CHAPTER - V

SUMMARY AND CONCLUSION

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. Additionally, it described the pods were simple, dehiscent dry follicle releasing seeds through a suture. The macroscopic examination of *C. tunicatum* offered a significant perspective on its taxonomy and ecology. It enhanced our knowledge of this species and contributed to its conservation. Further studies could investigate into its physiological adaptations and interactions within its ecosystem.

Microscopic observation played a pivotal role in plant anatomy, serving as a bridge between traditional plant sciences and modern disciplines. It provided essential insights into plant meristems, cell differentiation, and tissue dynamics, forming the foundation for developmental biology. Different magnification lens (10x, 40x and 100x) were used for microscopic analysis. 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. Sterilization technique is crucial in plant tissue culture to prevent contamination and ensure successful *in vitro* seed germination. The methodology developed using sequential treatments with sodium hypochlorite and mercuric chloride, offers an optimized approach for sterilizing seeds of *C. tunicatum*. The standardized protocols paved a way for further experiment, application in plant biotechnology and conservation efforts.

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. With the help of abiotic factors, the maximum germination rate (96%) was achieved. It highlighted their significant impact on seed germination percentage. The optimal ranges of these parameters on MS medium without PGRs showed the maximum percentage. It contributed to the sustainable utilization of *C. tunicatum* by enabling efficient propagation techniques that support conservation efforts, large-scale cultivation, potential use in pharmaceutical, ornamental, and ecological applications.

Embryo culture was analysed using the standardized protocol of abiotic factors from seed culture of *C. tunicatum*. Mature embryos were isolated from seedlings and inoculated on MS basal medium without plant growth hormone. 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. It results in dedifferentiation of cells and the formation of friable calli within six weeks. The most successful combination, yields the highest percentage of 89% at 2,4 D (2 mg L^{-1}) + IAA (2 mg L^{-1}). The successful callus formation of emphasized the importance of hormonal balance, particularly the synergistic effect observed with IAA and 2,4-D.

The *C. tunicatum* nodal explants of were cultured on MS basal medium using various plant growth regulators which results in both callogenesis, and organogenesis. Nodal explants were inoculated on BAP leads to the formation of single or multiple shoots, while IAA, IBA, NAA, and 2,4-D combination development of friable calli. 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. Additionally, other growth hormonal combinations consistently stimulated callus induction. The callogenesis of nodal explants using different PGRs exposed complexity hormonal interactions of *C. tunicatum*.

The *in vitro* internodal explants of *C. tunicatum* demonstrated callus proliferation within two weeks of inoculation. The maximum callus induction (90%) at IAA (2.0 mg L^{-1}) + 2,4 D (2.0 mg L^{-1}) on MS media. Various concentration of BAP on MS media led to the formation of

light brown compact calli, while other PGRs including IAA, 2,4 D, NAA and BAP induced friable calli. The rapid callus proliferation observed in internodal explants exhibited the potential of specific hormonal combinations of *C. tunicatum*.

The optimized protocol for *in vitro* callus induction was applied to all explants, in which root explant demonstrated maximum callogenesis of *C. tunicatum*. Within two weeks of inoculation, explants initiated to form an indistinguishable cell. 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 %. This hormonal combination facilitated early cell proliferation, leading to vigorous callus formation. 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 (1 mg L^{-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.* The established protocol for direct organogenesis offers a valuable approach for efficient shoot and root formation of *C. tunicatum*. The use of PGRs at specific concentration facilitated the direct regeneration and neglecting the process of callogenesis. It revealed the formation of rapid propagation, and conservation of rare medical plant which contributes to the biotechnological utilization.

Somatic embryogenesis of *C. tunicatum* was obtained from root-derived callus on MS medium supplemented with BAP (1.0 mg L^{-1}) + 2,4-D (1.5 mg L^{-1}). Cell morphology transitioned from isodiametric to ovate shapes. Globular, heart shape, and cotyledonary embryos were observed. Successful somatic embryogenesis of *C. tunicatum* offers avenues for mass propagation.

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. This substrate provided ideal nutrient availability required for the pre-

acclimatization. The acclimatized plantlets were transplanted into larger mud pots, which supported further root and shoot development. 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. Primordial cell clumps and intensive cell divisions were observed in embryonic calli. Non-embryogenic calli displayed irregular parenchymatous cells with visible intercellular spaces. 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 histological examination provided the cellular dynamics of embryogenesis of *C. tunicatum*. The observed stages of embryogenesis and organogenesis of *C. tunicatum* underscored the regeneration process.

The plant powder and callus powder were obtained from *C. tunicatum* and subjected to organoleptic study. It characterized by coarse texture, light green colour, light odour, and sweet taste, with a rough feel when touched. It provided fundamental understanding into the physical characteristics of both plant and callus powder, helps to identification and potential medicinal application. Healthy plants and callus powder were sequentially extracted using various organic solvents such as hexane, chloroform, ethyl acetate and methanol. Totally, eight extracts were acquired in both plant and callus extracts. 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.

Preliminary phytochemical screening of eight extracts such as PHE, PCE, PEAE, PME, CHE, CCE, CEAE and CME of *C. tunicatum* were determined. PEAE, PME, CEAE and CME revealed abundant phytoconstituents such as alkaloids, carbohydrates, glycosides, phenols, terpenoids, quinines, and phyto steroids. The preliminary screening exhibited diverse secondary metabolites of *C. tunicatum* extracts, particularly in ethyl acetate and methanol extracts. It supported further investigation into the antimicrobial, antioxidant and anticancer activity of *C. tunicatum*.

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.* Values were expressed in

atropine equivalent per mg of extract. Both methanolic extracts showed maximum alkaloid content compared to other extracts. The potential of alkaloid could be used for further pharmacological properties.

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. Values were expressed in rutin equivalent per mg of extract. The significant flavonoid content in PME and CME extracts of *C. tunicatum* endorsed their potential as a rich source 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. The highest phenolic content was observed in the CME compared to other extracts. Antioxidant property was significantly exhibited by phenolic compounds, it demonstrated the potential health benefits for anti-cancer activity.

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 rich sources of secondary metabolites were observed in PME and CME from *C. tunicatum*. These are known for their diverse pharmacological activities, indicated the potential therapeutic significance of *C. tunicatum* extracts in traditional medicine and pharmaceutical applications.

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. This assay confirmed that this species has a natural anti-fungal agent, which could be explored for the development of novel pharmaceutical therapies.

The MIC were scrutinized using PEAE, PME, CEAE and CME of *C. tunicatum* against five microbes including three bacteria and two fungi. The concentration of 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL of CME showed no turbidity against *Salmonella enterica.* The MIC value of CME against *S. enterica* was 6.25 µg/mL and PEAE against *Aspergillus niger* was 6.25 µg/mL.

The MBC and MFC were examined using PEAE, PME, CEAE and CME of *C. tunicatum* against five microbes. 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 significant antioxidant activity was observed in methanolic extracts of *C. tunicatum*, explored its use in folk medicine and helps to prevent radicle scavenging disorder.

The antioxidant activity of PEAE, PME, CEAE, CME of *C. tunicatum* was evaluated using FRAP assay, which is measured by FRAP. 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 maximum FRAP values were observed in methanolic extract of *C. tunicatum*, stated their ability as potent antioxidants.

The ABTS assay was determined using four extracts such as PEAE, PME, CEAE and CME of *C. tunicatum.* The percentage scavenging activity 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.

TAA assay of four extracts (PEAE, PME, CEAE, and CME) of *C. tunicatum* was evaluated using phosphomolybdate reagent with various concentrations. The highest antioxidant 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. Subsequently, the CEAE exhibited the second lowest mortality rate was observed as 17, 20, 27, 40, 53% at the concentrations of 100, 250, 500, 1000, 1500 µg/mL correspondingly.

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). This findings highlighted *C. tunicatum* as a promising candidate for future cancer drug development