Hybanthus enneaspermus (L.) F. Muell [syn. Ionidium suffrutosum (L.) Ging] is a medicinal herb of the family Violaceae. The species is distributed in the tropical and the subtropical regions of the world and in India, it is found in the warmer parts of the Deccan peninsula. The plant is popularly known as ‘pink ladies slipper’ in English and ‘padmacarini’ in Sanskrit. It has aphrodisiac, demulcent, tonic and diuretic properties and is used against urinary infections, diarrhoea, leucorrhoea, dysuria, inflammation and sterility. *H. enneaspermus* is widely used in Ayurveda, Siddha and other folklore health care practices. In Ayurveda, the botanical identity of ‘padmacarini’ is controversial. Some physicians have considered it as *Nervilia aragoana* or *Habenaria grandiflora*. However, Kirtikar and Basu (1918), Nadkarni (1954) and Chopra (1956) have described ‘padmacarini’ as *H. enneaspermus*. The plant is under threat in its natural habitat due to over exploitation, overgrazing, seasonal habitat, sporadic distribution and poor germination of seeds. The non-availability and controversy in botanical identity may lead to adulteration and decrease the efficacy of the raw drug. Therefore, a detailed authentication of the drug is quite essential which is needed for both pharmaceutical companies as well as the public health care sector and to ensure reproducible quality of herbal medicine. It is envisaged that development of mass propagation techniques and alternate methods for the production of raw drugs/metabolites should definitely improve the threat status of the plant. Previous scientific studies revealed the diverse biological activities of the herb. The species is reported to possess antidiabetic, antiplasmodial, antimicrobial, anticonvulsant, nephroprotective, aphrodisiac, hepatoprotective and anti-inflammatory activities. Considering the prospects of the multifarious properties of the herb, further scientific studies are necessary to exploit the
unknown activities. Hence, in the present study, investigations were conducted initially to authenticate the plant using various pharmacological tools, secondly, to standardise the micropropagation technique for rapid multiplication and production of biomass and finally to evaluate the bioactivities such as antioxidant capacity and anticancer property.

*Hybanthus enneaspermus* which was collected from Thanjavoor, Tamil Nadu and reared in the herbal garden of Navajyothi Sree Karunakara Guru Research Centre for Ayurveda and Siddha, Uzhavoor, Kottayam, Kerala, served as the study material. It is a sub-erect or erect herb with a somewhat woody base and spreading branches. Detailed macro and micro morphological features were studied. The distinguishing microscopic characteristics of the stem includes, the vascular cylinder consisting of 1 or 2 layers of discontinuous patches of perivascular sclerids, 4-7 layered phloem and closed dense cylinder of xylem. Physico-chemical parameters such as moisture content (67.5%w/w), solid content (32.5%w/w) total ash (14.8 %w/w), acid insoluble ash (50 % w/w), water soluble ash (19.1%w/w), water extractive (12% w/w), methanol extractive (8 %w/w), pH of 1% aqueous solution (6.33) and pH of 10% aqueous solution (6.30) were determined. Qualitative phytochemical tests were conducted with extracts obtained from successive soxhlet extraction of the dried, powdered plant samples using petroleum ether, chloroform and methanol. High performance liquid chromatography (HPTLC) profiling of the methanol extract detected the presence of 10 distinct bands ($R_f$: 0.03, 0.12, 0.20, 0.25, 0.37, 0.29, 0.53, 0.69, 0.78 and 0.84). However, compounds with $R_f$ values 0.03 and 0.53 have more intense bands. Results of the bioactivities as such as antioxidant (reducing power) and cytotoxic activities using *in vitro* methods
evolved during the present study can be used in the activity-based standardization of *H. enneaspermus*. The pharmacognostical standards evolved during the present study using macroscopic, microscopic, physicochemical, phytochemical and biological parameters can be utilized for identifying the genuine drug, *H. enneaspermus*.

A more efficient and reproducible *in vitro* direct regeneration protocol using nodal explants was developed in *H. enneaspermus*. Better regeneration rate and easy establishment of aseptic cultures have revealed the suitability of nodal segments in the *in vitro* propagation. Murashige and Skoog (MS) medium supplemented with N\(^6\)-benzyladenine (BA), kinetin (KIN), 2-iminopurine (2-iP), thidiazuron (TDZ), 2,4-dichlorophenoxyacetic acid (2,4-D) and \(\alpha\)-Naphthylacetic acid (NAA) either singly or in combination with BA or 2-iP with NAA or 2, 4-D were tested. Significant variations were found in the *in vitro* shoot induction response with respect to type and concentration of growth regulators. Among the different cytokinins 2-iP showed better shoot induction response, followed by BA, TDZ and KIN. Combination of NAA or 2, 4-D with 2-iP or BA significantly retarded the shoot regeneration response. Shoot regeneration was totally absent when the culture was treated singly with auxins (NAA or 2, 4-D). Instead it produced creamy, friable callus. It was also noted that 0.5 mg L\(^{-1}\) 2, 4-D is more suitable for callus induction. Observations on *in vitro* culture response using four different nutrient formulations such as MS, B\(_5\), Nitsch and Whites revealed that mineral nutrition has a controlling influence on shoot morphogenesis. Use of media like Whites and Nitsch significantly reduces the shoot induction response and the developed shoots were weak with narrow leaves and pale yellow colour. Cultures in full strength MS medium
produced highest shoot number and better shoot growth which revealed the aptness of MS medium for shoot morphogenesis in *H. enneaspermus*. The type and concentration of carbon source also affected the *in vitro* response of *H. enneaspermus*. A combination of glucose and fructose promotes the shoot induction response much better than individual treatments of glucose and fructose or treatment with disaccharide sucrose (contains glucose and fructose monomers). From the *in vitro* culture studies, it is concluded that MS medium containing 1.5% glucose and 1.5% fructose along with 3 mg L\(^{-1}\) 2-ip is the best combination of culture media for optimum multiplication (14.3 shoots after 60 days) of *H. enneaspermus* using nodal explants. Rooting of the regenerated shoots (12 roots per shoot after 30 days) was achieved in half strength MS medium supplemented with 0.5 mg L\(^{-1}\) IBA. The plantlets with well developed roots were hardened in net house showing 93% survival rate. The plants showed normal flowering and fruiting and were morphologically similar to wild plants.

Preliminary phytochemical screening of the extracts taken from dried powdered whole plants of *H. enneaspermus* in petroleum ether, chloroform and methanol were conducted and it was found that most of the constituents were present in methanol extract. Hence, the methanol extract was used for the bioactivity studies. *In vitro* antioxidant activities using various concentrations (5–500 μg/ml) of the methanol extract were tested. Reducing power of an extract is correlated with antioxidant activity and during the study, it was evident that the methanol extract of *H. enneaspermus* possesses potent antioxidant
activity. Hence, further investigations on detailed antioxidant evaluation with different reaction systems were conducted. It is observed that the plant has significant free radical scavenging activity towards DPPH, superoxide, nitric oxide and hydroxyl radicals. The amount of methanol extracts needed for 50% inhibition ($EC_{50}$) of DPPH, superoxide, nitric oxide and hydroxyl radicals were found as 471µg/ml, 66 µg/ml, 86 µg/ml and 93 µg/ml respectively. Based on the results obtained in the present study, it can be concluded that methanol extract of *H. enneaspermus* is a potent source of natural antioxidants which offers various therapeutic applications of the species.

Experiments were conducted to evaluate the cytotoxicity and anticancer properties of the methanol extract of *H. enneaspermus*. It showed significant *in vitro* short term cytotoxicity on DLA and EAC cell lines. 200 µg/ml extract showed 44% and 48% cell death in DLA and EAC cells respectively. During MTT cell viability assay, it was noticed that 10 µg/ml showed 26.18% cytotoxicity. The results revealed that the extract is able to produce metabolic and/or genetic changes in tumour cells which lead to cytotoxicity. Further, *in vivo* antitumour studies using solid tumour and ascites model revealed the antitumour property of the methanol extract of *H. enneaspermus*. A significant reduction in the tumor volume was noticed in drug treated animals. Tumour volume of the control group was 3.034 ± 0.70 mm$^3$ on the 30th day while the 200 mg extract treated group showed only 0.844 ± 0.14 mm$^3$, for the same duration. In ascites tumour model, the life span of extract treated group was found to be significantly increased. The 200 mg extract treated group survived for 20.5 days.
However, the control group survived only for 14.8 days after tumour induction. The result of the present investigation reveals that *H. enneaspermus* possesses antitumour activities and the property may be exploited for the treatment of cancer.

An attempt was also made to compare the antioxidant and cytotoxic potential of the methanol extracts of the field grown plant and *in vitro* grown calli samples of *H. enneaspermus*. EC$_{50}$ values of the samples obtained under *in vitro* stress conditions were lower compared to the normal field plants, showing that *in vitro* samples have more antioxidant potential. During qualitative phytochemical analysis, colour reactions of the samples revealed similar results for both the samples. However, HPTLC profile of the extracts showed variations in the number and intensity (area) of bands. These variations in the chemical constitution might have reflected in the disparities in their bioactivities.

The present study proposes an *in vitro* protocol for the rapid multiplication and techniques for *in vitro* biomass production of *H. enneaspermus*. It unveils the antioxidant potential of the herb and concludes that stress induced calli samples possess more efficacy than field grown samples. This study brings to the fore the anticancer property of the herb and suggests pharmacognostic markers for identifying the genuine drug.