CHAPTER- III

MATERIALS AND METHODS

The species *Cynanchum tunicatum* Retz. (Alston) is a rare medicinal plant, yet there is no research study reported till date. The entire study was endorsed in four phases.

Phase I – *In vitro* plant regeneration

Phase II – Phytochemical analysis, isolation and characterization

Phase III – *In vitro* biological activity of biomolecules

Phase IV – Molecular docking

In phase I focused on external and internal structure of plant parts. The germination percentage of the seed is very low because it is recalcitrant. Hence, conservation of *Cynanchum tunicatum* through *in vitro* studies were optimized for seed germination through plant tissue culture. Standardization of abiotic factors for the seed culture through Response surface methodology, and optimization of *in vitro* callus induction, somatic embryogenesis, direct, indirect organogenesis and acclimatization. Different PGRs including 2,4 D, IAA, BAP, NAA, TDZ and IBA were used to observe the morphological development in the callogenesis and organogenesis. Histological observations were carried out for callogenesis, somatic embryogenesis and organogenesis. This study helps to observe the cell development, multiplication, and different stages of somatic embryos and biomass of *in vitro* callus was calculated.

3.1. PLANT TISSUE CULTURE

3.1.1. Collection of plant

Fresh and healthy plants of *Cynanchum tunicatum* were collected from Sirumalai forest, Dindigul, Tamil Nadu (N:10º16’45.1; E: 77 º59’55.1). The collected plant saplings are maintained in the herbal garden, at Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India.

3.1.2. Morphological and microscopic observation

Cynanchum tunicatum was observed for morphological and anatomical studies. Transverse sections were obtained by hand sectioning using razorblade, and mounted on glass slides. It was stained with safranin; an image of tissue samples was observed under an electron microscope and micrographs were documented.

3.1.3. Sterilization of Glassware

3.1.3.1. Preparation of Chromic acid

The potassium dichromate (20 gm) dissolved in 200 mL of distilled water and 300 mL of Sulphuric acid, which was gradually added and made up to 1000 mL with distilled water. All the glassware such as amber bottles, beakers, petri dishes, glass rod, glass pipette, Erlenmeyer flasks, test tubes, measuring cylinder, funnels, and tissue culture bottles were immersed in chromic acid for an overnight period. Then the glassware's were washed with running tap water followed by a detergent solution and rinsed with double distilled water. The washed glassware's were dried in a hot air oven (60°C) for 2 hours.

3.1.4. Preparation and Composition of Plant Tissue Culture media

The Murashige & Skoog basal medium was used throughout the entire plant tissue culture study (Murashige & Skoog, 1962). Stock solutions were prepared separately for macronutrients I & II, micronutrients, Fe-EDTA, KI, vitamins and stored in amber bottles at 4°C (Table 1). Plant growth regulators (PGRs) and meso-inositol were prepared freshly at the time of media preparation. The culture media was prepared by required amount of stock solutions, 3% (w/v) sucrose, 0.1% (w/v) meso-inositol were added and makeup with final volume by sterilized double distilled water. The pH of the medium was adjusted to 5.6 using 1N HCl and NaOH. The 0.8% (w/v) agar was used as a gelling agent. The 20 mL of medium was poured into culture vials and autoclaved at 15 Ibs pressure for 15 minutes at 121°C.

Analytical grade chemicals were supplied by Precision, Hi-Media and Sigma chemicals.

Table 1. Chemical composition of Murashige and Skoog (1962) medium

S. No	Component	Stock conc(g)	Dissolved in water (mL)	For 1 litre (mL)
A.	(NH_3) No	16.500	500	50
	$KINo_3$	19.000		
	$CaCl_2.2H_2O$	4.400		
	$MgSo_4 7H_2O$	3.700		
	KH_2PO_4	1.700		
B.	$MnSo_4.4H_2O$	2.230	250	2.5
	$ZnSo_4.7H_2O$	0.860		
	H_3Bo_3	0.620		
C.	$Na_2\ MoO_4.4H_2O$	0.125	500	1.5
	$CuSo_4.5H_2O$	0.012		
	$CoCl_2.6H_2O$	0.012		
D.	KI	0.083	250	2.5
E.	**Iron**		250	5.0
	Na_2 EDTA	1.862		
	$FeSo_4.7H_2O$	1.392		
F.	**Vitamins**		100	1.0
	Thiamine HCl	0.010		
	Pyridoxine HCl	0.050		
	Nicotinic acid	0.050		
	Glycine	0.200		

3.1.5. Preparation of Hormone Stock Solution

Auxins - 2,4-D (2,4-dichloro phenoxy acetic acid), IAA-(Indole -3- acetic acid), IBA (Indole -3- butyric acid), NAA (Naphthalene acetic acid) and Cytokinin BAP (6 – benzylamino-purine), TDZ (Thidiazuron) were prepared and stored at 4°C.

- **2,4-D (2,4-dichlorophenoxyacetic acid)** (M.W: 221.0 g mol^{-1}), 100 mg was dissolved in 1.0 mL of 1N KOH and it was heated, water was added to make a final volume of 100 mL (1.0 mg / mL).

- **IAA (Indole -3- acetic acid)** (M.W:175.2 g mol^{-1}), 100 mg was dissolved in 1.0 mL 1N KOH, warm water was added to make final volume 100 mL (1.0 mg / mL).

- **IBA (Indole-3- butyric acid)** (M.W.203.2 g mol^{-1}), 100 mg was dissolved in 1.0 mL 1N NaOH, warm water was added to make a final volume of 100 mL (1.0 mg / mL)

- **NAA (α-Naphthalene acetic acid)** (M.W:186.2 g mol^{-1}),100 mg was dissolved in 1.0 mL 1N NaOH, water was added to make final volume of 100 mL (1.0 mg / mL).

- **BAP (Benzyl adenine)** (M.W:225.2 g mol^{-1}), 100 mg was dissolved in 1.0 mL of 1N HCl and heated slowly, water as added to make final volume of 100 mL (1.0 mg / mL).

- **TDZ (Thidiazuron)** (M.W:220.25 g mol^{-1}), 100 mg was dissolved in 1.0 mL of 1N NaOH, water was added to make a final volume of 100 mL (1.0 mg / mL).

3.1.6. Sterilization of Seeds

The healthy pods (fruits) were surface sterilized with liquid detergent (Tween 20) under running tap water for 20 minutes and rinsed with sterilized double distilled water. To avoid the interaction of microbes such as bacteria and fungi, the seeds were rinsed with sodium hypochlorite (1.0%, v,v) three times for 10-20 minutes each consecutively rinsed with double distilled water, and 0.1% (w,v) mercuric chloride for 1 – 3 minutes and washed with double distilled water to eliminate the trace elements of mercuric chloride in a laminar chamber.

3.1.7. Inoculation of Seeds

Sterilized seeds of *C. tunicatum* were inoculated on MS basal medium without any plant growth hormones for standardization of seed germination. Five seeds were inoculated in each culture medium. The germination process was observed regularly. The percentage of germination was calculated using the following formula.

Germination percentage (%) = Total number of seeds germinated/ Total number of seeds inoculated

3.1.8. Optimization of Abiotic Factors in Seed Culture through RSM

The analytical procedure of RSM was followed by (Nosratimovafagh et al., 2022) used by Design-Expert software version 13.0.8.0. Using numerical and graphical optimization methods were used to analyze the growth of seed culture-independent variables. The percentage of seed germination was calculated using a traditional graphical method. The experimental design was constructed employing a 3-variable, optimal design with 5 centre points. The three independent variables of abiotic factors were the pH (A), photoperiod (B), sucrose concentration (C) the details of the constituents of each factor and their upper and lower limits were determined from the graphical representation of the analysis of mean values from each level for a particular factor (Table 1).The abiotic factors such as pH (5.1-6.6), photoperiod (9/15)- (24/0), and sucrose concentration (2.3%- 3.8%) were optimized in *in vitro* seed culture using Response Surface Methodology.

3.1.9. Embryo Culture

Embryos were dissected from sterilized seeds under the aseptic condition in the laminar airflow chamber and inoculated on MS basal medium without any plant growth regulators. The cultures were observed regularly, and the germination percentage was calculated using the following formula.

Germination percentage (%) = A total number of embryos germinated/ A total number of embryos inoculated

3.1.10. Callus Induction

The optimization of callogenesis was carried out in four explants such as leaf, node, internode, and root of *C. tunicatum*. Five different PGRs and 24 different combinations were used for optimization of callus induction. The texture of the calli were observed regularly.

3.1.11. Leaf Explant

The leaf explants were acquired from *in vitro* sterile plantlets. The explants were inoculated on MS basal medium enriched with different combinations and concentrations of PGRs such as 2,4 D, IAA, NAA, BAP, and IBA.

3.1.12. Nodal and Internodal Explants

The *in vitro* nodal and internodal explants were obtained from the sterile plantlets used for callus and organogenesis. Explants were inoculated on MS basal medium fortified with different combinations and concentrations of PGRs such as 2,4 D, IAA, NAA, BAP, and IBA.

3.1.13. Root Explant

The *in vitro* root explants were obtained from sterile plantlets and inoculated on MS basal medium fortified with different combinations and concentrations of PGRs such as 2,4 D, IAA, NAA, BAP, and IBA.

3.1.14. Incubation and Culture Condition

The culture vials were maintained at 25± 2°C in incubation chamber. They were placed on the rack at uniform distance of 20 cm. A 16 / 8 h (light/dark) photoperiod of cool white light was provided from 2000 lux fluorescent tubes.

3.1.15. Direct Regeneration (Organogenesis)

In vitro direct regeneration of the nodal explant of *C. tunicatum* was experimented with the help of BAP, 2,4 D and IBA.

3.1.16. Somatic Embryogenesis

Stomatic embryogenesis was observed from the root-derived callus of *C. tunicatum*. Well-grown 1.0 g friable callus was used with different concentrations and combinations of PGRs such as 2,4 D, BAP and TDZ.

3.1.17. Indirect Regeneration (Organogenesis)

In vitro indirect organogenesis was optimized using nine different concentrations and combinations of PGRs such as BAP, TDZ, NAA and IBA.

3.1.18. Acclimatization

The regenerated plants were removed from the cultured bottles and rinsed in sterilized distilled water to remove the medium existing on the roots. They were transferred to hycopots containing vermiculate and organic manure (3:1) and kept in a net house under shade for 15-20 days. The plantlets were transferred to earthen pots and planted in the field. The percentage of survival was recorded after 6 weeks.

3.1.19. Histological analysis

3.1.19.1. Fixation

Histological studies were carried out for different stages of embryogenic calli. Fresh embryogenic calli were fixed in FAA solution (Formaldehyde (5mL), Acetic acid (5 mL), 70 % (v/v) ethanol (63 mL), and made up to 100 mL using distilled water) for one month.

3.1.19.2. Dehydration

The samples were immersed in the solution (xylene or TBA and 70 % (v/v) ethanol) for dehydration. The concentration of the solution increased every 12 hours to remove the excess water content.

3.1.19.3. Infiltration with wax

The sample material was placed in a mixture of xylene or TBA and wax (1:1 ratio) under 45°C in hot air oven for overnight. Repeat this process with wax until the TBA or xylene smell is completely removed from the samples.

3.1.19.4. Embedding

Melt the wax at 60°C, embed it in the wax-coated paper boat, the calli were embedded into the wax and pour a layer of wax on the sample.

3.1.19.5. Staining and microscopic observation

The wax-embedded section (10 μm thick) was cut on a microtome and was stained with 1% (w/v) safranin and 0.05% (w/v) fast green for microscopic observations.

3.1.20. Data collection and statistical analysis

The collected data were analyzed using one-way Analysis of Variance (ANOVA) and the significance of the results was determined statistically using DMRT ($p \leq 0.05$).

3.2. PHYTOCHEMICAL ANALYSIS

The preliminary phytochemicals were screened for the extracts of wild plants and *in vitro* calli. The extracts were taken sequentially, and organic solvents were chosen based on the polarity from hexane, chloroform, ethyl acetate to methanol. This phase involved the identification of metabolic profiling and secondary metabolite quantification through preliminary analysis and using chromatographic techniques. In GC-MS, methanolic extracts of both field-grown plants and *in vitro* callus obtained many similar bioactive compounds.

3.2.1. Extraction

The wild plant and *in vitro* calli were dried in shade and pulverized. The coarse powder was mixed with solvents in the ratio of 1:4, (w/v) and extracted through soxhlet apparatus and dried by rotary evaporator. The extracts were named as Plant hexane extract (PHE), Plant chloroform extract (PCE), Plant ethyl acetate extract (PEAE), Plant methanolic extract (PME), Callus hexane extract (CHE), Callus chloroform extract (CCE), Callus ethyl acetate extract (CEAE) and Callus methanolic extract (CME) and the yield of the extracts were calculated by the following formula.

The percentage yield of the extract (w/w) = W2/W1*100

W1 = Weight of the plant powder (grams)
W2 = Weight of the crude extract (grams)

3.2.2. Preliminary Qualitative Phytochemical Analysis

The qualitative analysis was carried out of all extracts such as PHE, PCE, PEAE, PME, CHE, CCE, CEAE and CME to detect various phytoconstituents (Harborne, 1998; Raaman, 2006).

3.2.2.1. Alkaloids

Crude extracts (50 mg) were stirred with a few mL of dilute hydrochloric acid and filtrate. The filtrate was tested carefully with different alkaloid reagents as follows:

- **Mayer’s test**

To a few mL of filtrate, a drop or two of Mayer reagents was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

- **Wagner’s test**

To a few drops of filtrate, few drops of Wagner’s reagent was added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

- **Hager’s test**

To a few mL of the filtrate, 1 or 2 mL of Hager’s reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicated the test as positive.

- **Dragendorff’s test**

To a few mL of filtrate, 1 or 2 mL of Dragendorff’s reagent was added. A prominent yellow precipitate indicated the test as positive.

3.2.2.2. Detection of Carbohydrates and Glycosides

The 100 mg of extract was dissolved in 5 mL of methanol and filtrate. The filtrate was subjected to the following tests.

- **Molisch’s test**

To 2 mL of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 mL of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

- **Fehling’s test**

One mL of filtrate was boiled in a water bath with 1 mL each of Fehling solutions I and II. A red precipitate indicated the presence of sugar.

- **Barfoed’s test**

To one mL filtrate, 1 mL of Barfoed’s reagent was added and heated on a boiling water bath for 2 mins, red precipitate indicated the presence of sugar.

- **Benedict test**

To 0.5 mL of filtrate, 0.5 mL of Benedict’s reagent was added. The mixture was heated in a boiling water bath for 2 min. A characteristic-colored precipitate indicated the presence of sugar.

- **Borntrager’s test**

To 2 mL of filtrate hydrolysate, 3 mL of chloroform layer was separated and 10 % *(v/v)* ammonia solution was added to it. The pink color indicated the presence of glycosides.

- **Legal’s test**

The extract (50 mg) was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% (w/v) sodium hydroxide. The formation of pink color indicated the presence of glycosides.

3.2.2.3. Detection of Saponins by Foam test

The 50 mg of extract was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 10 min. A two cm layer of foam indicated the presence of saponins.

3.2.2.4. Detection of Phytosterols

- **Liebermann Burchard's test**

The extract (50 mg) was dissolved in 2 mL of acetic anhydrate. To this, one or two drops of concentrated H_2SO_4 were added slowly along the sides of the test tube. An array of color changes showed the presence of phytosterols.

3.2.2.5. Detection of fixed oils and fats

- **Spot test**

A small quantity of extract was pressed between two filter papers. Oil stains on the paper indicated the presence of fixed oil.

- **Saponification test**

A few drops of 0.5 N alcoholic potassium hydroxide solution were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated in a water bath for 24 hrs. The formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

3.2.2.6. Detection of Phenolic Compounds and Tannins

- **Ferric Chloride**

The 50 mg of extract was dissolved in 5 mL of ferric chloride solution was added. A dark green color indicated the presence of phenolic compounds.

- **Gelatin test**

The 50 mg of extract was dissolved in 5 mL of methanol and 2 mL of 1 % (w/v) gelatin containing 10 % (w/v) sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

- **Lead acetate test**

The extract (50 mg) was dissolved in methanol and 3 mL of 10% (w/v) lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

- **Alkaline reagent test**

The extract was treated with 10% (v/v) ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

3.2.2.7. Detection of Magnesium and Hydrochloric Acid Reduction

The 50 mg of extract was dissolved in 5 mL of alcohol and a few fragments of magnesium ribbon and concentrated hydrochloric acid (drop-wise) were added. The presence of flavanol and glycosides was inferred by the development of pink to crimson color.

3.2.2.8. Detection of Quinones

To 1 mL of extract, 1 mL of conc. H_2SO_4 was added. The formulation of red color indicated the presence of quinones.

3.2.2.9. Detection of Cardiac Glycosides

To 0.5 mL of extract, 2 mL of glacial acetic acid and a few drops of 5% ferric chloride were added. This was under-layered with 1 mL of conc. H_2SO_4. The formation of a brown ring at the interface indicated the presence of cardiac glycosides.

3.2.2.10. Detection of Terpenoids (Salkowski test)

To 0.5 mL of extract, 2 mL of chloroform was added, and conc. H_2SO_4 is added carefully. The formation of a red-brown ring at the interface indicated the presence of terpenoids.

3.2.2.11. Detection of Coumarins

To 1 mL of extract, 1 mL of 10% (w/v) NaOH was added. The formation of yellow color indicated the presence of coumarines.

3.2.2.12. Detection of Phlobatannins

To 1 mL of plant extract, a few drops of 2% (v/v) HCl was added, the appearance of red color precipitate indicated the presence of phlobatannins.

3.2.2.13. Detection of Steroids

To 1 mL of plant extract equal volume of chloroform is added and subjected to a few drops of the concentrated sulfuric acid, the appearance of a brown ring indicated the presence of steroids.

3.2.2.14. Detection of Anthraquinones (Bontrager Test)

To 1 mL of plant extract, a few drops of 10% (v/v) ammonia solution were added, the appearance of a pink color precipitate indicated the presence of anthraquinones.

3.2.2.15. Detection of Flavonoids (Shinoda Test)

To 2 mL of plant extract, 1 mL of 2N sodium hydroxide was added. The presence of yellow color indicated the presence of flavonoids.

3.2.2.16. Detection of Protein (Xanthoproteic test)

One mL of the extract was taken and placed into a test tube. Then few drops of nitric acid were added and shaken. The emergence of yellow color indicated the presence of protein.

3.2.3. Quantitative analysis

The PHE, PCE, PEAE, PME, CHE, CCE, CEAE and CME of *C. tunicatum* were subjected to quantitative analysis of alkaloid, flavonoid, phenol and terpenoid.

3.2.3.1. Estimation of Total Alkaloid Content

Prepare a different concentration of atropine standard and plant samples (0.2, 0.4, 0.6, 0.8, and 1mL) and transfer it into separating funnels. Add 5 mL of phosphate buffer (pH 4.7) and 5 mL of bromocresol green solution to the separating funnel and mix well. Then separated with chloroform in parts (1 + 2 + 3 + 4 = 10 mL), it was collected in volumetric flask. The absorbance was recorded at 470 nm in UV spectrophotometer. The amount of alkaloid content was calculated from the calibration curve of atropine and the result was expressed as µg atropine equivalent per mg plant extract (Ajanal et al., 2012).

3.2.3.2. Estimation of Total Flavonoid Content

Aluminum chloride colorimetric method is used to estimate the total flavonoid content. Each extract was diluted with methanol (0.5 mL) followed by adding 10% (w/v) aluminium chloride (0.1 mL), potassium acetate (0.1 mL), and distilled water (2.8 mL). The reaction mixture is then kept for 30 min at room temperature and the optical density was measured at 415 nm. The value was represented as µg rutin equivalent (µg/RUT) per mg plant extract (Slinkard & Singleton, 1977).

3.2.3.3. Determination of Total Phenolic Content

Total phenolics were determined using Slinkard & Singleton method. Extracts (1mL) were mixed with diluted FC reagent (1.0 mL) and incubated for 5 mins at room temperature. Two percent sodium carbonate (3.0 mL) was added to the mixture and allowed to stand for 2 hrs at dark. The optical density was measured at 760 nm. Total phenolic content of the extracts was expressed in gallic acid equivalent (µg/GAE) per mg (Lu et al., 2011).

3.2.3.4. Determination of Total Terpenoid Content

The total terpenoid content was determined using colorimetry assay. Vanillin-glacial acetic acid solution (5%, v/v) was mixed with 1 mL of extract followed by the addition of perchloric acid (500 µL). The reaction mixture was incubated in water bath for 45 mins at 60°C. Add 2.25 mL of glacial acetic acid to the mixture and absorbance was measured at 548 nm. Terpenoid content was calculated as µg linalool equivalent per mg plant extract (Evans, 2009; Indumathi et al., 2014).

3.2.4. FTIR analysis

FTIR (Shimadzu Miracle 10) is used to identify functional groups with range of 400-4000 cm^{-1} and it's a resolution of 16 cm^{-1} in plant extracts and secondary metabolites**.** Spectral interpretation was used to analyse the functional groups or biomolecule characterization using all four crude extracts of *C. tunicatum.*

3.2.5. GC-MS analysis

GC-MS (Perkin Elmer Clarus 680 GC) analysis of plant and callus methanol extracts from *C. tunicatum* were determined. A filtered, diluted crude extract (1 µL) was injected into injector. Mass spectra were recorded across a scan range of 40–550 amu and the composition of each constituent was expressed as a percentage of the peak area. Based on retention time, the interpretation of the mass-spectra of GC-MS results were performed by NIST library database, which includes more than 62,000 reference patterns. Spectra of unknown components were aligned with the NIST library to determine the names, molecular weights, formula and structures of the compounds in the extracts.

3.2.6. HPTLC

3.2.6.1. Equipment

The HPTLC analysis was carried out using CAMAG system which include LINOMAT 5 applicator with syringe, scanner, WINCATS software for data analysis.

3.2.6.2. Solvents and Chemicals

All chemicals and solvents were obtained from analytical grade.

3.2.6.3. Sample Preparation

Methanol extract of *C. tunicatum* were dried, 3 g of dried powder was dissolved in 100 mL of grade methanol and filtered. The mixture was filter and used for HPTLC study.

3.2.6.4. Chromatographic parameter

The analysis was determined using precoated silica gel TLC plate without prior modification. The sample (100 mg/mL) was loaded in a development chamber with the following mobile phase composition.

- For Colchicine: Ethyl acetate: Methanol: Water (20:3:2)
- For Rutin : Ethyl acetate: methanol: formic acid: water (20:3:1:2)

The scanning was performed and visualized under white light, short UV (254 nm), long UV (366 nm). The bioactive compounds indicated as a dark spot against a bright background due to fluorescence light. The WINCATS software is used to detect the retention factor values, resolution and spectral data (Bhargava et al., 2021).

3.2.6.5. Column Chromatography

It is widely used method to isolate and purify the phytoconstituents from mixture of compounds present in plant extracts of *C. tunicatum.* It contained glass column measured with 50 mm in diameter and 50 cm in height with stop cock at bottom to facilitate gradual elution. Silica or alumina is used, and mixture of solvents is used for mobile phase (Ayafor et al., 2024).

3.2.6.6. Column packing

Silica gel was utilized as an adsorbent and it is mixed with hexane to create slurry. The slurry is carefully poured into the column and rinsed to ensure all the adsorbent is evenly packed. A flat surface at the bottom of the column is crucial for optimal separation. Excess solvents were drained.

3.2.6.7. Addition of Sample

After preparing the column, the solvent is drained until it covers the adsorbent layer. Then plant sample were added. The crude extract was dissolved in low eluting solvents. After the sample is loaded, again the solvents were drained until it reached adsorbent. Any residue on the column wall was rinsed using a small amount of solvent.

3.2.6.8. Elution

The sample were eluted using a gradient of solvent with increasing polarity followed by sample addition. It begins with solvent of low polarity such as hexane and gradually increased to polar solvent such as chloroform, ethyl acetate and methanol in specific mixture.

3.2.6.9. Collection of Fractions

The compounds being separated in different colours, their movement through the column can be visually monitored and fractions contained individual compounds were collected.

3.2.6.10. Fractions

The PME (3 g) of *C. tunicatum* were analysed in column chromatography using a series of solvents with increasing polarity. The sample was loaded onto the silica gel packed column in a minimum volume of 100% hexane, then eluted sequentially with the following solvent systems:

- Hexane = 100 mL
- Hexane: Chloroform = 80: 20 mL
- Hexane: Chloroform= 50: 50 mL
- Hexane: Chloroform = 20:80 mL
- Chloroform = 100 mL
- Chloroform: Ethyl acetate = 80: 20 mL

- Chloroform: Ethyl acetate = 50: 50 mL
- Chloroform: Ethyl acetate = 20: 80 mL
- Ethyl acetate = 100 mL
- Ethyl acetate: Methanol = 80: 20 mL
- Ethyl acetate: Methanol = 50: 50 mL
- Ethyl acetate: Methanol = 20: 80 mL
- Methanol = 100 mL

3.2.6.11. Thin Layer Chromatography

TLC plates (Merck) were performed using a fraction of methanol extract of *C. tunicatum.* Different solvent system was used to separate the compound (Raaman, 2006). UV chamber was used to detect the band and R_f value were calculated.

$$R_f = \text{Distance travelled by the compound}/\text{Distance travelled by solvent front}$$

3.2.6.12. Characterization

The separated compounds were subjected to various analytical techniques for characterization. UV (Labman UV-visible double beam spectrophotometer), FTIR (Shimadzu) and GC-MS/MS (Perkin Elmer Clarus 680 GC) were used to determine their structural, chemical and functional properties of isolated compounds.

3.3. BIOLOGICAL ACTIVITIES

The comparative analysis of biological assays of PEAE, PME, CEAE and CME were carried out in Phase III. Bioactive compounds are responsible for biological activity such as anti-microbial, anti-oxidant, anti-inflammatory, and anti-cancer activities.

3.3.1. Antimicrobial activity

- **Microorganisms and culture conditions**

The microbes were obtained from the Microbial type Culture Collection (MTCC), Chandigarh, India. The bacteria such as *Salmonella enterica* [MTCC- 3858] (Gram-positive bacteria), *Enterococcus faecalis* [MTCC - 439] (Gram-positive bacteria), *Escherichia coli* [MTCC - 433] (Gram-negative bacteria), and fungi such as *Candida albicans* [MTCC-183] and

Aspergillus niger [MTCC - 281] were used for antimicrobial activity. All the cultures were sub-cultured on Müeller-Hinton medium used for bacterial growth whereas potato dextrose agar medium for fungal culture at regular intervals and stored at 4°C for further studies.

3.3.1.1. Inoculum preparation

The mother culture (*Salmonella enterica, Escherichia coli* and *Enterococcus faecalis*) is maintained at 4°C and the culture is taken with a loop and culture were poured into a tube contained 5 mL of Müeller-Hinton broth. The liquid culture is maintained at 35°C until it attained turbidity (usually 2 to 6 hours).

3.3.2. Test for antifungal activity

Fungus (*Aspergillus niger* and *Candida albicans*) were cultured on PDA medium maintained at 25°C for 5 days. The plates were uniformly streaked with swab with suspension and maintained at 30°C for 7 days.

3.3.3. Agar well diffusion method

Using sterile glass spreader 150 µL of culture were spread uniformly and the nutrient agar plate. A 10 mm sterile cork-borer was utilized to make 6 uniform wells after 24 hours on all culture plates. The plant and callus extracts of *C. tunicatum* were diluted with DMSO and added to the wells according to their concentrations (25 – 100 µg/mL). Ampicillin (positive) and DMSO (negative) were used as a control. The inhibition zone was measured by mm in diameter (Janssen et al., 1987; Magaldi et al., 2004).

3.3.4. Determination of MIC

The standard serial dilution method was used to evaluate the antimicrobial activity of ethyl acetate and methanol of both plant and callus extracts in nutrient agar broth. The two-fold serial dilution of different extracts in various concentrations (50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL and 1.56 µg/mL) were analyzed against three bacteria such as *Escherichia coli, Salmonella enterica, Enterococcus faecalis,* and two fungi include *Aspergillus niger* and *Candida albicans*. The nutrient broth without extract was considered as a control which is incubated for a day. The MIC defined as the lowest suspension concentration with no visible growth and values was determined by turbidity before and after incubation (Sen & Batra, 2012).

3.3.1.5. Determination of MBC & MFC

After the determination of MIC of different extracts, 50 μL aliquots from all concentrations were streaked on agar plates and incubated for 24 hrs at 37ºC. When 99.9% of the bacterial population is killed at the lowest concentration of an antimicrobial agent, it is termed as MBC and MFC endpoint. This was carried out by observing pre- and post-incubated agar plates for the presence or absence of bacteria (Sen & Batra, 2012).

3.3.2. Determination of Antioxidant Activity

The antioxidant activity of various crude extracts such as PEAE, PME, CEAE and CME of *C. tunicatum* were evaluated using the DPPH assay (Hatano et al., 1988), FRAP assay (Benzie & Strain, 1996), ABTS (Shah & Modi, 2015) and TAA (Prieto et al., 1999).

3.3.2.1. Evaluation of DPPH Assay

The DPPH is used as reagent to determine the radicle scavenging activity of *C. tunicatum*. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The antioxidants can reduce the stable radical DPPH to the yellow-colored diphenyl picrylhydrazine. The DPPH free radical scavenging activity was determined by (Hatano et al., 1988). Different extracts with various concentrations (20, 40, 60, 80, and 100 μg/mL) of *C. tunicatum* were mixed with 2.7 mL of 0.1 mM methanolic solution of DPPH. The mixture was shaken vigorously and left to stand in the dark for 30 min at room temperature. Then, absorbance was measured at 517 nm. The control was taken without a sample with DPPH. The percentage inhibition was calculated as follows:

Radical scavenging activity (%) = [(Control absorbance – Sample absorbance)/Control absorbance] *100

The half maximum inhibition concentration (IC_{50}) value was calculated using linear regression plots.

3.3.2.2. Evaluation of FRAP assay

The FRAP (Benzie and Strain method) was measured using plant and callus extract of *C. tunicatum*. Different concentrations (20, 40, 60, 80, 100 μg/mL) of extracts was added to 3 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM 2,4,6-Tripyridyl-S-Triazine solution and 1 part of 20 mM $FeCl_3$ $6H_2O$ solution) and the reaction mixture was incubated in a water bath at 37°C for 30 minutes. Gallic acid was used as a standard. The absorbance was measured at 593 nm (Benzie & Strain, 1996).

FRAP Value = [(Sample absorbance – Sample blank)/(Sample control – Sample blank)]*2

3.3.2.3. Evaluation of ABTS assay

The stock solution ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) contained (Shah & Modi, 2015) aqueous solution (7 mM) with 2.45 mM of potassium persulfate (Merck, India) in equal amount. The mixture was maintained at room temperature in a dark for 12 hours before use. The working solution were prepared by 2.0 mL of ABTS●+ solution and 1 mL of extract at various concentrations (20, 40, 60, 80, 100 µg/mL). It was maintained in dark condition at room temperature for 10 minutes. The 2 mL of stock along with distilled water were used as a control and ascorbic acid were employed as a standard. The absorbance was determined at 734 nm.

Radical scavenging activity (%) = [(Ao-As)/ Ao] × 100

Where *Ao* is the absorption of control and *As* is the absorption of the tested extract solution.

3.3.2.4. Evaluation of Total Antioxidant Activity

The phosphomolybdenum method (Prieto et al., 1999) is a commonly used technique to assess the total antioxidant activity of *C. tunicatum* extracts. Its principle involved the reduction of Mo (VI) to Mo (V) by the antioxidants present in the sample. This reduction leads to the formation of a green-coloured phosphate/Mo (V) complex, which is then measured using spectrophotometry at 695 nm wavelength. The total antioxidant capacity of different extracts of *C. tunicatum* was evaluated by the phosphomolybdenum method. An aliquot of 0.5 mL of ethyl acetate and methanolic extract of both plant and callus with various concentrations (20, 40, 60, 80, 100 µg/mL) was mixed with 4.5 mL of phosphomolybdenum reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Ascorbic acid was used as a standard. The mixtures were incubated in a water bath at 95°C for 90 min and the absorbance was measured.

Antioxidant effect (%) = [(Control absorbance – Sample absorbance)/Control absorbance] *100

3.3.3. Evaluation of Anti-inflammatory Activity

The anti-inflammatory activity of ethyl acetate and methanol extracts (both plant and callus) of *C. tunicatum* was evaluated by bovine serum albumin denaturation assay (Chandra et al., 2012). The 5 mL reaction mixture consists of 0.2 mL of bovine serum albumin, 2.8 mL of phosphate-buffered saline (pH 6.4), and 2 mL of various concentrations (20, 40, 60, 80, 100 µg/mL) of plant extracts. The mixtures were then incubated at (37±2)°C in a BOD incubator for 15 min, followed by heating at 70°C for 5 min. The absorbance was measured at 660 nm. Diclofenac sodium was used as a standard. The inhibition percentage of protein denaturation was calculated by

Percentage inhibition (%) = [Sample absorbance/(Control absorbance - 1)] × 100

3.3.4. Determination of Anticancer Activity

3.3.4.1. Materials and Reagents

Minimal Essential Medium (MEM), 96-well microplates, 0.45 microns filter, MTT, Tissue culture flasks 25 cm^2, TPVG, 1X PBS, Hepes buffer (1M) and Ethidium bromide staining.

3.3.4.2. Preparation of Ingredients

- **Penicillin and Streptomycin Solution**

Crystalline penicillin G (1 X 106 units) and streptomycin sulphate (1 g) were dissolved in 100 mL of PBS. This solution was sterilized by filtration and stored at -20°C. From this stock solution, 1 mL was added to 100 mL of medium to obtain a final concentration of 100 units of penicillin and 100 µg of streptomycin per mL.

- **Kanamycin sulphate solution**

Kanamycin (1 mg) was mixed in 50 mL of double distilled water to obtain a final concentration of 20 mcg/ mL and stored at -20°C.

- **Fungizone solution**

Amphotericin B (50 mg) was dissolved in 5 mL of double distilled water. From this stock solution, 1 mL was diluted to 50 mL of double distilled water to obtain a final concentration of 20 mcg/ mL and stored at -20°C.

- **L-Glutamine solution (3%)**

L-Glutamine (6 g) was mixed in 200 mL of double distilled water and filtered through a 0.22-micron membrane filter and stored at - 20°C.

- **Fetal bovine serum**

Fetal bovine serum was thawed at room temperature and inactivated at 56°C in water bath for 30 min and cooled to room temperature. Floating particles were filtered through Seitz filter and stored at -20°C.

- **Sodium bicarbonate solution (7.5%)**

Sodium bicarbonate (15 g) was mixed in 200 mL of double distilled water, filtered through Whatmann filtered paper No. 4 and stored at -4°C.

- **TPVG**

Phosphate buffered saline NaCl - 8 g, KCl - 0.2 g, Na_2HPO_4 - 2.88 g, KH_2PO_4 - 0.2 g (pH - 7.4). All the above ingredients were dissolved in 1000 mL of double distilled water and filtered through Whatmann filter paper No. 4 and autoclaved at 15 lbs for 15 min.

- **EDTA (Versene 0.2%)**

EDTA (200 mg) was mixed in 100 mL of double distilled water and autoclaved at 15 lbs for 15min.

- **Glucose A.R. 10mL (10%)**

Glucose (1g) was dissolved in 10 mL of distilled water and filtered through Whatman filter paper and autoclaved at 10 lbs for 15 min.

- **TPVG – 1000 mL**

PBS - 840 mL, 2% Trypsin - 50 mL, 0.2% EDTA - 100 mL, 10% Glucose - 5 mL, P&S - 5 mL. All the ingredients were mixed, and pH was adjusted to 7.4 and stored at – 20°C.

- **Phenol Red (0.4%)**

Phenol red (0.04 g) was dissolved in 10 mL of double distilled water. Sterilized by autoclave at 15 lbs, 121°C, for 15 min.

- **Trypan Blue (0.1%)**

Trypan Blue (0.1 g) was dissolved in 100 mL of phosphate-buffered saline, filtered through Whatman filter paper No. 4 and stored at -4°C.

3.3.4.3. Preparation of Media for Cell Culture

The media preparation for cell culture is represented in Table 2. All the ingredients were mixed well by gentle shaking, pH was calibrated and adjusted to 7.2 to 7.4. HCT-116 cell line was cultured in open-vented 75 cm^2 culture vessels in a standard horizontal laminar flow hood and incubated in a CO_2 incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. HCT-116 cell lines were provided with growth media of 90% MEM media (Minimal Essential Medium Eagle) (ATCC, VA) and 10% fetal bovine serum (Gibco, CA) as well as 5% streptomycin - penicillin (Sigma-Aldrich). Media were refreshed at least three times a week (15 mL) under aseptic techniques. HCT-116 cell lines were sub-cultured when 80% confluency was reached. Sub-culture consists of dispensing old media with a vacuum and the addition of 5 mL of 0.25% Trypsin, 0.03% EDTA solution (Gibco) to the remaining cell layer followed by incubation for 6 minutes at 37°C. TPVG was removed and the bottle was incubated at 37°C until all the cells detach from the surface. The cells were resuspended in 5 mL of growth media. The suspensor was aspirated a few times to break cell clumps. The cell concentration was determined by counting the cells in the hemocytometer.

3.3.4.4. Sample preparation

To prepare a stock solution, 5 mg of ethyl acetate and methanol of plant and callus extract was dissolved in 0.5 mL of 2% MEM. From this working solution with various concentrations (10-100 μg/mL) were prepared 96 well plates for cytotoxic study.

Table 2. Preparation of animal cell culture media

Ingredients	Growth medium (10%)	Growth medium (5%)	Maintenance medium (2%)
MEM	870 mL	920 mL	95 mL
P&S (penicillin & streptomycin solution	1 mL	1 mL	1 mL
Phenol red (0.4 %) solution	1 mL	1 mL	1 mL
Kanamycin solution	1 mL	1 mL	1 mL
Fungizone solution	1 mL	1 mL	1 mL
L-glutamine solution (3%)	1 mL	1 mL	1 mL
Fetal Bovine serum	100 mL	50 mL	20 mL
Sodium bicarbonate solution (75%)	20 mL	20 mL	20 mL
Hepes buffer	5 mL	5 mL	5 mL
Total volume	**1000 mL**	**1000 mL**	**1000 mL**

3.3.4.5. MTT method

MTT is a calorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can occur in metabolically active cells the level of activity is a measure of the viability of the cells.

This method is used to quantitatively detect living cells. In brief approximately 2×10^4 cells / well were seeded onto 96 well plates, 100 µL of MEM medium was added and incubated at 37°C for24 hours. Then, the medium was discarded and fresh medium was added of extract and compounds. The setup was incubated for 1-3 hours at 37°C in a CO_2 incubator. Fresh medium was added with 10 µL of MTT (5 kg/mL) after 2 hours of incubation, the medium was discarded and 100 µL of DMSO was added to dissolve the formazan crystals. Then, the absorbance was noted using a spectrophotometer at 570 nm.

$$\textit{Viable cells (\%)} = \textit{Test OD/ Control OD} \times 100$$

3.3.5. Statistical analysis

All samples were analyzed in triplicates. Data are presented as mean ± standard error mean. Differences were evaluated by one-way analysis of variance (ANOVA) test completed by DMRT. Differences were considered significant at $p \leq 0.005$.

3.3.6. Brine Shrimp Assay

The lethality assay was determined using ethyl acetate and methanol of plant callus extract. The different samples were taken in varying volumes of 100µL, 250µL, 500µL, 1000µL, and 1500µL. The concentration of the original samples was determined before the dilution process. The final concentration of the stock solution was predetermined based on the experimental requirements and the desired concentration range for the saline solution. The dilution factor for the sample was calculated by dividing the final volume of the stock solution by the initial volume of the respective sample. The final volume of the stock solution was obtained by adding the volume of the sample to volume of distilled water used for dilution. Following accurate measurements of the appropriate volumes, the dilution process was carried out, and each stock solution was thoroughly mixed to ensure homogeneity. Subsequently, aliquots of the prepared stock solutions were transferred to individual beakers, each containing saline solution. These beakers were then ready for further analysis using the respective stock solution concentrations (Meyer et al., 1982).

3.3.7. MOLECULAR DOCKING

The metabolites present in the extracts are responsible for the biological activity and this was confirmed through the *in-silico* studies which were evaluated in Phase IV.

3.3.7.1. Preparation of Protein Structure

The crystal structure of the HCT-116 Cell line (PDB ID: 6 GUE, resolution 1.9 Å) was obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). The enzyme was 3D protonated and hydrogen atoms were added in their standard positions. The system was then optimized, and partial charges were computed. Co-crystallized water molecules were removed, and the binding pocket was isolated and defined.

3.3.7.2. Preparation of Ligand Structure

Some of the compounds (GCMS) retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) for 3D structure. Optimization of ligand will be docked into distinguished model using Ligand Fit theory. Structure files will be converted to the required format by using Open Babel.

3.3.7.3. *In silico* Docking analysis

Docking studies were performed using Glide, a Schrödinger software. Before the docking experiment, all the co-crystallized ligands were removed from the respective protein structure. The ligands such as 5-(Hydrox ymethyl)-2-Furaldehyde, 4H-Pyran-4-one, Megastigmatrienone, Furfural, Phenol were docked against protein 6 GUE (HCT-116 Cell line). Inhibitors were minimized using the ligand prep module of Schrödinger. The ligands were docked in extra precision (XP) mode and intra-molecular hydrogen bonds were rewarded while calculating the docking score.