 270 nm for gallic acid and 370 nm for quercetin.

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

**Comparative GC MS analysis of wild and tissue cultured extract of *E. fluctuans:*** The crude extract of tissue cultured sample (TK) and wild variety (W) were dissolved in methanol (HPLC grade), and GCMS analysis was performed using a Clarus 680 GC and Clarus 600C MS, both from PerkinElmer, USA; a liquid autosampler with a capillary column 'Elite- 5MS' having dimensions of length 60 m, ID 0.25 mm, and film thickness 0.25 µm, and the stationary phase being 5% diphenyl 95% dimethyl polysiloxane, at Guwahati Biotech Park, Assam. The operating conditions of the GC-MS set for the analysis were as follows: Oven temperature at 60 °C for 3 minutes, then 200 °C at 5°C/min for 3 minutes, and finally 300 °C at 60 °C/min for 10 minutes. With the help of the Perkin Elmer Programmable Split/Splitless Injector (PSSI), the initial set point of temperature is 280 °C and initially holds for 999.00 minutes. The sample injection was 1 µL, and the carrier gas was helium at 1 mL/min. The ionisation of the sample components was carried out at 70 eV. The total running time of the GC was 39 minutes. The chromatogram ranges from 50 to 600 Da in a column length of 60.0m with a 250µm diameter. The interpretation of mass spectral data for GC-MS was conducted using the database of the National Institute of Standards and Technology (Library Software: Turbo Mass NIST 2014). The spectra of the unknown components were compared with the spectra of known components stored in the NIST library.

**Results and Discussion**

Plant tissue culture is an essential and powerful tool in plant biotechnology for plant research and plant germplasm conservation. It comprises various culturing methods for plant organs and tissues, including different methods for plant modifications and improvements. Successful *in vitro* propagation techniques for *E. fluctuans*have been standardised.

**Explant selection and surface sterilization:** The disease-free apical meristem and rapidly growing plant parts were the best choices for the successful establishment of contamination-free explant culture. After the selection of explants for *in vitro culture, the* explant surface sterilisation step was the most crucial step in the entire process. Contaminations were observed in the cultures after 7–20 days of culture initiation; sometimes contaminations arose after shoot formation.

0.1% mercuric chloride was used for the explant surface sterilisation of *Enydrafluctuans.* After 30 min of treatment with 0.5% Bavistin, the explants were introduced into 0.1% mercuric chloride for 0–5 minutes. Successful surface sterilisation was observed in 0.1% mercuric chloride treatment (3–4 minutes), with an 80% explant survival rate after 28 days of culture initiation. In the case of a 4-minute treatment with mercuric chloride, 80% of the explants survived. The explant survival rate was only 20%, while 1 min of treatment with 0.1% mercuric chloride and 2 min of treatment with 0.1% mercuric chloride resulted in a 40% explant survival rate after 28 days of culture initiation. While 5 minutes of treatment with 0.1% mercuric chloride resulted in 60% of explant survival with no contamination in the tissues.

**Explant initiation and shoot multiplication and rooting:** After the successful surface sterilisation of explants, the well-responded explants were induced for multiple shoots and rooting *in vitro* innew 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 *E. fluctuans*were observed in the MS medium with 2 mg/L BAP (BM7), which is an average of 12 shoots and an average shoot length of 2cm. The highest average shoot length was obtained in the media MS with 1 mg/L BAP and 0.6 mg/L NAA (BM5). Also, the most effective shoot multiplication and the highest average shoot length were observed (Fig. I), with an average of 10 shoots and an average of 6 cm of shoot length. The lowest average shoot lengths were observed in BM9 medium (Table 1).

| Sl no | Basal media | Growth regulators (mg/L) | | Number of explant culture initiation | Rate (%) of explant producing shoots | number of shoots per explant (mean+SE) |
|---|---|---|---|---|---|---|
| | | BAP | NAA | | | |
| 1 | Control | 0.0 | 0.0 | 06 | 100 | 8±1.5 |
| 2 | BM1 | 1.0 | 0.0 | 06 | 100 | 8±1.4 |
| 3 | BM2 | 1.0 | 0.2 | 06 | 100 | 15±1.51 |
| 4 | BM4 | 1.0 | 0.4 | 06 | 100 | 11±0.83 |
| 5 | BM5 | 1.0 | 0.6 | 06 | 100 | 10±1.30 |
| 6 | BM6 | 1.0 | 1.0 | 06 | 100 | 17±1.14 |
| 7 | BM7 | 2.0 | 0.0 | 06 | 100 | 15±1.30 |
| 8 | BM8 | 2.0 | 0.2 | 06 | 100 | 10±1.14 |
| 9 | BM9 | 2.0 | 0.4 | 06 | 100 | 11±1.40 |
| 10 | BM10 | 2.0 | 0.6 | 06 | 100 | 12±1.51 |
| 11 | BM11 | 2.0 | 2.0 | 06 | 100 | 8±1.14 |
| 12 | BM12 | 0.0 | 0.0 1.0 | 0.0 06 | 100 | 9±1.51 |
| 13 | BM13 | 0.0 | 0.0 0.0 | 0.2 06 | 100 | 8±1.30 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14 | BM14 | 0.0 | 0.0 | 0.0 | 0.4 | 06 | 100 | 7±1.51 |
| 15 | BM16 | 0.0 | 0.0 | 0.0 | 1.0 | 06 | 100 | 6±0.83 |

***Table 1:****Effect of different concentrations of PGRs on callus induction, shoot proliferation, elongation and rooting from nodal explants of Enydrafluctuans in vitro.*

**Fig I:***in-vitro* propagation of *Enydrafluctuans*

***In vitro*** **rooting and acclimatization:** Well-developed multiple shoots of *E. fluctuans were* subcultured into the new fresh medium at different concentrations of IBA and IAA. After completely developing into plants, they were taken out of the culture jars and grown in a coco pit and soil mixture inside a greenhouse at 28±2ºC temperature and 65% relative humidity. After 15 days, the plants were taken out and successfully grown in the field.

**RAPD Assay:** The *in vitro* propagated plants may cause numerous changes in the phenotype as well as the genomic content of the plant as compared to the mother plant. The detection of these variants are important after propagation. Molecular markers can be a useful technique for detection of somaclones. The RAPD assay is a useful tool for analysis and detection of changes in DNA and repair, which may be directly linked to the survivability and improvement of specific species or organisms. This method can also be used to detect changes in the genome induced by chemicals (Atienzar, F. A., & Jha, A. N., 2004). In this RAPD assay, nine distinct random primers (OPC 01, OPC 02, OPC 03, OPC 04, OPC 05, OPC 06, OPC 07, OPC 08, and OPC 09) were used for the detection of the somaclones in the micropropagated plants. Out of the nine RAPD primers, two (OPC 05 and OPC 07) showed somaclonal variation (Fig. II). The wild plant formed four distinct bands with OPC 05 primers, while the micropropagated plant formed only 3; with OPC 07 primers, the wild plant formed 2 distinct DNA bands, while the micropropagated plant formed 3 bands (Table 2). From the experiment, it can be concluded that the micropropagated plant genome underwent variation in the process of *in vitro* propagation.

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

***Table 2****: Table showing number of amplified bands using RAPD assay in wild and micropropagated E. fluctuans.*

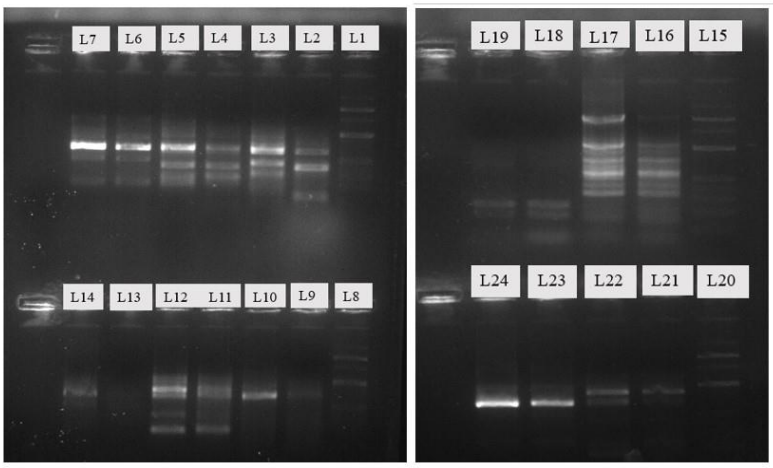


**Fig II**: RAPD assay; Lane 1,8,15&20: 100 bp marker; lane 2&3: OPC 01; lane 4&5: OPC 02; lane 6&7: OPC03; lane 9&10: OPC 04; lane 11&12: OPC05; lane 13&14: OPC06; lane 16&17: OPC07; lane 18&19: OPC08; lane 21&22: OPC09; lane 23&24: OPC10.

**Comparative *in-vitro* antioxidant tests of wild and tissue cultured extract of *E. fluctuans:*** After successful standardisation of the *in vitro* propagation method for *E. fluctuans*, 1 mg/mL of the plant extracts were prepared in 70% methanol (for both wild and tissue-cultured plants) for the following tests using a UV-vis spectrophotometer.

**Total Phenol Content:**The total phenol content of the extracts of *E. fluctuans*was determined using the FCR method (21) and determined using the regression curve ($y = 2.21x + 0.0856$, $R^2 = 0.994$) and expressed as mg of gallic acid equivalents (GAE). In present study, the total phenolic content of wild and tissue cultured plant extracts of *E. fluctuans*was varied; the wild extract showed 54.7 ±5.3 mg, and the tissue cultured extract showed 61 ±3.4 mg GAE/g powder weight.

**Total flavonoid content:** The total flavonoid content in *E. fluctuans*extract was determined by the regression curve ($y = 1.446x - 0.0728$, $R^2 = 0.9966$) expressed as mg of quercetin equivalents (QE). The total flavonoid content was 24±7.7 mg QE/g in the tissue cultured extract and 20±7 mg/g in the wild extract of *E. fluctuans.*

**Total antioxidant capacity:** The total antioxidant capacity of *E. fluctuans*extract was determined using the ammonium phosphomolybdate method (4). The total antioxidant capacity was measured using regression curve ($y = 1.7089x - 0.0556$; $R2 = 0.9922$)and found to be 114.7±7.2 mg ascorbic acid equivalent (AEE/g) of dried extracts in tissue cultured extract and 94±7.2 mg AEE/g in wild extract.

**Comparative quantitative HPLC analysis for the detection of gallic acid and quercetin:** The estimation method for gallic acid and quercetin was successfully validated and standardised for *E. fluctuans.* To standardise HPLC protocol for *E. fluctuans* plant extract, the mobile phase was tuned. The system was stabilized before the injection of the sample to bring the mobile phase into equilibrium. 30% (v/v) HPLC-grade water and 70% (v/v) methanol were found effective mobile phases for the separation of quercetin and gallic acid at a flow rate of 1 mL/min using the Waters RP-18 column. Satisfactory results of quercetin and gallic acid in wild and micropropagated *E. fluctuans*were obtained using this technique. Standard curves and chromatograms of gallic acid and quercetin were drawn from the data obtained in the extracts of *E. fluctuans*, and the data are tabulated below in the **table 3.** Gallic acid and quercetin had retention times of 2.4 and 4.42 min, respectively. According to the findings of the experiment, the gallic acid contents of tissue-cultured methanolic extracts of *L. pusilla* were comparatively higher than the wild plant, and the quercetin content of the tissue-cultured extract was comparatively lower than that of the wild plant. **(Table 3).** 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.

| **Extracts** | **Peak name** | **RT** | **Area** | **% Area** | **Height** | **Concentration in µg/10mg of dried extract(Mean +SE)** |
|---|---|---|---|---|---|---|
| Tissue cultured *E. fluctuans* | Gallic Acid | 2.463 | 21375374 | 67.41 | 2936952 | 101.934±1.18 |
| Wild *E. fluctuans* | Gallic Acid | 2.389 | 16900835 | 69.58 | 2334686 | 80.59±0.65 |
| Tissue cultured *E. fluctuans* | Quercetin | 4.498 | 1754045 | 1.47 | 80402 | 51.292±1.08 |
| Wild *E. fluctuans* | Quercetin | 4.491 | 690760 | 0.91 | 52157 | 20.199±1.09 |

***Table 3****: Accuracy of Gallic acid and Quercetin content in E. fluctuans extract using HPLC*

**Comparative GC-MS compound analysis in tissue cultured and wild extracts of L. pusilla:** The bioactive compounds were plant extracts of tissue cultured and wild *E. fluctuans* plants studied using GC-MS analysis. The GC-MS chromatogram of the aqueous methanolic extract of *L. pusilla* is shown in Figure III (A&B). The compound search was carried out by virtue of 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 in Table 4. The tissue cultured extracts carried many important bioactive compounds, like L-Norvaline, Metoprolol (used to treat high blood pressure patients), L-Glutamic Acid, Adenine, and 9-(2,3-dideoxy-.beta. D-glycero-pent-2-enofuranosyl) (an anti-HIV drug). Again, the wild extract of *E. fluctuans*also carries important bioactive compounds like Z,z-6,28-heptatriactontadien-2-one and hentriacontane, which have anti-inflammatory, anti-tumour, and antimicrobial properties.

| A:: Compounds in the Tissue cultured *E. fluctuans* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sl no | Compound name | Retention time (RT) | Area (%) | Reverse search index | Forward search index | Molecular weight (MW) | Molecular Formula | Importance/remarks |
| 1 | L-Norvaline | 20.008 | 1.379 | 620 | 556 | 245 | $C_5H_{11}NO_2$ | It has a role of bacterial metabolite, a hypoglycemic agent and a neuro protective agent. |
| 2 | Metoprolol | 20.008 | 1.379 | 608 | 415 | 411 | C15H25NO3 | 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. |
| 3 | 2-Propenoic Acid | 21.149 | 0.400 | 637 | 489 | 282 | $C_3H_4O_2$ | Used as cross-linking agent pharmaceutical industry |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 4 | Ethyl Nipecotate | 21.149 | 0.400 | 585 | 517 | 157 | C8H15NO2 | Ethyl nipecotate is an enantiopure cholinergic drug that has been shown to have antinociceptive and anti-inflammatory properties. |
| 5 | Ritalinic Acid | 25.330 | 0.754 | 725 | 515 | 291 | C13H17NO2 | The drug commonly prescribed to children for treatment of attention-deficit hyperactivity disorder is the ester dl-methylphenidate (Ritalin) |
| 6 | L-Glutamic Acid | 25.330 | 0.754 | 691 | 562 | 147 | C5H9NO4 | Help treat epilepsy and muscular dystrophy. . Treat low blood sugar (hypoglycemia) in people with diabetes. |
| 7 | Adenine, 9-(2,3-dideoxy-beta.-D-glycero-pent-2-enofuranosyl) | 32.543 | 1.215 | 664 | 357 | 233 | C10H11N5O2 | Anti-HIV activity |
| **B:: Compounds in wild *E. fluctuans*** | | | | | | | | |
| **Sl no** | **Compound name** | **Retention time (RT)** | **Area (%)** | **Reverse search index** | **Forward search index** | **Molecular weight (MW)** | **Molecular Formula** | **Importance/remarks** |
| 1 | Hentriacontane | 32.233 | 12.363 | 953 | 880 | 436 | CH3(CH2)29CH3 | anti-inflammatory, antitumor and antimicrobial activities |
| 2 | Z,z-6,28-heptatriacontadien-2-one | 29.857 | 0.221 | 804 | 620 | 530 | C37H70O | Vasodilator, larvicidal effects on A. aegypti and A. stephensi |
| 3 | 2-Azetidinecarboxylic Acid | 16.322 | 0.483 | 736 | 634 | 101.104 | C4H7NO2 | Teratogenic agent, causes the production of abnormal proteins with impaired biological activity |
| 4 | L-Norvaline | 20.008 | 1.379 | 620 | 556 | 245 | $C_5H_{11}NO_2$ | It has a role of bacterial metabolite, a hypoglycemic agent and a neuro protective agent. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 5 | Ritalinic Acid | 25.330 | 0.754 | 725 | 515 | 291 | C13H17NO2 | The drug commonly prescribed to children for treatment of attention-deficit hyperactivity disorder is the ester dl-methylphenidate (Ritalin) |

***Table 4::****Bioactive compounds found in aqueous methanolic extract of E. fluctuans A: wild plant B: Tissue cultured plant*

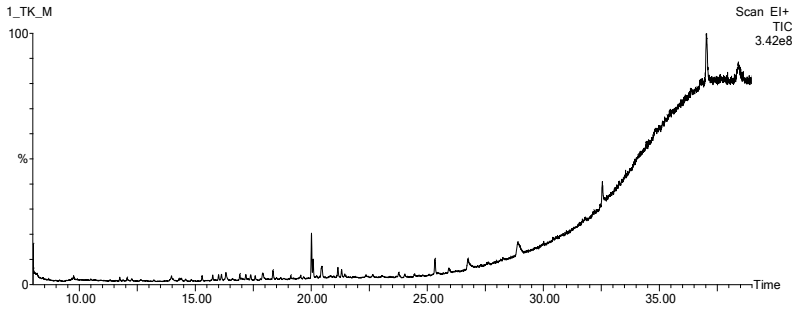


**Fig.III(A):** GC-MS spectrum of tissue cultured *E. fluctuans*plant in methanol extract

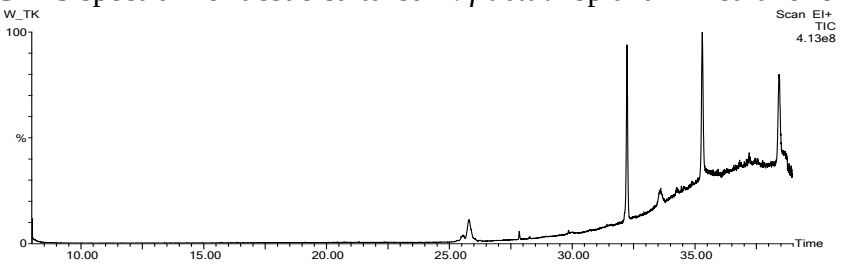


**Fig III(B)**: GC-MS spectrum of wild *E. fluctuans*species in methanol extract.

**Conclusion:** In the experiment, an efficient *in vitro* mass propagation protocol has been developed for *E. fluctuans* species. Using this technique large-scale propagation of *E. fluctuans* is possible in MS media supplemented with MS medium with 2 mg/L BAP (BM7). Also, the RAPD study revealed that *E. fluctuans*propagated in plants by tissue culture underwent somaclonal variation. From the study of comparative anti-oxidant properties of wild and tissue cultured *L. pusilla*, it was observed that the tissue cultured extracts carried higher concentrations of phenolics, flavonoids, and anti-oxidant properties than in the tissue cultured extracts of *E. fluctuans.* Chromatographic studies for the determination of gallic acid and quercetin in the wild and tissue cultured extracts showed a higher concentration of gallic acid and quercetin in the tissue cultured extracts than the wild extracts. The GC-MS study revealed some important bioactive compounds in the plant extract, like adenine and 9-(2,3-dideoxy-beta). D-glycero-pent-2-enofuranosyl) used against HIV; metoprolol used to treat high blood pressure in tissue cultured extract; and hentriacontane compounds having properties of anti-inflammatory, anti-tumour, and antimicrobial activities found in wild extracts of *E. fluctuans.*

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