7. SUMMARY AND CONCLUSION

In India number of traditional systems of medicine are practiced since centuries. These include Ayurveda, Sidha, Unani etc. apart from those having ethnobotanical usage of herbs at regional level by the inhabitants or tribes, as medicine. Although these systems possess rich heritage as health care, these were confined to Vaidyas, Hakeems etc. and were not reached to industrial level of manufacture and distribution of different formulation practiced, due to lack of documentation of scientific facts regarding the properties of drugs and medicaments used. Investigation on plants for their biological utility therefore have become important task throughout the world on a scientific basis.

The present study was envisaged to systematically validate two selected medicinal plants named *Hybanthus enneaspermus* and *Cardiospermum halicacabum* used as tonic and nervine tonic in traditional medicine and reported to used as tonic, aphrodisiac, to cure earache, rheumatism etc., but no systematic and detail studies for anti-dementic potential have been reported yet. The present study was therefore, undertaken to explore these two plants in the light of pharmacognostical significance for identity of parts, phytochemical investigation and pharmacological evaluation for antidermic activity.

The aerial parts of both plants were collected in July from New Vallabh Vidyanagar Campus and authenticated by a Taxonomist, Dept. of Biosciences, Sardar Patel University, Vallabh Vidyanagar, India.

The selected plant materials were first evaluated for their correct identity by studying their pharmacognostic features. Various quality control parameters like foreign matter, ash values, extractive values, loss on drying were determined to ensure the quality of raw materials. The coarsely powdered plant materials were then successively extracted with solvents of increasing polarity viz., petroleum ether, benzene, chloroform, methanol and water. The successive extracts were then subjected to preliminary phytochemical test for
the presence of secondary metabolites. A complete thin layer chromatography profile of the secondary metabolites comprising of the $R_f$ values in the extracts were recorded and documented. Isolated compounds from both the plants were analysed and developed validated HPTLC quantification methods for content markers.

In order to derive scientific evidences to the reported traditional claims, antidementic activity was performed for defatted methanolic extracts.

**Hybanthus enneaspermus**

Some important morphological features for the identification of *Hybanthus enneaspermus* stem/leaves/petiole were observed. Leaves were small, dorsiventral, simple, alternate, linear with serrate margin, acute apex, pleasant aromatic smell with slightly sweet taste. Flowers were solitary pink-purple and spade shaped. Fruits were capsular with 5-12 ribbed seeds.

Microscopical evaluation remains indispensable and cost effective tool of conventional analytical Pharmacognosy. So, important microscopic features of stem/leaf/petiole of *Hybanthus enneaspermus* have been documented in the present study.

Microscopic features of stem showed circular with uneven outline and revealed the presence of thick cuticle, compact layer of epidermis, trichomes, distinguished cortex, endodermis, sclerenchyma patches (sclerids), a cambium tissue separating phloem outside and wider secondary xylem and primary narrow xylem cells towards inside. Abundant pith (parenchyma) enclosed the centre.

Leaf was dorsiventral and transcurrent. The T.S. of fresh leaf showed presence of the upper and lower epidermis enclosing the compact mesophyll. The midrib was prominent projecting unequally on adaxial and abaxial side. On adaxial surface mid rib was bluntly conical and on abaxial side it was hemispherical.
The centre of the mid rib was occupied by a well developed collateral vascular bundle. Mesophyll was differentiated in 2-3 layers of palisade and 3-5 layers of spongy tissues. The palisade tissue was transcurrent across the mid rib. Along the margin of lamina unicellular trichomes were present. Anisocytic type of stomata were present on both lower and upper surface of the leaf.

In cross sectional view, the petiole was triangular in outline/spindle shaped with three lateral wings. In the proximal part of petiole three vascular bundles were present. In the distal part of petiole the vascular bundles were fused to form a single ring of xylem and phloem.

Microscopical examination of powder showed the presence of xylem vessels which were lignified associated with fibres in bundles. There were unbranched unicellular trichomes, presence of testa of the seeds, thick and thin walled cork cells.

Fluorescence analysis of the powdered drugs /various extracts was separately carried out under day light as well as UV light according to prescribed method. Various parameters of proximate analysis viz total ash value, acid insoluble ash value, water soluble ash value, alcoholic soluble extractive value and water soluble extractive value were determined.

The preliminary phytochemical screening, evaluation based on ash value, extractive values help to lead identity of plant properly.

The phytochemical qualitative chemical tests gave the idea about the nature of chemical constituents present in the drugs which further helped for detection of specific constituent.

Qualitative tests for different extracts revealed presence of carbohydrates, alkaloids, coumarins and phenolic compounds in methanolic extract. Phytosterol, alkaloids and carbohydrates were present only in some other extracts.
Thus, in continuation of our studies on qualitative analysis of secondary metabolites present in successive extracts of aerial parts of *Hybanthus enneaspermus*, thin layer chromatography (TLC) studies was performed. A complete TLC profile of the successive extracts comprises of Rf values for chemical constituents present in the extracts were recorded and documented.

GC–MS results shown that at least 9 compounds were found to be present in methanolic extract. These were identified as 3-Carene,4-acetyl; 5,8-epoxy-3H-2-benzopyran,4,4a,5,8-tetrahydro-5,8-dim; 4-Methoxy-6-methyl-6,7-dihydro-4h-furo(3,2-C)pyran; Tridecanoic acid, methyl ester; Eicosanoic acid, methyl ester; 2H- Pyran-2-one, tetrahydro; Pthalic acid; Thujapsene and Humulane.

Four phytoconstituents were isolated from unsaponified fraction of *Hybanthus enneaspermus* and their structure elucidation was carried out by applying various modern analytical techniques like UV, IR, TLC, NMR, MS. These isolated phytoconstituents were named as HEI-1, HEI-2, HEI-3 & HEI-4 and their structure was elucidated as 28-hydroxy, 3-oxo friedelane; 29-formyl, 3-hydroxy friedelane; 3β-28 dihydroxy friedelane and 28-hydroxyfriedelane-3-one-29-oic acid respectively. These structures are reported first time in the aerial parts of this plant.

The pharmacological screening was carried out for antidementic activity of defatted methanolic extract of plant (MHE) using different models on animals.

In elevated plus maze apparatus MHE extract (50, 100, 200 mg/kg, p.o.) successfully reversed memory deficits induced by scopolamine by reducing the TL to 46.04, 38.52 & 26.44 sec. respectively in mice. Standard nootropic agent piracetam (200 mg/kg, i.p) more significantly improved memory as is evident by decrease in TL to 20.17 sec. All the results were statistically significant as compared to scopolamine treated group indicating significant improvement in memory on administration of MHE at all dose levels. Highly significant improvement in memory was observed at dose 200mg/kg, p.o. as compared to other.
In passive avoidance test Administration of MHE (50, 100 & 200 mg/kg, p.o.) significantly (p <0.05) reduced the latency to reach SFZ to 28.37, 23.26 & 18.81 sec. respectively. As compared to scopolamine administration of 200mg/kg MHE produced more significant reduction (p<0.01, 18.81sec) in latency to reach SFZ which was comparable with latency of 13.42 sec to reach SFZ induced by standard nootropic agent piracetam (200 mg/kg).

In clonidine induced hypothermia MHE extract (200 mg/kg) did not significantly modify hypothermia. Hence we conclude that MHE extract exhibited nootropic activity without involvement of noradrenergic function.

Mice treated with MHE (100 & 200, mg/kg, p.o.) were able to maintain equilibrium on the rotating rod for more than 180 sec., whereas the animal treated with diazepam exhibited incordination and fall off time was significantly (p<0.05) reduced to 43.62±0.10 sec. The results of these findings revealed that extract of MHE did not have neurotoxic effect.

In brain cholinesterase activity standard drug piracetam (200 mg/kg) significantly (p<0.01) lowered AChE activity to 0.126 µmol/min/mg protein as compared to AchE activity in phenytoin treated group, administration of MHE (50, 100, 200 mg/kg, p.o.) significantly reduced the AchE activity to 0.215, 0.180 & 0.158 µmol/ min/ mg protein, while piracetam reduced to 0.126, indicating counteracting action of these drugs on cholinergic system.

**Cardiospermum halicacabum**

Some important morphological features for the identification of *Cardiospermum halicacabum* aerial parts were observed and documented herein. The leaves were alternate, twice ternately compound. Leaflets beared lobed margin, lanceolate in shape and pubescent with pinnate venation. Flowers were panicles, irregular and pentamerous. Each flower beared four sepals, two large and two small, four whitish petals 4mm long. Fruits were green, balloon like papery capsules with three chambers, 3-4 cm in diameters.
Seeds were black, opaque smooth with a white heart shaped spot at the micropyle.

Microscopally, *Cardiospermum halicacabum* exhibited an outer most layer of epidermis, a band of collenchymatous tissue consisting of 2 to 4 rows of chlorenchymatous tissue lies underneath this, each collenchymatous tissue being located under the ridge, the remaining tissue of cortex being parenchymatous. The stellar tissue consists of secondary xylem ring in connection with primary bundles, pith was parenchymatous, very wide, encircled by small groups of perimedullary phloem placed beneath the protoxylem of primary bundles.

The midrib was broadly hemispherical on the abaxial side with short hump on the adaxial side. Covering unicellular trichomes were present on either sides of midrib. Inverted vascular bundles (i.e., xylem towards the dorsal surface and phloem towards the ventral surface) was the characteristic of the leaflet. Lamina was dorsiventral. Mesophyll was differentiated into 1-2 layers of palisade followed by 3-4 layered spongy parenchyma. Lower epidermis consisted of single layer of rectangular cells rosette crystals of calcium oxalate traversed throughout the parenchymatous tissue.

Transverse section of petiule was polygonal in outline with two winged projections at the upper side. Epidermis was single layered and was composed of horizontally flattened cells. The cells were compactly arranged and showed presence of cuticle. The peripheral layers in the ground tissue were composed of collenchymatous and chlorenchymatous cells. These cells occurred as bands and they alternate with each other thus forming a continuous layer next to epidermis. Vascular bundles were more than five and collateral in ground tissue. The rest of ground tissue was parenchymatous.

Proximate analysis was performed to help setting certain standards for dried drugs in order to avoid batch-to-batch variation and to judge their quality and
purity. Parameters like total ash, acid insoluble ash, water soluble ash, water soluble extractive value and alcohol soluble extractive values and fluorescence analysis were studied under proximate analysis.

Methanol soluble extractive value was higher than any other organic solvent during successive extraction for aerial parts of *Cardiospermum halicacabum* which indicated the presence of polar chemical constituents.

Physicochemical analysis provided an idea regarding the probable content of various inorganic metal ions as well as the nature of the constituents present. Total ash value (5 %) indicated the presence of silicates and oxalates present in drug.

The colour of the plant extract in UV light is mainly due to its chemical composition. The same extract may appear in different colours at wavelength of light (366 nm) and these colors are characteristic for the particular drug or different parts of same drug. Therefore, fluorescence studies were done on powdered aerial parts of *Cardiospermum halicacabum*.

Successive extracts were prepared by subjecting coarsely powdered plant materials to the solvents of increasing polarity viz., petroleum ether, benzene, chloroform, ethyl acetate, methanol and water. The successive extracts were subjected to various qualitative chemical tests to determine the presence of various phytoconstituents.

Qualitative tests for different extracts of *Cardiospermum halicacabum* showed maximum constituents were positive for methanolic extracts such as carbohydrates, alkaloids, proteins flavanoids and phenolic compounds. Saponins, phytosterols and fixed oil were present only in some extracts.

Thus, in continuation of our studies on qualitative analysis of secondary metabolites present in successive extracts of aerial parts of *Cardiospermum halicacabum*, thin layer chromatography (TLC) studies was performed. A
complete TLC profile of the successive extracts comprises of $R_f$ values for chemical constituents present in the extracts were recorded and documented.

Three compounds CHI-1, CHI-2 and CHI-3 were isolated by column chromatography from flavanoid rich fraction of aerial parts *Cardiospermum halicacabum*. These isolated compounds were then subjected to determination of various physical characteristic like melting point, UV, FT-IR, $^{13}$C NMR and Mass spectra to elucidate the chemical structure. Compounds CHI-1, CHI-2 and CHI-3 were identified as naringenin, dihydroquercetin and quercetin respectively and reported first time in the aerial parts of this plant.

Apigenin and luteolin are marker constituent of *Cardiospermum halicacabum* were quantified in aerial parts by validated HPTLC method. Toluene: ethyl acetate: formic acid (10:9:1) was used as mobile phase. Apigenin and luteolin content were found to be 0.45% & 0.50% w/w respectively. $R_f$ values, quantitative parameters and system suitability parameters were also recorded. This method was found to be accurate, precise, specific and repeatable. This was the first time quantification of apigenin and luteolin in *cardiospermum halicacabum* using mobile phase toluene: ethyl acetate: formic acid (10:9:1) was carried out.

In EPM model administration of MCH extract (50, 100 & 200 mg/kg, p.o.) showed significant (p<0.05) reduction in TL on 9$^{th}$ day to 42.05, 31.10 and 22.32 respectively when compared with TL of 60.57 sec. in scopolamine treated group, suggesting improvement in memory.

In Passive avoidance model MCH (50,100 & 200 mg/kg, p.o.) administered for 8 days, significantly (p<0.05) reversed the amnesia induced by scopolamine and improved memory as evidenced by dose dependent decrease in latency to reach SFZ to 25.72, 20.50 and 15.97 sec. respectively.

In clonidine induced hypothermia MCH (200 mg/kg, p.o.) did not potentiate clonidine induced hypothermia. Infact at 60 minutes MCH (200 mg/kg, p.o.)
increase the rectal temperature of rats and the difference was found to be statistically insignificant (p>0.05).

In neurotoxicity test MCH extract (100 & 200, mg/kg, p.o.) were able to maintain equilibrium on the rotating rod or more than 180 sec., whereas the animal treated with diazepam exhibited incoordination and fall off time was significantly (p<0.05) reduced to 29.83±1.5 sec. The results of these findings revealed that MCH did not have neurotoxic effect.

In brain acetyl cholinesterase activity on administration of MCH extract (50, 100 and 200 mg/kg, p.o) treated groups showed significant reduction of acetylcholinesterase activity to 0.206, 0.179, 0.134 µmol/min/mg protein respectively in brain indicating the counteracting actions of these drugs on the cholinergic system. Standard nootropic agent piracetam (200mg/kg, i.p.) also reduced AchE activity to 0.126 µmol/min/mg protein. Thus MCH acts as a potential anticholinesterase agent similar to piracetam.

CONCLUSION

The selected plants Hybanthus enneaspermus and Cardiospermum halicacabum were successfully evaluated for their correct identity by studying their pharmacognostic features.

Parameters like, ash value, extractive value and loss on drying were determined, for assessing their quality standards.

GC–MS results shown that at least 10 compounds were found to be present in methanolic extract and were identified as 3-Carene,4-acetyl; 5,8-epoxy-3H-2-benzopyran,4,4a,5,8-tetrahydro-5,8-dim; 4-Methoxy-6-methyl-6,7-dihydro-4h-furo(3,2-C)pyran; Tridecanoic acid, methyl ester; Eicosanoic acid, methyl ester; 2H- Pyran-2-one, tetrahydro; Pthalic acid; Thujapsene & Humulane.

Four compounds MHI-1, MHI-2, MHI-3 & MHI-4 were elucidated as 28-hydroxy, 3-oxo friedelane; 29-formyl, 3-hydroxy friedelane; 3β-28 dihydroxy
friedelane and 28-hydroxyfriedelane-3-one-29-oic acid respectively and to be reported first time in aerial parts of *Hybanthus enneaspermus*.

Validated HPTLC method was developed for quantification of apigenin and luteolin first time in aerial parts of *Cardiospermum halicacabum*.

The chemical structure of three flavanoid compound CHI-1, CHI-2 & CHI-3 were elucidated as naringenin, dihydroquercetin and quercetin respectively and to be reported first time in aerial parts of *Cardiospermum halicacabum*.

Pharmacological studies of methanolic extract of aerial parts of *Hybanthus enneaspermus* and *Cardiospermum halicacabum* possess good potential for antidementic activity. These results could be attributable to the presence of flavanoids and phenolic compounds present in defatted methanolic extract of *Hybanthus enneaspermus* and *Cardiospermum halicacabum*. This has spurred research, including epidemiologic studies, on potential role of flavonoids found in the daily diet, in preventive therapy for cancers and neurological degeneracy as Parkinson's and Alzheimer's disease.209,210

Cognitive dysfunction has been shown to be associated with impaired cholinergic function, and the facilities of central cholinergic activity are related to memory improvement. Cholinergic hypothesis postulates that low synaptic levels of acetylcholine resulting from loss of cholinergic neurons in the nucleus basalis magnocellularis leads to cognitive decline. Inhibition of acetylcholinesterase, the key enzyme in breakdown of acetylcholine is considered as promising strategy for the treatement of neurological disorders.211,212

Plants traditionally used in Ayurvedic medicine to boost mental ability in old age have been found to have the same action as conventional drugs used in the treatment of Alzhemier’s disease. Immunohistochemical studies suggested the existence of chronic inflammation in certain regions of brain in pathogenesis of Alzhemier’s disease. It has been observed that patient with the prolonged use
of certain non steroidal anti inflammatory (NSAID) drugs such as ibuprofen have a reduced risk of developing the symptoms of dementia.\textsuperscript{213} \textit{Hybanthus enneaspermus} and \textit{Cardiospermum halicacabum} both are reported for their anti-inflammatory activity.\textsuperscript{214, 215} They are found to inhibit cyclooxygenase (Cox-2) also, which would certainly help dementic patients by reducing the inflammatory components of Alzhemier 's disease.

Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and in the pathogenesis of dementia.\textsuperscript{216} \textit{Hybanthus enneaspermus} and \textit{Cardiospermum halicacabum} have also been reported to possess antioxidant properties.\textsuperscript{217, 218} The neuroprotective effect of MHE and MCH may be attributed to their antioxidant properties, which results in susceptible brain cell being exposed to less oxidant stress, reduced brain damage and improved neuronal function.

Thus the combination of anticholinesterase, anti-inflammatory and antioxidant effects exhibited by MHE & MCH may be eventually responsible for the memory enhancing effect observed in this study.

Hence, through the present studies on both the selected plants, \textit{Hybanthus enneaspermus} and \textit{Cardiospermum halicacabum}, their phytopharmacological evaluation could be validated to a reasonably level. The results can be used as scientific validation for both the plants regarding their uses mentioned in traditional literature.

Based on comprehensive therapeutic characteristics associated with \textit{Hybanthus enneaspermus} and \textit{Cardiospermum halicacabum} can be utilized for treatment of ailments like dementia or can be included as an ingredient for antidementic herbal preparations after further studies on development of its proper formulation and confirmation for its clinical efficacy.