CHAPTER II

REWIEW OF LITERATURE

2. Review of Literature

Centella asiatica comprises of some 33 species, inhabiting tropical and subtropical regions (Patra *et al*., 1998). This genus belongs to the family Apiaceae (previously known as Umbelliferae) and includes the most ubiquitous species *Centella asiatica* (Liu *et al*., 2003). *Centella* is also known as Pennywort, Asiatic pennywort, Indian pennywort, gotakola and brahmi. This plant is a perennial creeper flourishes abundantly in moist areas and is a small herbaceous annual plant. It is occurring in swampy areas of South East Asia, India, Sri Lanka, China, Madagascar, South Africa, Thailand, Mexico, Malaysia and Eastern South America (Satyavati, 1976; Chopra *et al*., 1956; Kirtikar and Basu, 1987).

Centella is clonally propagated by producing stolons that are characterized by long nodes and internodes. The stems are green to reddish green in colour, long, interconnecting to one another, creeping and rooting at the nodes. Leaves are simple, green, smooth texture with palmately netted veins, 2.10 fascicled at the node, orbicular, reinform, 1-7 cm long, 1.5-9 cm broad. The flower are small (less than 1mm) pinkish to red umbel shape. Each flower is partly enclosed in 2-3 green bracts, 5 sepals, 5 petals which are 1-1.5 cm long. Fruits are flattened, 2-3 mm long, 3-4 mm broad. Seeds are very small in size (on average 2.5 mm long and 1.7 mm broad) and mass (1.3 mg seed-1). On germination, the seeds produce seedlings and germinate during the months of September to February (Sushma *et al*., 2011; Sakshi *et al*., 2010).

Centella is a wild medicinal plant that has probably been used since prehistoric times and has been reported to have been used for various medicinal and cosmetic purposes, therefore thus becoming an important commercial product. For that reason, this plant is listed as a drug in the Indian Herbal Pharmacopoeia, the German Homeopathic Pharmacopoeia, the European Pharmacopoeia, and the Pharmacopoeia of the People's Republic of China (Schanerberg *et al*., 2003). According to World Health Organisation (WHO) monographs, Herbae *Centellae* should not contain less than 2% of the triterpene ester glycosides asiaticoside and madecassoside (World Health Organisation, 1999). Availability of natural genetic

resources of the plant for variation in morphological, growth and yield contributing characters in the cultivars of this plant is low and it has become a major limiting factor for genetic improvement of *Centella asiatica*. For that reason we have tried to improve the genetic improvement for high yield saponins contains in *Centella asiatica* by applying the colchicine induction methods. Available reports on morphological variation, microscopic variation, chromosomal variation, medicinal importance and saponins contains and genetic improvement for saponins in *Centella asiatica* and other related plants employing induction of autotetraploidy, and induction of somaclonal variations are reviewed here.

2.1. Plant Description

Indian Pennywort or *Centella asiatica* (L.) Urban is a genus of the plant family Apiaceae (Umbelliferare). This medicinal herb is known as Brahmi in Unani medicine, Mandookaparni in Ayurvedia and Gotu Kola in the Western World. In India, plant was earlier confused for *Bacopa monnieri* Wettest., as both were sold in the market by the name Brahmi. However, the controversy has been resolved and it is concluded that Brahmi is *Bacopa monnieri* and Mandookaparni is *Centella asiatica* (The Wealth of India, 1992; Sakshi *et al*., 2010; Shakir *et al*.,2007). *Centella* herb, which has a mildly bitter taste also commonly known as *Hydrocotyle asiatica* L., Indian Pennywort or *Hydrocotyle asiatique* in france (Ling *et. al*., 2000). Other names of Brhami include 'Luci Gong Gen' or 'Tung Chain' in China, 'Vallarai' for tamil nadu in India and 'Daun Kaki Kuda' in Indonesia (Perry, 1998; Goh *et al*., 1985).

Brhami can be found easily in moist habitats or wet swampy area throughout India, Malaysia, Madagascar, China, Southern United State America and Middle Africa (Perry, 1998). *Centella asiatica* (L.) is a prostrate, faintly aromatic, stoloniferous, perennial, creeper herb, attains height up to 15cm (6 inches). The stem is glabrous, striated, rooting at the nodes. *Centella* flourishes extensively in shady, marshy, damp and wet places such as paddy fields, river banks forming a dense green carpet. The leaves, 1-3 from each node of stems, long petioled, 2- 6cm long and 1.5-5cm wide, orbicular-renniform, sheathing leaf base, crenate margins, glabrous on both sides. Flowers are in fascicled umbels, each

umbel consisting of 3-4 white to purple or pink flowers, flowering occurs in the month of April-June. Fruits are borne throughout the growing season in approx 2 inches long, oblong, globular in shape and strongly thickened pericarp. Seeds have pedulous embryo which are laterally compressed (Anonymous, 1992; Shakir *et al*., 2007; Sakshi *et al*., 2010). The whole plant including leaves, stem and root are consumed as 'ulam' and therapeutic agents (Brinkhaus, 2000; Indu Bala and Ng, 1999).

2.2. Geographical Source of *Centella*

The *Centella* plant material and its products are being used for health care since ages. It is important traditional socio-economic uses of this marvellous herb *Centella asiatica* in different countries and in different area. The genus *Centella asiatica* comprised of about 33 species of herbs (Patra *et al*., 1998) is widely found throughout tropical and sub-tropical regions of India up to an altitude of 600 m (The Wealth of India, 1992; Kirtikar and Basu, 1987; Physicians Desk Reference for Herbal Drugs, 2000; Gupta and Sharma, 2007). The plant has been reported to occur also at high altitudes of 1550 m in Sikkim and 1200 m in Mount Abu (Cacho *et al*., 1991; Aiyer and Kolammal, 1964; Sakshi *et al*., 2010). It flourishes in and around water and damp swampy area but it is often observed growing along the stone wall or other rocky sunny area (Ohwi, 1965). The plant is native to parts of India, South-East Asia, Sri- Lanka, parts of China, the Western South Sea Islands, Madagascar, South Africa, South East USA, Mexico, Venezuela, Columbia and Eastern South America (Satyavati, 1976; Subban *et al*., 2008). It is found to be available naturally in some parts of India. It is also cultivated in India, Madagascar and Sri Lanka for commercial purposes.

2.3. Cytological Variations in *Centella* Species

Cytological studies on *Centella asiatica* have been reported that, they have revealed the presence of chromosome number was different in different ecotypic races of this species. However, the previous cytological data have revealed the somatic chromosome number 2n=18 chromosome forming regular nine bivalents (Sharma and Ghosh, 1954; Sharma and Bhattacharya, 1959, 1960; Bell and

Constance, 1960; Liu, *et al*., 1961; Joshi and Raghuvanshi, 1970; Subramanian, 1986). Some conflicting report regarding chromosome number i.e., 2n=22 and 2n=33 have been reported by Sharma and Sharma, (1957) and Mitsukuri and Kurahori, (1959), respectively. Chromosomal races differing in number of chromosomes and presence of accessory B-chromosomes has also been reported in plants from Lucknow, West Bengal and Kerala (Joshi and Raghuvanshi, 1970; Nagendra Prasad and Janakiammal, 1985). The existence of chromosomal variation among accessions of *C.asiatica* suggests the presence of diversity in form and structure.

2.4. Conservation Status of the Plant

According to the reports of Export and Import Bank of India *Centella asiatica* is one of the important medicinal plants in the International market of medicinal plant trade (Kameshwara, 2000). In the year 1990 alone the estimated annual requirement of *Centella* was around 12,700 tonnes of dry biomass valued at Rs.1.5 billion (Ahmad, 1993). However, the wild stock of this plant species has been markedly depleted, because of large scale and unrestricted exploitation of this natural resource coupled with limited cultivation and insufficient attempts for its replacement has been made. Moreover, now it has been listed as Threatened plant species by the International Union for Conservation of Nature and National Resources (IUCN) (Pandey *et al*., 1993) and endangered species (Singh, 1989; Sharma and Kumar, 1998 and Dora and Khatri, 2011). According to Joshi (1988), *Centella asiatica* is a rare and threatened species in Gujarat.

2.5. Medicinal Importance of the Plant

Centella asiatica is used as a traditional medicinal plant that has probably been used since prehistoric times. It has been reported that, have been used for various medicinal and cosmetic purposes, thus becoming an important commercial product. It is an ethenomedical plant used in different continents by diverse ancient cultures and tribe groups (Nadkarni, 1986). The plant is highly valued for various diseases and it is used in Ayurveda, Siddha and Unani System of medicine. Therefore, this plant is listed as a drug in the Indian Herbal Pharmacopoeia, the

German Homeopathic Pharmacopoeia, the European Pharmacopoeia, and the pharmacopeia of the People's Republic of China (Schaneberg *et al*., 2003; Jacinda and Ian, 2009).

In India *Centella asiatica* L. is important herbal medicinal plant used for various applications (Jacinda and Ian, 2009). Since ancient times, *Centella asiatica* has been used in traditional Indian medicine for various pathological disorders, and in particular for healing wounds and for leprosy. In the Ayurvedic system of medicine it is also recommended in chronic diseases and as a nerve tonic (Singh *et al*., 2008; Dora and Khatri, 2011). Utilization of *Centella asiatica* have been known for many years in treating all kind of diseases such as gastrointestinal disease, gastric ulcer, asthma, wound healing and eczema (Brinkhaus *et al*., 2000; Chew *et al*., 2011; Sushma *et al*., 2011).

It has been traditionally used as an antipyretic, diuretic and treatment for skin inflammations (Merce Bonfill *et al*., 2006). In South Africa, it is used of the treatment of fever and syphilis and it is also used for the treatment of acnes and allergy (Van Wky *et al*., 1997). According to Chopra *et al*., (1985) it's more common use is wound healing. In Europe an extract of *C. asiatica* was used for many years for the treatment of wounds (Pointel *et al*., 1987; Maquart *et al*., 1999).

C. asiatica is well reputed as a brain tonic for the mentally retarded patients (Karting, 1988; Appa Rao *et al*., 1973). Significant improvement in general mental ability and mental concentration, overcoming stress and fatigue has been reported by Appa Rao, *et al*., (1977). The plant extracts are used popularly in memory enhancing tonics and for the treatment of mental and stress-related disorders (Sharan and Khare, 1991; Moharana and Moharana, 1994).

It is listed officially in the Chinese Pharmacopoeia and used as an antipyretic, diuretic, and antidote in the treatment of icterus, heat stoke, diarrhea, ulcerations, eczema and traumatic diseases (Sakshi *et al*., 2010). It was historically known as "Snow plant" for the reason of its cooling properties (William, 1985). The *Centella* leaves crushed and root extract is applied to the affected parts to kill germs from wounds and cure leprotic wounds (Joshi and Joshi, 2007).

It has been claimed in Thai traditional recipes as a poultice for the wound healing (Farnsworth and Bunyapraphatsara, 1992) and mostly used as a vegetable and tonic (Peiris and Kays, 1996). This plant occupies an important place in indigenous system of Indian medicine as tonic in diseases of skin, nerves (Datta and Basu, 1962; Rao and Seshadri, 1969), leprosy, bloods (Datta and Basu, 1962), anti-inflammatory, cure in leprosy and syphilis (Shakir *et al*.,2007). Leaves are used as tonic and for improving memory (Sakina and Dandiya, 1990). Seeds are used for dysentery, fever and headache (Shakir *et al*., 2007).

The use of *Centella* in food and beverages has increased over the years basically due to its health benefits such as antioxidant (Abdul-Hamid *et al*., 2002; Vimala *et al*., 2003; Ullah *et al*., 2009), as anti-inflammatory and wound healing (Shakir *et al*.,2007) memory enhancing property (Subathra *et al*., 2005) and many others. The potential of *Centella* as an alternative natural antioxidant especially of plant origin and its protection against age-related changes in brain antioxidant defense system, have notably increased in recent years (Subathra *et al*., 2005). Free radicals have been claimed to play an important role in ageing process and capable of damaging many cellular components (Jacinda and Ian, 2009). These changes will affect the brain as it is particularly vulnerable to oxidative damage; as such many studies on its neuroprotection activity have been reported.

The herb also finds use in the cosmetic industry for the preparation of hair oils, tonics and shampoos. The plant has also been used in the traditional Indian and Chinese systems of medicine for treatment of diseases such as leprosy and psoriasis, insanity acnes, allergies and cancer (Bose, 1932; Kan, 1986; Sastri, 1950; Van Wky *et al*., 1997). It has been used as a nervine tonic, cardio-tonic and also for the treatment of varicose, ulcers, lupus and certain eczemas, stomachic, carminative, diuretic and laryngitis, (Kartnig, 1988; Sharma *et al*., 2000).

Maquart *et al*., (1990) have reported elevated collagen synthesis in fibroblasts by asiaticoside *in vitro*. It is used as a remedy for sodation and stabilization and against lepra, anabrosis (Chopra *et al*., 1956; Maquart *et al*., 1990; Yoshinori *et al*., 1982). The triterpenic fraction of *Centella* is effective for treating venous insufficiency and venous hypertension (Belcaro *et al*., 1990; Cesarone *et*

al., 1992).

Extensive clinical investigation on *C. asiatica* has led to the use of the plant in managing diabetics (Cesarone *et al*., 2001), hypertension and edema (De Sanctis *et al*., 2001; Incandela *et al*., 2001a, b) in addition to its evaluations in wound treatment. The antitumor and cytotoxic properties of the crude extract and partially purified fractions were reported by Babu *et al*., (1995). According to the authors, the partially purified extract was more effective on tumor cells than the crude extracts. Dermatologically, extracts of *C. asiatica* has been used in scar management and in cosmetic formulation (Martelli *et al*., 2000; Widgerow *et al*., 2000).

Antitumor and cytotoxic properties of the crude extract and partially purified fractions were reported by Babu *et al*., (1995). Antibacterial activity of the plant against Gram positive and gram negative strains of bacteria is reported by Oyedeji and Afolayan, (2005). The plant is also known for its antistress, antitubercular properties (Chakraborty *et al*., 1996). Some pharmacological activities, like antiprotozoal, psasmolytic, alterative, astringent, anti-inflammatory, antifertility, sedative, CNS depresent, antitubercular, antileprotic, hepatoprotective, antispasmodic, antiamoebic and hypotensive, of *C. asiatica*, have been reported by Sharma *et al*., (2000). Modern drugs comprising pharmacologically active triterpenoid fractions and glycosides such as asiaticoside and madecassoside are being currently used in the treatment of burns, ulcers of the duodenum, skin and cornea, tuberculosis and venous diseases (Boiteau *et al*., 1949; King, 1950,a,b; Boiteau and Ratsimamanga, 1956; Ratsimamanga *et al*., 1958; Boiteau and Ratsimamanga, 1959; Allegra *et al*., 1981; Giardina e*t al*., 1987).

C. asiatica is one of the spiritual herbs for improved meditation in Ayurveda (Shakir et al., 2007). This herb is used by Yog is to increase their meditation abilities through better concentration, focus and alertness. This herb has also shown great promise in curing mental retardation and increasing IQ (Annonymous, 2004). The result of double blind trial of mandookparni on mentally retarded children showed a very significant increase in both general ability and behavioral pattern (Sharma *et al*., 2000).

2.6. Chemical Constituents of the *Centella asiatica*

Centella asiatica (Linn), is commonly used in the ayruvedic system of medicine as well as being a leafy vegetable various studies have shown that it is rich in triterpenoids, volatile oils and flavonoids. Over several decades, there has been an increasing interest in various compounds obtained from different sources of *Centella*. The different reported compounds may be due to place of origin of the materials or to the differences in variety of the plant (Sakshi *et al*., 2010). Jacinda and Ian (2009) reported that the active compound is composed of four related chemical extracts they are asiatic acid (constituting 29-30%), madecassic acid (29-30%), madecassoside (1%) and asiaticoside (40%). They are the biologically active constituents in *Centella* that have a potential to be promoted as commercial products (Indu Bala and Ng, 1999). In addition, they contain asiaticoside (1-8%) and total phenolics (23,000 mg/100 g) (Brinkhaus *et al*., 2000; Fezah *et al*., 2000). Flavonoid components including apigenin, kaempferol, quercetin and rutin have been detected in *Centella*. The yield of apigenin was found to be hightest followed by quercetin, kaempferol and rutin (Radzali *et al*., 2001). In addition, Koo and Suhaila (2001) found that the concentrations of quercetin and kaempferol in dried pennywort were 423.5 and 20.5 mg/kg, respectively. Kaempferol-3-glucoside and quercetin-3-glucoside are flavone derivatives isolated from leaves of *Centella* (Prum *et al*., 1983). Earlier work on this plant has led to the isolation of more than 70 constituents, such as triterpenoids saponins (Jiang *et al*., 2005; Kuroda *et al*., 2001), polyacetylenes (Schulte *et al*., 1973), flavones (Prum *et al*., 1983), sterols and lipids (Kapoor *et al*., 2003).

First report of studies on the chemical constituents of *Centella asiatica* goes to back 1956, when Bhattacharyya, S.C, reported a triterpenic constituent with the name indocentoic acid and a water soluble glucoside indocelloside. Rastogi *et al*., (1960) isolated two triterpene acids, brahmic acid and isobrahmic acid along with two saponins brahmoside and brahminoside from the alcoholic extract of the air-dried plant powder. Si-Qi and Huei-Fang, (1980), reported that they isolated another saponin asiaticoside from *Centella asiatica*. Later, Prum *et al*., (1983) they isolated 3-O-glucosyl quercetin and 3-glucosyl kaemferol from the leaves of

C.asiatica. Jacinda and Ian (2009) undertook studies on the chemical constituents of the whole plant of *Centella asiatica* using various chromatographic techniques. They reported the isolation and structures of 14 constituents which included β-sitosterol, hexacosanol octanaoate, kaempferol, quercetin, daucosterol, vanilic acid, succinic acid, asiatic acid, madecassic acid, terminolic acid, asiaticoside, asiaticodiglycoside, madecassoside and asiaticoside B. The plant is reported to contain tannins, sugars, inorganic acids 35 and resin 31, amino-acids, viz. aspartic acid, glycine, glutamic acid, α-alanine and phenylalanine36. The total ash contains chloride, sulphate, phosphate, iron, calcium, magnesium, sodium and potassium. The leaves are rich in vitamins such as vit.B, vit.C37 and vit.G18. Asiatic acid, asiaticoside, madecossoside and madecassic acid are the biologically active constituents in *Centella* that have a potential to be promoted as commercial product (Indu Bala and Ng, 2000).

2.7. Triterpenoid Saponins: Active Compounds of *Centella asiatica*

Saponins are a vast group of glycosides, widely distributed in higher plants. A number of different plant species synthesize triterpenoid saponins as part of normal growth and development with the most predominant group being pentacyclic triterpenes derivatives and their sapogenins (Haralampidis *et al*., 2002). Their surface-active properties are what distinguish these amphiphilic compounds from other glycosides (Sparg *et al*., 2004). The triterpenoid structure (aglycone) is hydrophobic and contains a hydrophilic sugar chain (glycone) and these characteristics are responsible for the biological activity of saponins (Jacinda and Ian, 2009).

The main active components of the *Centella asiatica* are believed to be triterpenoid saponins. Several studies have revealed the triterpenoid saponins of *Centella asiatica* using different techniques (Diallo *et al*., 1991; Du *et al*., 2001). Its active metabolites, triterpenoid saponins, such as madecassoside, asiatic acid, madecassic acid and the three asiaticosides, asiaticoside, asiaticoside A and asiaticoside B are reported to have many medicinal and therapeutic properties (Sing and Rastogi, 1969; Inamdar *et al*., 1996; Brinkhaus *et al*., 2000). *Centella*

contains not less than 2% triterpenes ester glycosides, asiaticoside and madecassoside (Kartnig, 1988). Schaneberg *et al*., (2003) have reported that the isolation of other triterpenes with healing potential, namely, terminolic acid, madecassoside, and asiaticosdie-B. Earlier work on this plant has led to the isolation of more than70 constituents, such as triterpenoid saponins, polyacetylenes, flavones, sterols and lipids (Jiang *et al*., 2005; Matsuda *et al*., 2001; Kuroda *et al*., 2001; Schulte *et al*., 1973; Prum *et al*., 1983; Kapoor *et al*., 2003).

Recently, Devkota *et al*., (2010 A) obtained data about the variations in secondary metabolite in different geographical areas of Nepal selecting high producing triterpene plants for possible cultivation. In addition several phytochemical constituents were reported in *C.asiatica* such as chicoric acid, chlorogenic acid, rosmarinic acid, quercetin, quercetin 3-0-glucuronide, kaempferol, brahmoside and brahminoside (both saponin glycosides), madecassic acid,centelloside, indcentelloside, thankuniside, isothankuniside, brahmic acid, betulic acid, thiamine, riboflavin, pyridoxine, vitamin K, glutamate, serine, threonine, alanine, lysine, histidine, magnesium, calcium and sodium (Devkota *et al*., 2010B; Anonymous 2004; Bhattacharya, S.C., 1956; Datta and Basu, 1962). Significant difference in contents of active constituent have been observed in samples of *C.asiatica* originating from different countries, such as India and Madagascar (Das and Mallick, 1991; Rouillard-Guellec *et al*., 1997). Comparative study by Das and Mallick (1991), in 10 ecotypes of *Centella asiatica* from different regions of India, showed a correlation between genomic diversity and asiaticoside content. Pick Kiong, (2004) reported that the asiatic acid content was high in *Centella asiatica* of Malaysia.

However, previous studies also showed that *Centella asiatica* has a high level of phenolic compounds and these compounds are believed to be responsible for the antioxidative activity (Hussin *et al*., 2009). The herb was reported to have abundant phenolic compounds that are majorly from flavonoids such as quercetin, catechin, epicatechin, rutin, luteolin, myricetin, kaempferol and naringenin (Hussin *et al*., 2009; Mustafa *et al*., 2010;Chew *et al*., 2011). The nutritional value of *Centella asiatica* is promising, as it is rich in carotenoids and vitamins B and C

(Paramageetham *et al*., 2004). Essential oil composition of *C. asiatica* was studied by Oyedeji and Afolayan (2005). They found that the plant possessed 11 monoterpenoid hydrocarbons (20.20%), nine oxygenated monoterpenoids (5.46%), 14 sesquiterpenoid hydrocarbons (68.80%), five oxygenated sesquiterpenoids (3.90%), one sulfide sesquiterpenoid (0.76%), a-Humulene (21.06%), b-caryophyllene (19.08%), bicyclogermacrene (11.22%), germacrene B (6.29%), and myrcene (6.55%).

2.8. Nature of the Polyploidy

Polyploid organisms contain more than two complete sets of chromosomes in the nucleus and occur frequently in three of the four eukaryotic kingdoms (Storchova and Pellman, 2004). In the five kingdom classification system, polyploids have naturally risen in three kingdoms: plantae, protista and animalia (Baatout, 1999). In the plant kingdom polyploids are common in angiosperms and ferns, (Schuettpelz *et al*., 2008) but are rare in gymnosperms (Briggs and Walters, 1997). Furthermore, genome duplication or polyploidy is believed to have occurred during the evolution of 30 – 70 percent of angiosperms (Masterson, 1994). Although polyploids occur in animals they are rare among mammals but more common in other classes. Algae and bryophytes are not clearly defined in the five kingdoms system; some of these species are polyploids (Briggs and Walters, 1997).

Ploidy is usually represented by the notation nx, where a nucleus with two complete sets of chromosomes is referred to as a diploid (2x), three complete sets of chromosomes is a triploid (3x), four complete sets a tetraploid (4x), five complete sets a pentaploid (5x) and so on. The highest level of ploidy currently known in angiosperms is an 80-ploid (80x) stonecrop (*Sedum suaveolens* Kimnach) (Otto and Whitton, 2000). The wide range of chromosome number observed in plants suggests a role in plant evolution (Leitch and Bennett, 1997). A ploidy series can exist within a species (*Fraxinus americana* Linnaeus) ploidy changes can exhibit morphological, genetic or geographical differences but they do not warrant taxonomic reclassification (Wright, 1944).

Polyploids can have multiple origins that are related to the nature of chromosomal duplication. For example, an autopolyploid is formed through the replication of chromosomes within a species (genome denoted e.g., AA → AAAA). In contrast to an autopolyploid, an allopolyploid arises from the duplication of chromosomes contributed from different species (genome denoted e.g., AABB). Autoallopolyploids contain duplicated chromosomes from one species and at least one set from another species (genome denoted e.g., AAAABB) (Stebbins, 1971 and Grant, 1981). Polyploids frequently occur within plants used in the fruit industry. Polyploid fruit crops include sour cherries (*Prunus cerasus L.*) (Tavaud, *et al*., 2004), strawberries (*Fragaria spp. L.*) (Ahokas, 1999), kiwi (*Actinidia deliciosa Liang and Ferguson*) (Udall and Wendel, 2006), blueberries (*Vaccinium spp. L.*) (Decroocq, 2004), European plums (*Prunus domestica L.*) (Decroocq, 2004), persimmons (*Diospyros kaki Thunb.*) (Tao *et al*., 2009) and watermelons (*Citrullus lanatus Thunberg*) (Love *et al*., 1986). Agronomic crops such as some selections of wheat (*Triticum aestivum L.*) (Udall and Wendel, 2006), potato (*Solanum tuberosum L.*) (McGregor *et al.,* 2000), cotton (*Gossypium spp. L.*) (Udall and Wendel, 2006), alfalfa (*Medicago sativa L.*) (Udall and Wendel, 2006) and sugar cane (*Saccharum spp. L.*) (Cordeiro *et al.,* 2000) may also be polyploids. Polyploids often have improved horticultural or agronomic traits such as larger fruit, thicker leaves and robust stems (Kehr, 1996), so plant breeders sometimes favor polyploids in breeding programs. Polyploid plants are also found in the ornamental horticulture industry and they may have thicker flower petals that last longer than their diploid counter-parts (Kehr, 1996). Common polyploid bedding plants include dahlia (*Dahlia spp. Cav.*), pansies (*Viola spp. L.*) and chrysanthemum (*Chrysanthemum spp. L.*). Naturally occurring polyploids are less common in mammals. The red Viscacha rat (*Tympanoctomys barrerae Lawrence*) is an aneuploid that represents the first known naturally occurring mammal with tetraploid chromosomes for all but the sex-chromosome (Gallardo *et al.,* 1999).

2.9. Induction of Polyploidy

Artificial polyploidy is a technique through which desired changes can be brought about in plant by altering the chromosome numbers. Polyploids can arise

spontaneously within plants during somatic cell division (mitosis) which can result in an autopolyploid shoot often noticeable by its enlarged "gigas" condition (Stebbins, 1971). Autopolyploids can also arise during the meiotic process through the union of unreduced gametes. Allopolyploids are much more common than autopolyploids and are the function of the hybridization of separate species that contain separate sets of non- homologous chromosomes (Stebbins, 1971;Soltis and Soltis, 2000). This can be carried out using various chemical agents. In the late 1930s it was discovered that colchicine inhibited the formation of spindle fibres and effectively arrested mitosis at the metaphase stage. At this point, the chromosomes have multiplied but cell division has not yet taken place resulting in polyploidy cells. In later years, other methods of inducing polyploids include treatments with mitotic inhibiting chemicals such as ethyl methyl sulphonate (VanTuyl *et al.,* 1992), Oryzalin (Alberts *et al.,* 1994), trifluralin (Eeckhaut *et al.,* 2004) and N2O (Kitamura *et al.,* 2009).

Methods for applying these agents vary. One of the easiest and most effective methods is to work with a large number of seedlings with small, actively growing meristems. Seedlings can be soaked or the apical meristems can be submerged or sprayed with different concentrations (ranging from 0.1% to 0.5%) durations (ranging from 2 hours to 96 hours) or frequencies of a given doubling agent. Shoots on older plants can be treated, but it is often less successful. Treatment of smaller axillary or sub-axillary meristems is sometimes more effective. Chemical solutions can be applied to bud using cotton, agar or lanolin or by dipping branch tips into a solution for a few hours or days. Surfactants, wetting agents, and other carriers (dimethyl sulphoxide) are sometimes used to enhance efficacy.

Colchicine is an alkaloid extracted from seeds or corms of *Colchicum autumnale L.* (autumn crocus or meadow saffron) and was first isolated in 1820 by the French chemists P.S. Pelletier and J. Caventon (Pelletier and Caventon, 1820). Colchicine blocks inflammation caused by uric acid crystals and is used to treat acute gouty arthritis in humans (Eustice and Eustice, 2007). Medicinal colchicine is used as a 0.5 mg and 0.6 mg oral tablets, or as an intravenous injection, but the treatments have a high risk of serious toxicity (Eustice and Eustice, 2007).

Fatalities have been reported after ingestion of 7 mg to 12 mg of colchicine in adult humans (Stapczynski *et al.,* 1981). The Environmental Protection Agency (EPA) mandates a "toxic" label on all containers containing colchicine because of this toxicity. Ploidy levels have been manipulated in animals and plants (Derman, 1940) by submerging the specimen in a solution of colchicine.

The most effective range of treatment ranges from micro to millimolar concentration of colchicine with the optimal concentration for a species needing to be determined empirically. Oryzalin [3,5-dinitro-N4, N4-dipropylsulfanilamide] is the active ingredient (a.i.) of the pre-emergence herbicide Surflan® (Sourthern, 1998) and is also used to induce polyploids in plants. Oryzalin binds to plant tubulin heterodimers only during metaphase through a pH-dependent interaction forming a rapid and reversible tubulin – oryzalin (TO) complex (Hugdahl and Morejohn, 1993). Further research concluded that the oryzalin binding site is under the N loop of the α-tubulin and consistently docks to Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243 and Phe244 sites (Morrissette *et al*., 2004). It is suggested that the oryzalin or the TO complex binds to the microtublin positive (+) end leading to the disruption of the polymerization of microtublin (Hugdahl and Morejohn, 1993). The spindle fibers, which are composed of microtubules, function to pull the sister chromatids to opposite poles of the cell and without their action the mitotic process is disrupted (Alberts *et al.,* 1994). This disruption can result in DNA replication without cell division when oryzalin is used at low concentrations (Bartels and Hilton, 1973).

The cytoskeleton of plant cells is also composed of microtubules which when treated with oryzalin can lead to abnormalities of cytoskeleton function such as maintaining cell shape, maintaining cell protection and cell motion. Although a LD 50 has been established in animals, oryzalin does not disrupt animal microtubules due to the absence of an oryzalin – binding site on mammalian tubulin (Hugdahl and Morejohn, 1993), therefore, chromosome numbers in mammals are not altered (Bartels and Hilton, 1973). The ploidy level of several plant species have been altered using oryzalin. This includes *Miscanthus sinensis* Anderson (Petersen *et al*., 2002), *Pyrus L.* (Bouvie *et al*., 2002), *Solanum L*. (Chauvin *et al.,* 2003), *Rosa L.* (Kermani *et al.,* 2003), *Lilium L.* (Van Tuyl *et al.,*

1992) and *Tulipa L.* (Chauvin *et al.,* 2005). Research with *M. sinensis* determined that treating shoot apices in 15 μM oryzalin solutions for a period of 96 hours was the most effective treatment for inducing polyploids (Petersen *et al*., 2002). Petersen *et al*. (2002) also found that 60 μM oryzalin prevented callus initiation of immature inflorescences of *M. sinensis* that were cultured *in vitro*. Bouvie *et al.* (2002) found that 200 μM – 300 μM concentrations of oryzalin were required to induce polyploidy in *Pyrus L*. In *Solanum L.* the most effective treatment for producing tetraploids was a 24 hour treatment with 28.8 μM oryzalin solution applied to apical buds (Chauvin *et al*., 2003). From prior research it is evident that the optimal oryzalin concentration and treatment duration for polyploid induction varies among species and must be determined empirically.

Trifluralin [2, 6-dinitro-N, N-din-propyl-4-trifluomethyl aniline] has also been successfully used to manipulate ploidy levels. Approximately 5% of the anther filaments of *Spathiphyllum wallisii* Regal 'Speedy' treated with 10 μM trifluralin or oryzalin became polyploids (Eeckhaut *et al*., 2004). The Eechhaut *et al.* (2004) study suggested that both of these chemicals, (both structurally similar dinitroanilines) could effectively replace colchicine as an anti-mitotic agent, thus removing the risk of colchicine exposure in laboratory procedures (Eeckhaut *et al*., 2004). Exposure to N2O gas has also been reported to alter the ploidy level although the mode of action, in plants, is not known (Kitamura *et al.,* 2009). N2O has been used on *Zea mays L.* (Kato and Birchler, 2006) and *Triticum dicoccum* Khapli (Kihair and Tsunewaki, 1960).

For all the reports described above, only a few cases of polyploid medicinal plants have been reported. For medicinal plants, polyploids are usually more valuable because they exhibit increased biomass and content of effective compounds (Gao *et al*., 1996). Within the last 30 years, the world has experienced an increased trend towards healthy diet and natural products. This has led to a growing demand for medicinal and aromatic plants (Gabler, 2002). Together with requirements for safety, efficacy and stability of medicinal plant products, the need for high quality raw materials is increasing. The induction of artificial polyploidy may prove useful in increasing the quality and quantity of important medicinal compounds (Dhawan and Lavania, 1996). A number of published papers have

reported that artificial tetraploidy in medicinal plants increases the amount of biomass or phytochemicals, such as *Artemisia annua* (Wallaart *et al*., 1999), *Salvia miltiorrhiza* (Gao *et al*., 1996), *Papaver somniferum* (Mishra *et al*., 2010), and *Scutellaria baicalensis* (Gao *et al.,* 2002).

2.10. Effects of Environmental Variation on the Content of Bioactive Components

Habitat factor may impose significant impact on accumulation of important bioactive components in *Centella asiatica*. Significant difference in contents of active constituent have been observed in samples of *Centella asiatica* originating from different countries, such as India and Madagascar (Das and Mallick 1991; Rouillard-Guellec *et al*.,1997; Datta and Basu 1962). They have found that *Centella asiatica* collected from different regions have differed in presence of saponins, such as asiaticoside, madecassoside , isothankuniside, brahmoside and centelloside. Devkota *et al*., (2010B) have studied the a comparative quantitative analysis of chemical constituents in *Centella asiatica* samples collected from three different habitats in Nepal was carried out by HPLC to evaluate the variability in the important constituents. There was marked variability in asiaticoside, asiatic acid and quercetin 3-O-glucuronide content among the samples collected from different habitats.

Generally, all *C.asiatica* samples showed relatively higher amount of asiaticoside than asiatic acid. This is in accordance with large amount of triterpene glycosides and trace of triterpenic acids from plants of Thailand, Costa Rica and Bahamas (Booncong, 1989). However, high asiatic acid content was reported in *C. asiatica* of Malaysia (Pick Kiong, 2004). Interestingly, Gupta *et al*., (1999) also reported variable asiaticoside content in 5 lines of *C. asiatica* collected from a field trial in India, with the mean content varying from 0.42-1.17%. Asakawa *et al*., (1982) and Jayatilake and Macleod, (1987) have analysed essential oils and other chemical constituents in *Centella asiatica* plants collected from Japan, Malaysia and Sri Lanka and observed variation in the bitter substances like aglucoside - asiaticoside, fatty oils, sitosterol and tannins and essential oils, extracted from the herb. Rao and Seshadri (1969), studied variation in the chemical composition of

the Indian samples of *C. asiatica*. They have reported variations in the yield of saponins depending on habitat of the plant.

2.11. Effect of Polyploidy on the Content of Bioactive Components

Reza *et al*., (2010) reported that the physiological effects of polyploidy are not generally predictable, and the responses are often species-specific, doubling the sizes of stomata, pollen grains, phenotypic characters and chromosome number of a plant increases the number of genes. And thus changes enzymatic activity and isozyme diversity. This can affect the biosynthetic pathways of secondary metabolites. Induction of artificial autotetraploidy in medicinal plants has often increased quantities of secondary metabolites and also altered them in a qualitative manner (Bertea *et al*., 2005; Dijkestra and Speckmann, 1980; Saharkhiz, 2007).

Tanavat *et al*., (2011) induced polyploidy in the traditional Thailand herbal medicinal plant, *Centella asiatica* using colchicine. He reported that the induced polyploidy plant showed increase of 11% in total triterpenes over that of the diploid plant. Mishra *et al*., (2010) have been also induced autotetraploid *Papaver somniferum* plant and they observed 25-50% higher morphine content as compared to normal diploid plant. Similarly, *Artemisia annua* plant was induced polyploidy by using colchicine solution and it showed increased artemisinin content of 38% (Wallaart *et al*., 1999). However, Gao *et al*., (1996, 2002) have reported that the amount of active constituents also depended on the plant genotype. Two lines of *Salvia miltiorrhiza* showed an increase of 8.90-78.74% in total tanshinones and cryptotanshinone, whereas the other lines showed decrease (Gao *et al*., 1996). In *Scutellaria baicalensis*, one tetraploid line exhibited an increase in baicalin of 4.6%, whereas an additional 19 lines showed reduced baicalin production (Gao *et al*., 2002).

Reza *et al*., 2010 has produced autotetraploid plant of Dragonhead by the treatment of their apical meristem of seedlings with colchicine solution. Results of studying stomata morphology, phenotypic characters, chromosome count in root tip meristem and using flow cytometry profiles indicated that the application of

colchicine induced tetraploidy in seedlings. Tetraploid plants of *Dracocephalum moldavica Linn*., was healthy in appearance and produced more dry weight of leaf per plant as compared to diploid control plants. He reported that induction of autotetraploidy in dragonhead had a significant effect on content of essential oil. In this plant, the essential oil content of the vegetable organs increased by 27.5% in tetraploid plants.

2.12. Induction of Somaclonal Variation

Somaclonal variation is defined as genetic and phenotypic variation among clonally propagated plants of a single donor clone (Sunderland, 1973; D'Amato, 1977, 1985; Bayliss, 1980; Larkin and Scowcroft, 1981, 1983; Orton, 1984; Ahloowalia, 1986; Larkin, 1987; Lee and Phillips, 1988; Sun and Zheng, 1990; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993; Duncan, 1997; Kaeppler *et al.*, 1998; Veilleux and Johnson, 1998; Olhoft and Phillips, 1999). Somaclonal variation caused by the process of tissue culture is also called tissue culture-induced variation to more specifically define the inducing environment. Somaclonal variation can be manifested as either somatically or meiotically stable events. Somatically stable variation includes phenotypes such as habituation of cultures and physiologically induced variation observed among primary regenerants. The source for the somaclonal variation may be the change in the structure and number of chromosomes, chromosomal mosaicism, DNA methylation, altered sequence copy number, transposable elements and seems to be influenced by the genotype, explant type, culture medium or genetic disorders in the cells participating in regeneration process (Veilleux and Johnson, 1998; Jain *et al*., 1998). The advantage of this technique includes the possibility of greater variation comparable to that obtained through induced mutations. The variation may occur either in cytoplasmic genomes or in nuclear genome or in both (Jain, 2000).

2.13. Effect of Somaclonal Variation on the Content of Chromosomal Variation

In vitro induction of polyploidy plants has been of considerable interest for

researchers and has been used for obtaining new plant characteristics (Cheng and Korban, 2011). *In vitro* induction of tetraploids was first achieved in tobacco by Murashige and Nakano (1966) which paved the way for other plant species. The success for any in vitro approach depends very much on the existence of a reliable regeneration system, based either on organogenesis or embryogenesis (Carvalho *et al*. 2005). *In vitro* chromosome doubling can be induced by several antimitotic agents (Dhooghe *et al*., 2011). The most commonly used are colchicines such as in *Centella asiatica* (Tanavat *et al*., 2011), *Lagerstroemia indica* (Zhang *et al*., 2010), *Paulownia tomentosa* (Tang *et al*., 2010). However, oryzalin is often preferred to colchicine as a result of its reduced toxicity, higher affinity to plant tubulins, effectiveness at lower concentrations, and higher survival of plantlets (Hansen and Andersen, 1996; Ramulu *et al*., 1991 and Van Tuyl *et al*., 1992). Oryzalin has also been used successfully for *in vitro* ploidy manipulation in several genera, including *Smallanthus sonchifolius*, *Buddleia*, *Hypericum L*. *Miscanthus*, and *Rosa L*. (Dunn and Lindstrom, 2007; Kermani *et al*., 2003; Meyer *et al*., 2009; Petersen *et al*., 2003) and also trifluralin antimitotic agents used in *Ranunculus* (Dhooghe *et al*., 2009).

In vitro colchicine treatment of haploid calli was effective in inducing chromosome doublings, through which doubled haploid maize and wheat plantlets were obtained successfully (Wan *et al*., 1989; Hassawi and Liang, 1991), respectively. But colchicine also has a negative toxic effect, decreasing the survival rate and regeneration rate of tissue culture (Jahne and Lorz, 1995; Cohen and Yao, 1996; Song *et al*., 1997). Chromosomal variation has been observed in several in vitro culture derived plant species and their progenies (Ahloowalia, 1975, 1976, 1983; Duncan, 1997; Roth *et al*., 1997). Creissen and Karp (1985) reported that the high ploidy and high-chromosome explants show more variability than low ploidy and low – chromosome number species.

In vitro polyploid induction using colchicine has been achieved successfully in many fruit species such as apple (Shi *et al*., 1992), banana (Ganga and Chezhiyan, 2002), grapevine (Motosugi *et al*., 2002), citrus (Zeng *et al*., 2006), and pear (Kadota and Niimi, 2002). When colchicine was added to the medium, a long time was required for the induction of adventitious regeneration

(Kadota and Niimi, 2002). Because of their long juvenile periods, polyploid induction from zygotic embryos of fruit crops is not beneficial for shortening the breeding period. Polyploid plants induced directly during shoot regeneration from leaf explants have not been reported. Polyploidy in tissue culture-derived plants is generally results from endopolyploidization or nuclear fusion (Sunderland, 1977; Bayliss, 1980). The altered karyotypes in somaclones include chromosomal rearrangements as well as aneuploidy and euploidy. Aneuploidy may be caused by non-disjunction, aberrant spindles, lagging chromosomes, chromosome breakage that produces dicentric and acentric chromosomes (Sunderland, 1977).

2.14. Effect of Somaclonal Variation on the Content of Bioactive Components

Plants are capable of synthesizing an overwhelming variety of small organic molecules, which called secondary metabolites. In recent years considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products, normally secondary metabolites, for prevention and cure of different human diseases including microbial infections (Dubey *et al*., 2004). Moreover, the continuous and non-organized exploitation has resulted in many plants becoming rare or extinct. In view of commercial importance given to secondary metabolites, efficient production of bioactive compounds by tissue culture technology has gained popularity (Vaniserce *et al*., 2004). In modern medicine, plants are used as sources of direct therapeutic agents, as models for new synthetic compounds, and as a taxonomic marker for discovery of new compounds. They serve as a raw material base for the elaboration of more complex semisynthetic chemical compounds (Akerele, 1992; Anonymous, 2001). The synthesis of bioactive compounds chemically is difficult because of their complex structure and high cost (Anonymous, 2001). The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. To overcome this limitation, biotechnological approaches specifically plant tissue culture technology plays a

vital role in search for alternatives to production of desirable medicinal compounds from plants (Smetanska, 2008; Rao and Ravishankar, 2002).

For effective induction of secondary metabolite production, plant growth regulators (PGRs) are required (Al–Sane *et al*., 2005; Shilpashree and Ravishankar, 2009), since they have significant effects on the metabolism of secondary metabolites. The quality, as well as the quantity, of plant growth regulators, plays a major role in the production capability of a given *in vitro* culture. Plant growth regulators affect cell multiplication and division, which increases production of secondary metabolites (Staba, 1980; Weathers *et al*., 2005; Khan *et al*., 2008).

Aziz *et al*., (2007) was reported that the difference in distribution of asiaticoside and madecassoside between somoaclonal-derived plants and glasshouse–grown material. Roots of tissue culture–derived plants of the F-line, accumulated more of these compounds, compared with their glasshouse–grown counterparts. Conversely, for the S-line, the leaves of *in vitro* plants accumulated more of both compounds, compared with glasshouse–grown plants. However, production of triterpenes in leaf derived callus and cell suspension cultures of *Centella asiatica* was enhanced by the feeding of amino acids. In the callus culture manifold increase of asiaticoside accumulation was reported with the addition of leucien (Kiong *et al*., 2005).

Several researchers have carried out in the somaclonal studies of different medicinal plants, there was an increase in secondary growth compound, such as has been reported for *Bacopa monnieri* (Naik *et al*., 2010), *Centella asiatica* (Baek, 1997; Kim *et al*., 2004, 2002; Nath and Buragohain, 2005; Mangas *et al*.,2008). Baek, (1997) has reported 50% increase in the content of asiaticoside in micro-propagated shoots of *C. asiatica* as compared to those of field grown plants. Kim, *et al*., (2002) have reported that whole plants of *C. asiatica* derived from nodes are richer in asiaticoside. Manickam *et al*., (2000), Anjali *et al*., (2000) and Kulkarni *et al*., (1996) have cultured *Withania somnifera in vitro* with an objective of micropropagation.

Centella asiatica is an important herb in Indian Ayurvedic medicine and is popular as a nerve tonic to promote relaxation and to enhance memory. These properties have been ascribed to the active principles that belong to the β-amyrin ursolic acid group – asiatic acid, asiaticoside, madecassic acid and madecassoside. Over exploitation of certain plants for their medicinal application lead to the severe threat to the biodiversity and necessitated the implementation of modern conservation steps, in order to protect from extinction. Therefore application of tissue culture approaches for rapid multiplication of elite clones and germplasm conservation is of vital importance. Equally important is the generation of bioactive secondary products form this species. *C. asiatica* regeneration has been achieved using leaf derived callus (Mercy *et al*., 2011; Banerjee *et al*., 1999), stem segments (Patra *et al*., 1998) and nodal segments as explants (Kunta and Mani, 2011; Tiwari *et al*., 2000). The present finding which reveal the presence of asiaticoside in leaf callus culture to obtain maximum production of asiaticoside from first subcultured callus culture has been developed by Sholapur and Dasankoppa, (2011), Aziz *et al*., (2007), Nath and Buragohain, (2005) and Kim et al., (2004). Genetic transformation of *Centella asiatica* using Agrobacterium rhizogenes followed by elicitation with methyl jasmonate could enhance the bioaccumulation of asiaticoside in hairy roots (Kim *et al*., 2007). Recently, cDNA clones of genes associated with asiaticoside biosynthesis such as squalene synthase and cycloartenol synthase was studied in *C. asiatica* (Kim *et al*., 2007).

2.15. Methods for Assessing Triterpene Glycosides

Sharma and Arora, (2006) have reported in India most of the traditional knowledge about medicinal plants was in the form of oral knowledge that has been eroded or distorted due to the persistent invasions and cultural adaptations. There is a drastic reduction in Monographs on crude drugs and plant products in the Indian Pharmacopoeia 1955 to 1996. It is one of the reasons is not doing the estimation and validation of the adopted processes. Now-a-days the importance is not based on the isolation of a compound and its assay but also on the validation of the method which play an important role by the analyst in quality control or quality assurance of the product. The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceutics is

suitable for its intended use (Japanese Pharmacopeia, 2004). HPLC is a modern technique and a chemical standardize technique which is much more reliable and reproducible method for the standardization of both single and compound herbal formulation. It is the most often involved method for estimation of medicinal plant constituents. The marker constituent in the HPLC profile can be used as a method of identifying the presence of a particular compound in preparations (Chen *et al*., 2003). A question often asked by the growers is whether a tissue cultured and polyploidy plant has the same spectrum of active constituents as the plants growing in normal nature. This is one method by means of which genuinity of the drug and product uniformity can be confirmed.

Along with the use of *Centella asiatica* in medicine, the plant is also finding acceptance as a vegetable. *Centella* mainly contains asiatic acid, madecassic acid, terminolic acid, vanillic acid, succinic acid, asiaticoside, asiaticoside-B and madecassoside. The main active components of the plant are believed to be triterpenoids. Several studies have revealed the triterpenoid derivatives of *Centella asiatica* using HPLC techniques (Burnouf-Radosevich and Delfel, 1996, Varma *et al*., 1999; Indian Herbal Pharmacopoeia Revised, 2002; Kim *et al*., 2004; Rafamantanana *et al*., 2009). Gunther and Wagner (1996) also develop new HPLC method for isolation and determination of triterpene. The detection is done using the reversed-phase (RP) separation system with wavelength of 205 nm. A combination solvent of acetonitrile and water is used on RP column. In other investigation, combination of water (0.1%TFA), acetonitrile (0.1%TFA) and methyl tert-butyl ether (0.1%TFA) as gradient mobile phase were applied using Phenomenex Aqua 5mu C18 (Schaneberg *et al*., 2003).

The quantitative determination of triterpene saponin and aglycone extract from *Centella* plant, which is used for treatment of cellulitis, is widely reported in many studies. Phytochemical analysis is performed using reversed-phase high performance liquid chromatography (HPLC) coupled with photodiode array detector at 200nm (Morganti *et al*., 1999; Burnouf-Radosevich and Delfel, 1996). The phosphoric acid solution at 0.3% and acetonitrile has been used for efficient separation. Methanol and aqueous methanol effectively used for the extraction of triterpenes glycosides (Ling *et al*, 2000; Inamdar *et al*., 1996). The extraction of

asiaticoside is efficient in methanol with the amount of 0.36% dry weight compared to chloroform (0.30%), ethyl acetate (0.3%) and water (0.04%) (Varma *et al*., 1999).

Naik *et al*., (2010), Murthy *et al*., (2006) and Deepak *et al*., (2005) have reported that the extractions and high performance liquid chromatography (HPLC) analysis of *Bacopa monnieri* contain of bacoside A. Recently, the observations and determinations variations in secondary metabolite in different geographical areas of Nepal selecting high producing triterpene plants for possible cultivations (Devkota *et al*., 2010 A,B). Sharma *et al*., (2011) have also reported that rapid and validated method based on HPLC has been developed for quantitative determination of the compound asiatic acid in the whole blood of plant extract of *Centella asiatica*. Furthermore, HPLC analysis the morphological and chromosomal charactestics, the biomass, and the secondary metabolites accumulation of *Centella asiatica* at different ploidy levels were assessed (Tanavat *et al*., 2011; Rafamantanana *et al*., 2009; Randriamampionona *et al*., 2007) and from *in vitro* cultured *C. asiatica* (James *et al*., 2008; Nath and Buragohain, 2005).