SPECIMEN FORMAT FOR THESES OF MONTH

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Department	**:**	Botany
Branch/ Area:	**:**	Plant tissue culture and Biological activities
Sub Subject Heading:	**:**	Medicinal plants
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Title of the thesis	**:**	Optimization of *in vitro* Micropropagation, Characterization of Bioactive Compounds and Biological activities of *Cynanchum tunicatum* (Retz.) Alston – A Rare Medicinal Plant
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Abstract within 300 words:

Cynanchum tunicatum (Retz.) Alston is a rare medicinal plant known for its therapeutic potential, has significant attention due to its biologically active compounds with various pharmacological properties. In this preent study, macroscopic charectic feature and internl structure of leaf, stem and root were analysed. Optimization of sterilization techniques used for to overcome the low germination percentage. Hence, optimiztion enhanced the *in vitro* seed germination of *C. tunicatum* with different concentration of sodium hypochlorite ($NaHCl_3$) and mercuric chloride ($HgCl_2$). Abiotic factors were also standardized using Response Surface Methodology (RSM). The seeds of *C. tunicatum* were inoculated on MS medium absence of PGRs. With the help of abiotic factors, the maximum germination rate (96%) was achieved. *In vitro* explants such as leaves, nodes, internodes and root were produced friable and organogenic calli, these calli were used for mico-propagation. Somatic embryos were obtained and different stages were analysed with the help of microtome sectioning. The *in vitro* seed germination and micropropagation studies have been proven capable of promoting disease free plants.

This study explored the phytochemical profiling, biological activities, and potential applications of *C. tunicatum* extracts in pharmaceutical and biotechnological fields. Qualitative and quantitative phytochemical analysis revealed the presence of alkaloids, flavonoids, terpenoids and phenolic compounds in both wild plant and *in vitro* calli, which contribute to its antioxidant, anti-microbial, anti-inflammatory and anti-cancer properties. Molecular docking revealed the discovery of new drug from *C. tunicatum*. Bioactive compounds were obtained from GCMS analysis of *C. tunicatum* associated with the primary drug pathway for human colorectal cancer. The highest affinity score was achieved between chromone and 6GUE protein responsible for cancer. The findings underscored the potential of *C. tunicatum* as a promising candidate for future drug formulation for cancer.

i) Major objectives :

- To optimize *in vitro* propagation for *Cynanchum tunicatum* (Retz.) Alston
- To evaluate the phytochemical profiling of *Cynanchum tunicatum* (Retz.) Alston
- Comparative studies of biological activities of wild plant and *in vitro* callus extracts of *Cynanchum tunicatum* (Retz.) Alston

- To analyze the *in silico studies* of bioactive compounds of *Cynanchum tunicatum* (Retz.) Alston

ii) Hypothesis:

It is hypothesized that *in vitro* cultures of *Cynanchum tunicatum* (Retz.) Alston can serve as a sustainable source of bioactive compounds with comparable or enhanced biological activities to those of the wild plant.

iii) Methodology:

Phase I

Morphological and histological examination was conducted

Optimization of Abiotic Factors in Seed Culture through RSM by (Nosratimovafagh et al., 2022). 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.

The callus was induced with 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. 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.

The culture vials were incubated at 25± 2℃ 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.

In vitro direct regeneration of the nodal explant of *C. tunicatum* was experimented with the help of BAP, 2,4 D and IBA. *In vitro* indirect organogenesis was optimized using nine different concentrations and combinations of PGRs such as BAP, TDZ, NAA and IBA.

The regenerated plants were acclimatized in 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.

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. The samples were immersed in the solution (xylene or TBA and 70 % (v/v) ethanol) for dehydration. 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.

Phase II

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).

The quantification of secondary metabolites was carried out for alkaloids, flavonoid, phenol and terpenoid for eight extracts. Estimation of Total Alkaloid Content: Ajanal et al. (2012). Estimation of Total Flavonoid Content: Slinkard & Singleton (1977). Estimation of Total Phenolic Content: Lu et al. (2011). Determination of Total Terpenoid Content: Evans 2009; Indumathi et al. (2014).

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.*

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. 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.

The HPTLC 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).

The column chromatography was examined using plant methanolic extract (PME). It is widely used method to isolate and purify the phytoconstituents from mixture of compounds present in plant extracts of *C. tunicatum* (Ayafor et al., 2024). 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. 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.

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 respective solvents. 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).

The eluted fraction were characterized by 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.

Phase III

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. The antimicrobial activities were evaluated using *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].

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. In 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. 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).

The determination of MIC using 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 were analyzed against three bacteria such as *Escherichia coli, Salmonella enterica, Enterococcus faecalis*, and two fungi include *Aspergillus niger* and *Candida albicans*. 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).

The 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).

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).

The evaluation of anti-inflammatory activity using 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 determination of Anticancer Activity using ethyl acetate and methanol extracts (both plant and callus) of *C. tunicatum* by MTT assay by mitochondrial succinate dehydrogenase. 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℃ 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℃ in a CO_2 incubator.

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 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 (Meyer et al., 1982).

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 $p \leq 0.005$.

Phase IV

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/). 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.

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-(Hydroxymethyl)-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 ere docked in extra precision (XP) mode and intra-molecular hydrogen bonds were rewarded while calculating the docking score.

iv) Findings:

Phase I

An ethnobotanical survey was investigated in the Sirumalai forest, Dindigul district, Tamil Nadu. It revealed that *Cynanchum tunicatum* was used as folk medicine such as antifebrile, antitumor, diuretic, anodyne, tonic, and effective against chronic hepatitis by

indigenous people. It sheds light on the traditional medicinal uses of *C. tunicatum* in the Sirumalai forest region.

Cynanchum tunicatum, commonly known as Dog Strangling Vine or Milkweed. Macroscopic characteristics of *C. tunicatum* observed the perennial nature, twinning growth pattern, and distinctive morphological features such as heart-shaped leaves, white latex secretion, and pedunculate flowers with umbels. The detailed anatomical observation of *C. tunicatum* were examined microscopically including the cell size, shape, arrangement, distribution, types of crystal, and starch distribution. The significance of microscopic observation in unraveling the intricacies of plant anatomy.

The surface sterilization of *C. tunicatum* is used to remove the contaminants from explants. The optimization of sterilization techniques for *in vitro* seed germination of *C. tunicatum* with different concentration of sodium hypochlorite ($NaHCl_3$) and mercuric chloride ($HgCl_2$). The maximum germination rate (96.6%) was achieved when $NaHCl_3$ rinsed for 20 minutes and $HgCl_2$ for 3 minutes. The optimization of *in vitro* seed germination of *C. tunicatum* with various abiotic factors were investigated using Response Surface Methodology (RSM). The seeds of *C. tunicatum* were inoculated on MS medium absence of PGRs.

The *in vitro* zygotic embryos were germinated at 15 days post-inoculation and plantlets were achieved by 50^{th} day which results in 98% of germination rate. The successful embryo culture of *C. tunicatum* highlighted the efficacy of promoting germination of plantlets. It facilitated the advancement of embryo culture and hold promise for conservation and propagation of this species.

The optimization of callus induction from *C. tunicatum* leaf explants through different concentration and combinations of PGRs (IAA, 2,4D, NAA, IBA and BAP) on MS medium. most successful combination, yields the highest percentage of 89% at 2,4 D (2 mg L^{-1}) + IAA (2 mg L^{-1}). The maximum percentage of nodal explants reached 85% in the combination of 2,4 D (2.0 mg L^{-1}) + IAA (2.0 mg L^{-1}), showed the excellent growth rates. The maximum callus induction (90%) at IAA (2.0 mg L^{-1}) + 2,4 D (2.0 mg L^{-1}) on MS media. The maximum callus induction of root (94%) was achieved at 2,4 D (2.0 mg L^{-1}) + IAA (2.0 mg L^{-1}), closely followed by 2,4 D (2.0 mg $L^{-1)}$ + IAA (1.0 mg L^{-1}) stimulated 89.43 %. The explants of *C. tunicatum* exhibited maximum callus development under the optimized protocol, offering various advanced study in biotechnological applications.

In vitro C. tunicatum nodal explants were cultured on MS basal medium with BAP (1mgL^{-1}) and 2,4-D (1.5 mg L^{-1}). It leads to the proliferation of caulogenesis within three weeks of culture. From the shoot, rhizogenesis were induced using IBA (2 mg L^{-1}) of *C. tunicatum.*Cell morphology transitioned from isodiametric to ovate shapes. Globular, heart shape, and cotyledonary embryos were observed.

The shoots were transferred to rooting media containing various concentration of plant growth regulators (NAA, BAP, TDZ and IBA). The maximum shoot proliferation (93.3%) was attained on MS medium with BAP (0.5 mg L^{-1}) + TDZ (2 mg L^{-1}). The maximum rhizogenesis (97.6%) was reached on MS medium supplemented with IBA (2 mg L^{-1}). It suggested an established method for the mass propagation of *C. tunicatum.*

In vitro propagated plantlets of *C. tunicatum* were aseptically excised from the culture medium and removed to pots contained a pre-sterilized substrate composed of soil and organic manure in 3:1 ratio. After transplantation, the plantlets were achieved 100% survival rates under *ex vitro* conditions. Histological analysis of *C. tunicatum* revealed distinct stages of embryogenesis and organogenesis. Compact meristematic cells formed somatic embryos which leads to the development of globular, heart with suspensor-like structures, torpedo and cotyledonary shaped somatic embryos. The anatomical features of caulogenesis and rhizogenesis were observed.

The plant powder and callus powder were obtained from *C. tunicatum* and subjected to organoleptic study. The highest extraction yield obtained in CME and PME showed 4.26% and 4.86% respectively. The other extracts such as PHE, PCE, PEAE, CHE, CCE, and CEAE showed 1.22%, 1.43%, 2.97%, 1.32%, 1.62%, 3.54%, and 4.86% respectively.PEAE, PME, CEAE and CME revealed abundant phytoconstituents such as alkaloids, carbohydrates, glycosides, phenols, terpenoids, quinines, and phyto steroids. The amount of alkaloid of *C. tunicatum* was quantified in various extracts such as PHE, PCE, PEAE, PME, CHE, CCE, CEAE and CME. The maximum alkaloid was observed in the PME (1.37±0.03 µg/mL) and CME (1.074 µg/mL) of *C. tunicatum.*

C. tunicatum extracts were quantified flavonoid content in various extracts of using aluminum chloride method. The PME (3.58±0.02 µg/mL), and CME (3.36±0.05 µg/mL) showed the maximum number of flavonoids. The number of phenolic compounds of *C. tunicatum* were quantified, in which PME and CME exhibited the maximum value of 2.32±0.037 µg/mL and 3.80±0.08 µg/mL, respectively. Terpenoid content of *C. tunicatum*

extracts was analyzed using FC method. Among all extracts, PME exhibited highest terpenoid content at 1.60±0.02 µg/mL, followed by PEAE at 1.57±0.14 µg/mL. The significant amount of terpenoid observed in PME and PEAE extracts asserted that these extracts were rich sources of terpenoids from *C. tunicatum*.

The FTIR analysis of PME and CME from *C. tunicatum* were analysed to identify different functional groups including carboxylic acid, alkane, and aromatic alkenes. A rich chemical profile with potential pharmacological properties were determined. The FTIR analysis exposed the diverse chemical composition of *C. tunicatum*, spanning alkane to aromatic alkenes. GC-MS analysis of PME and CME from *C. tunicatum* were evaluated. Based on these analysis, 42 compounds were identified from different groups. Major compounds such as Beta-Amyrin (10.614%), 1-Hexacosene (0.145%), 1,3-Dioxolane (0.382%), Colchicine (8.129%), Furfural (1.73%), 4H-Pyran-4-one, n-Hexadecanoic acid (13.76%), Phthalic acid (4.69%), Rutin (4.54%), 9-Octadecenoic acid (Z)- (2.76%), 5-Cholestene-3-ol (5.243), Bis(2-ethylhexyl) phthalate (3.42%), 4H-1-Benzopyran-4-one (2.82%), 3-Butoxypropylamine (6.29%), 1,5-Pentandiol (7.89%), Acetoxyacetic acid (1.88%), Furazane (1.14%), Piperazine (4.35%) and 2,5-Furandione (2.92%). The biological activities of these compounds from *C. tunicatum* were elucidated their therapeutic potential and contributed for novel (cancer) drug development.

HPTLC profile of PME and CME of *C. tunicatum* were evaluated, to quantify the alkaloid and flavonoid compounds. The Colchicine and Rutin were quantified using mobile phases of Ethyl acetate: Methanol: Water (20:3:2), and Ethyl acetate: methanol: formic acid: water (20:3:1:2) respectively on HPTLC plates. The colchicine was observed as 0.12 µg and 0.033 µg while rutin was calculated as 0.211 µg and 0.12 µg in 1 µL of PME and CME respectively.

The PME of *C. tunicatum* were evaluated by column chromatography to isolate and characterize the bioactive compounds. As a result, 80 fractions were collected based on various solvent system. The chloroform and ethyl acetate fraction exhibited a single band in TLC plate with Retention factor of 0.7. The isolated fraction was further subjected to characterization using UV, FTIR and GC-MS analysis. The UV analysis showed a peak at 290 nm and the peak represented an organic compound. FTIR analysis exhibited various peaks such as 1735 cm^{-1}, 1242 cm^{-1}, and 1041 cm^{-1} which characterize the functional group of carboxyl, amine and sulfoxide. GC-MS spectra identified the isolated fraction as 1,3

Benzene dicarboxylic compound, with its molecular formula is $C_8H_4O_3$ and its molecular weight is 148.11 g/mol.

The antibacterial assay of PME, PEAE, CEAE and CME from *C. tunicatum* was assessed using agar well-diffusion method against *Salmonella enterica* and *Enterococcus faecalis* and *Escherichia coli*. CME exhibited a maximum inhibition zone (32.5±1.8 mm) against *Salmonella enterica* at a concentration of 100 µL. These results evidenced for the therapeutic relevance of *C. tunicatum* in combating bacterial infections.

The anti-fungal assay of PME, PEAE, CEAE and CME from *C. tunicatum* was investigated against *Aspergillus niger* and *Candida albicans*. The highest inhibition zone of 15.3±0.9 mm was observed at a concentration of 100 µL against *Aspergillus niger*, followed by a maximum inhibition zone of 13.7±1.4 mm at the same concentration against *Candida albicans* in CME. The MIC value of CME against *S. enterica* was 6.25 µg/mL and PEAE against *Aspergillus niger* was 6.25 µg/mL. All extracts of *C. tunicatum* against *Escherichia coli*, *Enterococcus faecalis* and *Salmonella enterica* at 25 µg/mL showed minimum inhibitory concentration. On the other hand, the PEAE showed MBC at the concentration of 50 µg/mL against *Enterococcus faecalis.* The PEAE of MFC at 12.5 µg/mL against *Candida albicans.*

The antioxidant activity of PEAE, PME, CEAE and CME of *C. tunicatum* were evaluated using DPPH method. PME exhibited the highest inhibition percentage 86.82% (IC_{50}= 53.1 µg/mL), followed by CME showed 84.08% (IC_{50} =52.2 µg/mL).

The highest FRAP values were observed at a concentration of 100 µg/mL, as an inference of CME (1.6 µg/mL) and PME (1.27 µg/mL) respectively. The percentage scavenging activity in ABTS assay of all four extracts showed CME (89.69%), followed by CEAE (65.5%), PME (57.07%), PEAE (56.42%) and ascorbic acid showed 51.39% exhibited at 100 µg/mL. The IC_{50} values of PEAE, PME, CEAE, CME and ascorbic acid showed 99.58, 72.99, 75.73, 60.24 and 51.39 µg/mL respectively. The TAA assay activity was achieved at 100 µg/mL, CME exhibited 83.68%, followed by PME at 81.83%. The IC_{50} values of PME, CME showed 47.11 µg/mL, 53.38 µg/mL respectively. These results indicated the significance of *C. tunicatum* in natural antioxidant properties, identification of key bioactive compounds which leads to cure cancer.

The extracts such as PEAE, PME, CEAE and CME of *C. tunicatum* were evaluated by bovine serum albumin denaturation assay. The CME showed maximum inhibition percentage 310.74% and PME showed 85.71, with corresponding IC_{50} values of 59.23 µg/mL and 57.03 µg/mL. The methanolic extracts of *C. tunicatum* from both plant and callus extracts demonstrated the highest inhibition concentration against protein denaturation.

The cell viability and cytotoxicity of PEAE, PME, CEAE and CME of *C. tunicatum* were evaluated using MTT (Methylthiazolyldiphenyl-tetrazolium bromide) against the HCT-116 at concentrations ranging 1.56 to 50 µg/mL. The maximum percentage of cytotoxicity of CEAE was observed as 88 % at the concentrations of 50 µg/mL. Followed by, CME exhibited 84 % of cytotoxicity. The IC_{50} value for CEAE and CME was determined to be 17.81 µg/mL and 16.71 µg/mL respectively. When concentration increases, the inhibition percentage of cell viability decreases. It demonstrated the potential cytotoxic effects of CME against HCT-116 cells, indicated its utility as an anti-cancer agent.

The Lethality Assay of PEAE, PME, CEAE and CME of *C. tunicatum* was assessed using Brine shrimp. The lowest toxicity was observed in the CME, percentage mortality was calculated after 24 hours observed as 53%. The percentage showed as 17, 20, 27, 40 and 53% at the concentration of 100, 250, 500, 1000, 1500 µg/mL respectively.

The protein structure (PDB ID: 6GUE) of the human cyclin-dependent kinase 2 enzyme were docked with multiple compounds/ ligands. The binding score of ligands such as Chromone (−7.62 Kcal/mol), 5-(Hydroxymethyl)-2-Furaldehyde (-6.231 Kcal/mol), 4H-Pyran-4-one (-6.09 Kcal/mol), Megastigmatrienone (-5.905 Kcal/mol), Furfural (-5.859 Kcal/mol) and Phenol (-5.686 Kcal/mol). These findings highlighted *C. tunicatum* as a promising candidate for future cancer drug development.

V) Examiners

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