Chapter 1

INTRODUCTION
“Memory is a part of the present. It builds us up inside; it knits our bones to our muscles and keeps our hearts pumping. It is memory that reminds our bodies and spirits to work, it keeps us who we are.”

— Gregory Maguire

1.1 General introduction

We learn during each and every moment of our life. Learning is an endless phenomenon. It is imperative for us to learn and excel in several skills to achieve success in our life. But, it is a fact that learning is futile without memory. Memory consists of learning, retaining, recalling and recognising. “Memory is the mental capacity to store, and later recall or recognise, events that were previously experienced” (Zombarbo, 1988). Memory is a vast mental reservoir. Everyday, a river of circumstances, facts and emotions flows through our brain and it records them ceaselessly and automatically most of the times. There are endless numbers of facts we constantly want to remember. Every one is interested in enhancing the performance of his memory.

Cognitive functions comprise of learning and memory, along with attention, creativity and intelligence. Cognitive dysfunction is one of the most functionally debilitating aspects of many neuropsychiatric disorders and neurodegenerative disorders, such as schizophrenia, depression, Alzheimer's disease, dementia, cerebrovascular impairment, seizure disorders, head injury and Parkinsonism (Ceskova, 2005). Ageing also plays an important role in the development of cognitive dysfunction. Cognitive enhancement means the amplification or extension of core capacities of the mind through improvement or augmentation of internal or external information processing systems (Bostrom and Sandberg, 2009).

Medicinal plants have been a source of health and wealth for humans since times immemorial. Several plants have been used to boost the memory of healthy individuals and to mitigate the memory impairments of the people bearing the agony of cognitive disorders (Stafford et al., 2008; Gomes et al., 2009). Moreover, many plants are also employed to assuage the effects of brain-aging. Indian, Chinese, European, African, South American and the other traditional systems of medicine advocate the use of various plants for neuroprotection and cognitive enhancement since ages. People using the traditional remedies may not understand the scientific rationale behind them, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses. Since, today we have a
better understanding of functioning of our body, we can very well understand the healing potential of plants for treating several diseases. Medicinal plants contain different chemical compounds that may act individually, additively or in synergy to improve health (Gurib-Fakim, 2006). A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant, anti-bacterial and anti-fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being. Modern allopathic system of medicine usually aims to develop a patentable single compound or a magic bullet to treat specific conditions. Traditional medicine often aims to restore balance by using chemically complex plants, or by mixing together several different plants in order to maximize a synergistic effect or to improve the likelihood of an interaction with a relevant molecular target. In most societies today, allopathic and traditional systems of medicine occur side by side in a complimentary way. The former treats serious acute conditions while the latter is used for chronic illnesses, to reduce symptoms and improve the quality of life in a cost-effective way (Gurib-Fakim, 2006).

The latest scientific studies and clinical trials carried out on several medicinal plants are also in agreement with these traditional systems of medicine. Several promising plants and their significant phytochemical compounds have also been identified by employing the modern scientific techniques. Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio friendly plant-based products for the prevention and cure of different human diseases. Considering the adverse effects of synthetic drugs, the Western population is looking for natural remedies which are safe and effective. In the same line of reasoning, the words of Schultes (1990) aptly apply here:

“People whom we have to consider members of less advanced societies have consistently looked to the Plant Kingdom . . . for the betterment of life. Should we as chemists, pharmacologists and botanists—with so many and varied means at our disposal—not take a lesson from them?”

It is documented that 80% of the world’s population has faith in traditional medicine, particularly plant drugs for their primary healthcare. India has a treasure of
well-recorded and traditionally well-practised knowledge of herbal medicine. Our country is perhaps the largest producer of medicinal herbs and is rightly called the botanical garden of the world. India officially recognizes over 3000 plants for their medicinal value. It is generally estimated that over 6000 plants in India are in use in traditional, folk and herbal medicine, representing about 75% of the medicinal needs of the Third World countries.

Plants are sophisticated organisms consisting of thousands of organic compounds. When using herbs therapeutically, we are usually interested in one or more of these compounds which has biological activity. The importance of plant secondary metabolites in medicine, agriculture and industry has led to numerous studies on the synthesis, biosynthesis and biological activity of these substances. It has been estimated that over 40% of medicines have their origins in these active natural products (Hanson, 2003).

With the ‘Back to Nature’ movement gaining momentum, Herbal Drug market is increasing at a very fast pace. The global demand for herbal medicine is not only large, but growing (Farnsworth and Soejarto, 1991). The natural resources are improperly utilized to meet this huge demand. This has forced many important species to become rare or endangered. Biotechnology comes to the rescue of Mother Nature in this scenario. Biotechnology, in recent years has created unprecedented opportunities, not only for the manipulation of biological systems for the benefit of mankind, but also for undertaking studies to understand the fundamental life processes. The biotechnological tools are important to select, multiply, improve and analyse medicinal plants. Production of plant secondary metabolites in vitro has been reported from various medicinal plants. Various in vitro techniques support the conservation of valuable species and also minimise the burden on the natural resources. Meticulous research and development works followed by bio prospecting endeavour are keys to exploit the valuable medicinal properties of these natural resources.

*Centella asiatica* (L.) Urban (Indian Pennywort/Mandookaparni/Gotu Kola), is an important medicinal herb known to enhance memory, youth and longevity. This plant, called Mandookaparni in Ayurveda is highly prized as a *medhya* (memory and intellect promoting) plant and one that prevents ageing (*vayahsthaapanami*), from the days of *Charaka* and *Sushruta Samhita*. Modern scientific investigations have
confirmed the classical claims. Its memory enhancing and wound-healing properties have been proved clinically.

1.2 Information about *Centella asiatica*

1.2.1 Systematic Position

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1.2.2 Distribution

*Centella asiatica* L. is commonly known as ‘Indian Pennywort’ or ‘Mandookaparni’. *Centella*, a genus of about 50 species of herbs is found in tropical & subtropical regions of the world (James and Dubery, 2009). The generic name has been derived from the Latin word “Centum” meaning hundred, referring to profusely branched prostrate herb which is a native of Madagascar. *Centella asiatica* is a small, herbaceous annual plant occurring in swampy areas of India, Sri Lanka, Madagascar, Africa, Australia, China, Indonesia, Malaysia, Australia and Southern and Central Africa. It flourishes abundantly in moist areas (Schaneberg *et al*., 2003; Verma *et al*., 1999).

1.2.3 Description

The stem of this perennial herb is slender, prostrate, creeping with long stolons and is nearly glabrous when young (Plate 3.1 a-d). The leaves are cordate or hastate or orbicular or reniform or sub entire or palmately lobed, consisting of long petiole and small stipules. Crowded leaves can be seen at nodes, consisting of very long stalks and sheathing leaf bases. Leaf blades are dentate, crenate with thick radiate veins and green in colour. Leaves are glabrous on both surfaces. Flowers are small, sessile and
dark pink in colour. They arise as simple umbels of 3 – 6 flowers at the ends of slender peduncles arising from the axils of leaves and much shorter than petioles supported below by an involucre of 2 boat shaped membranous persistent bracts. The fruit is a schizocarp around 3-4 mm in size, laterally compressed, readily separating into 2 indehiscent halves (mericarps) united by a very narrow plane of junction. Vittae are seen in the furrows of each mericarp. The seed has a hard oily endosperm and a small embryo. The plant propagates clonally by producing stolons that are characterized by long nodes and internodes. Depending on environmental conditions, the form and shape of the C. asiatica plant can differ greatly (Adamson, 1950).

1.2.4 Chemical constituents

Analytical studies suggest that Centella asiatica is a good source of triterpenoid saponins. Centella terpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, sceffoleoside, centellose, and asiatic, brahmic, centellic and madecasic acids (Aziz et al., 2007). Asiatic acid and its glycoside Asiaticoside were first isolated from the plant Centella asiatica in the 1940's. The leaves are rich in carotenoids, vitamin B and C (Dutta and Basu, 1968; Singh and Rastogi, 1969). The key constituents are ‘asiaticosides’ which have effect on skin diseases and psychotropic disease. Recent studies have led to the isolation of other triterpenoids with healing abilities, namely terminolic acid, madecassoside and asiaticoside-B (Schaneberg et al., 2003).

The chemical, pharmacological and clinical profiling has been done by Booncong et al., (1995); Brinkhaus et al., (2000) and Zainol et al., (2008). Analysis of biologically active constituents in Centella asiatica by microwave-assisted extraction combined with LC–MS was performed by Shen et al., (2009). James and Dubery (2009) have given a detailed account of the pentacyclic triterpenoids from Centella asiatica (L.) Urban. The chemical constituents of C.asiatica are classified into main groups including essential oil, flavone derivatives, triterpenic steroids, triterpenic acids and triterpenic acid sugar ester or saponin (Brinkhaus et al., 2000). It also contains various important constituents for clinical and pharmaceutical uses (Bonte et al., 1993). Chemicals that were previously investigated are brahmic acid, brahminoside, brahmoside, centellic acid, centelloside, hydrocotyline, 3-glucosylkaempferol, 3-glucosyl-quercetin, indocentelloside, isobrahmic acid, isothankunic acid, isothankuniside, madasiatic acid, madecassol, meso-inositol,
oxyasiaticoside, thankunic acid, vallerine; alkaloid, fatty acids, flavonols, polyphenols, saponins, sterols, sugars, tannins, terpenoids, triterpenes (Goh et al., 1995). Asiatic acid, asiaticoside, madecossoside and madecassic acid are the biologically active constituents in Mandookaparni that have a potential to be promoted as commercial product.

Mechanism of action has been studied and it is believed that antioxidant and neuroprotective mechanisms are involved. *C. asiatica* causes an overall decrease in the turnover of central monoamines norepinephrine, dopamine and serotonin, known to be implicated in learning and memory process (Nalini et al., 1992). Asiatic acid, the principal triterpenoid constituent of *C. asiatica* has been demonstrated to reduce hydrogen peroxide - induced cell death and lower intracellular free radical concentration (Mook-Jung et al., 1999). Asiaticoside, asiatic acid and its derivatives are subject matter of several patents covering treatment of dementia, ageing, and skin disorders (Sukhdev, 2006).

### 1.3 Importance of *Centella asiatica*

The topical properties of *C. asiatica* extracts have been well documented. It is used in western medicine as a cicatrising ointment (Bonte et al., 1993; Inamdar et al., 1996; Shukla et al., 1999). In the Indian system of medicine, Ayurveda, *Centella asiatica* has been used in various parts of India to cure different ailments like headache, body aches, insanity, asthma, leprosy, ulcers, eczemas and wound healing (Chatterjee et al., 1992; Shukla et al., 1999; Suguna et al., 1996). It is a reputed nervine tonic useful for improving the memory, voice, appetite, and for curing bronchitis, leucoderma, fever, biliousness, stuttering, anaemia, and smallpox. It is used for treatment of elephantiasis, kidney troubles, urethritis and in the preparation of senile pruritis drug ‘Geriforte’. *C. asiatica* is reputed to restore youth, memory and longevity (Kapoor, 1990).

The whole plant and leaves in particular, are useful as stomachic, carminative, soporific, sedative for nerves, tonic, cardiotonic and headache (Kirtikar and Basu, 1984). The plant possesses antileprotic, antifilarial, antifeedant, adaptogenic, antiviral, and antibacterial properties. The plant is also useful against anxiety neurosis, against peptic ulcer, to cure general debility and to improve the quality of memory in mentally retarded children (Apparao et al., 1973).
Centella asiatica, a plant mentioned in Indian literature has been described to possess CNS effects such as stimulatory-nervine tonic, rejuvenant, sedative, tranquilizer and intelligence promoting property (Kumar and Gupta 2002). The plant is also used as neutraceutical in the form of Brahmigrittha, a medicated ghee and as syrup by the brand name ‘Mentat’. The whole plant is used as a nervine tonic in various brain related diseases and given to children as syrup to increase the memory. It is thought to be effective for memory disorders, impaired intelligence and as a rejuvenator and blood purifier. In parts of India it is given with milk to improve memory against dementia and aging. It is also reported to improve general mental ability of mentally retarded children (Apparao et al., 1973).

A large number of reports suggest that many clinical researches have been carried out to prove the role of Centella asiatica in neuroprotection and cognition enhancement. The triterpene asiatic acid and its derivatives have been shown to protect cortical neurons from glutamate-induced excitotoxicity in vitro (Lee et al., 2000). The nootropic potentialities of C. asiatica have been assessed in pharmacological and behavioral trials in rats. The administration of aqueous or ethanolic extract showed an antistress activity by reducing stress-induced ulcerization and by inducing a state of non-specifically increased resistance. These effects were comparable to those of diazepam (Sarma et al., 1995, 1996; Valsala, 1998). A sedative and anticonvulsivant effect beside a low toxicity has been noticed, indicating a high therapeutic index (De Lucia et al., 1997). The findings of Kumar and Gupta (2002) indicate that the aqueous extract of C. asiatica has cognitive enhancing effect and an antioxidant mechanism is involved. The findings of Gupta et al., (2003) suggest the potential of aqueous extract of C.asiatica as adjuvant to antiepileptic drugs with an added advantage of preventing cognitive impairment. Rao et al., (2005) showed that treatment of mice during postnatal development with C.asiatica extracts influences the neuronal morphology and promotes higher brain function later on in life. Positive modulation of cognition and mood in the healthy elderly volunteers following the administration of Centella asiatica was found by Wattanathorn (2008).

Report of Export and Import Bank of India suggests that Centella asiatica is one of the important medicinal plants in the International market of medicinal plant trade. Therefore, the demand for high quality C.asiatica raw material has increased. The biomass of C. asiatica is important for preparation of herbal medicine mainly for improving the memory. In a report to Government of India; Technology Information
Forecasting and Assessment Council (TIFAC) had specifically recommended the list of few medicinal plants for immediate attention, *C. asiatica* being one of them. An increase in demand of biomass of *Centella asiatica* by 20.1% per annum has been predicted. As in the case of most plant-derived pharmacologically active compounds, pentacyclic triterpenoids have complex structures, making chemical synthesis an economically uncompetitive option. As a result, presently the entire ever-growing demand is met from the natural populations. Because of large scale and unrestricted exploitation of this natural resource coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this species has been markedly depleted.

Plant tissue culture provides the best means to elicit the cellular totipotency of plant cells and, therefore, it forms the backbone of the modern approach to crop improvement (Chaturvedi *et al.*, 2003). *In vitro* cultures have been used for rapid multiplication of elite clones and to increase the production of bio-active secondary metabolites of pharmaceutical interest (Giri, 2000; Gaines, 2004). The production of secondary metabolites *in vitro* is possible through plant cell culture. A particular important benefit is the potential ability to manipulate and improve the production of desired compounds in these cultures through experimentation. Recent progress has also showed great potential for the production of induced polyploid plants through *in vitro* culture. Polyploidy is the heritable condition in which cells contain more than two complete sets of chromosomes. Production of polyploid plants has been of considerable interest for researchers and has been used for obtaining new plant characteristics. *In vitro* chromosome doubling can be induced by using antimitotic agents like Colchicine and Oryzalin. The polyploid plants reportedly possess vigorous vegetative growth and capability of producing more biomass with higher organic content as compared to their diploid counterparts. Compared to diploids, polyploid plants have different morphological, physiological and biochemical parameters. Observed differences in the quantity and spectrum of active substances in diploid plants and induced polyploid plants (Haskell, 1968; Hiraoka, 1998) have provoked an interest in polyploidization of medicinal plants. The *in vitro* chromosome doubling technique has been used to produce tetraploid plants artificially and has been successful in several plant species (Predieri, 2001). It has been reported that induced tetraploidy in medicinal plants often generates variants with favourable characters like sturdiness, high productivity and increased amount of biomass and phytochemicals.
Their potential for photosynthesis is also greater as compared to diploids (İlarslan, 1990). Moreover, as compared to diploid plants, comparatively less biomass of tetraploid plants can help satisfy the demands of Ayurvedic and Pharmaceutical industry for preparation of drugs.

Traditionally, induction of tetraploidy is performed on plants established in soil, such as soaking roots or whole plants in a colchicine solution, or culturing shoots in colchicine-containing medium in the greenhouse. Other alternatives are injecting colchicine into the secondary buds or applying a colchicine-soaked cotton swab to axillary buds (Pei, 1985). However, low production efficiency of polyploid plants and a high frequency of chimeras are often associated with this method. Moreover, several cycles are required to separate chimeras by propagating axillary shoots (Pei, 1985). Producing tetraploids by using these methods is not only cumbersome but also uneconomical. Moreover, the treatment is lengthy and laborious. Once tetraploids have been produced, their survival rate is also quite low. In contrast, the in vitro polyploidization of plants is a better alternative. The tetraploids produced in vitro can also be rapidly bulked up by micropropagation. Besides, it offers many unique advantages over conventional propagation methods such as production of disease-free plants, non-seasonal, round the year production along with germplasm conservation.

The widespread application of in vitro propagation depends on its cost-competitiveness and is profitable only when there is an associated advantage over conventional propagation methods (Govil and Gupta, 1997). Though C.asiatica is medicinally important, meagre information is available on its improvement. Literature is available on the cultivation and utilization of C.asiatica; however very little efforts have been made for selection and cultivation of elite clones and improvement of species by using traditional as well as genetic engineering approaches. The success rate in both above methods for improvement of plant species is very low and cost intensive. Therefore the development of an efficient protocol will play a significant role in improving the biosynthesis of active metabolites on application of biotechnological approaches. In order to ensure the availability of raw material of superior quality and also to minimize the burden on its natural populations, this investigation was carried out on methods of improvement of C. asiatica through in vitro culture, focussing on the following objectives:

2. Induction of callus, organogenesis and somatic embryogenesis from different
explants (viz., root, stem, leaf and fruit).

3. Induction of tetraploidy by using chemicals like Colchicine and Oryzalin.

4. Acclimatization of in vitro produced diploid and tetraploid plantlets to natural conditions.

5. Establishment of diploid and tetraploid callus and cell culture.

6. Cytological, molecular and bio-chemical characterization of diploid and tetraploid cells, callus and regenerated plantlets.