CHAPTER 1

INTRODUCTION

1.1 MEDICINAL PLANTS AND THEIR REQUIREMENT WORLDWIDE

Medicinal plants have been used as a source for medicines since prehistoric years and the first physical evidence was reported from a cave in northern Iraq in a burial site of a Neanderthal man and it is believed to be sixty thousand years old (Solecki, 1975). Despite of many advanced techniques, still plants are being used for discovery of new drug as many compounds are either difficult to synthesize or the cost of synthesis is high (Newman et al., 2000, 2003; Butler, 2004; Oksman-Caldentey and Inzé, 2004). The process of synthetic drug discovery and development in USA takes around 12 years and approximately $ 230 million, whereas on the other hand plant based drugs take a comparatively less time and expenses (Ramakrishnappa, 2002). World Health Organization (WHO) reported that around 80% of the world's population, especially in developing countries, still relies on plant based medicines (Hegde, 2003; Vines, 2004; WHO, 2004; Gurib-Fakim, 2006).

Natural drugs have gained importance as the synthetic drugs have less efficacy and curative treatment for many chronic diseases have side-effects as well as costly to synthesize (Samuelsson, 2004; Petrovska, 2012; Pandey et al., 2013). Shu (1998) has reported that almost 60% of anti-tumour and anti-infectious drugs in the market are of natural origin. It is assumed that around 40% of the compounds which are being used in pharmaceutical industries and one quarter of the best selling drugs are directly or indirectly derived from plants (Rout et al., 2000; Butler, 2004). Worldwide requirement of herbal medicines as reported by WHO was $ 60 billion (KIT, 2003), which has now increased to around $ 120 billion and will reach $ 5 trillion by the year 2050 (Ganesan et al., 2016). 'WHO traditional medicine strategy 2014-2023' had been launched by WHO (2013) to promote the use of traditional and complementary medicine along with to ensure the safety, quality and effectiveness of herbal medicines.

However the immense use and overexploitation of medicinal plants has resulted in an increased threat on their existence, as more than 75% of the requirement is fulfilled by wild populations (Nalawade et al., 2003; Cole et al., 2007; Ali et al., 2016). Due to indiscriminate harvesting of medicinal plants approximately 20% of wild resources have been exhausted (Ross, 2005). There are around 50,000 to 80,000 plant species that are used for medicinal purposes worldwide out of which about 15,000 species have been extinct or threatened (Bentley, 2010). Plant based medicines for primary healthcare is used by many countries e.g. nearly 80% of population of Africa and 30-50% of China uses it. Whereas

75% of the patients in San Francisco, London and South Africa use traditional/complementary medicine for treatment of HIV/AIDS (Sen and Chakraborty, 2017). China and India are reported to have highest number of medicinal plants and hence majority of pharmaceutical industries rely on these two top exporters for botanical materials (Wakdikar, 2004).

1.2 INDIAN MEDICINAL PLANTS

India is a country with different religions and cultures, because of which several medicinal systems have developed within the country, and several other systems were introduced from outside (Sen and Chakraborty, 2017). The rich history of traditional system of medicines are mainly based on Ayurveda, Siddha, Unani, Homeopathy, Yoga and Naturopathy, out of which Ayurveda is the most ancient which is widely accepted and practiced (Mukherjee, 2001). The development of Indian traditional systems of medicines is because of the rich diversity of plants, and India is one of the 12 mega biodiversity centres and harbours nearly 8% of the global biodiversity (Wakdikar, 2004). India ranks first in percentage of plants having active medicinal ingredient and a total of 20% plant species are being used which is higher than the world's average i.e. 12.5% (Schippmann et al., 2002; Wakdikar, 2004). In India, more than 70% of rural areas still use traditional medicines, however the exact number of medicinal plants being used may vary but around 7000-7500 medicinal plants have been reported for the same (Pandey et al., 2013; Chen et al., 2016). There are approximately 7800 manufacturing units in India which produces plant based drugs and the market for Ayurvedic medicines is expanding at the rate of 20% (Subrat, 2002; Pandey et al., 2013). The trade of medicinal plant in India is worth Rs. 1000 crore/year and National Medicinal Plants Boards (NMPB) has documented that annual demand of botanical raw drugs is 3,19,500 MT of DW (Kala, 2004; Ved and Goraya, 2007). India was reported to export different herbal products with an annual growth rate of 16.8%, which increased from US$ 69 million in 2005-06 to US$ 128 million in 2009-10 (Scindia, 2010).

Despite of large annual requirement, still 90% of the plants are collected from the wild and only 10% are cultivated which has created gap between demand and supply of the plant material (Natesh, 2000; Rajasekharan and Ganeshan, 2002; Maiti, 2004). A report of Gujarat government stated that there is a huge gap between demand and supply of many

species in the state (Fig. 1, Vibrant Gujarat, 2017). It is observed that maximum gap between demand and supply is for climbers and herbs.

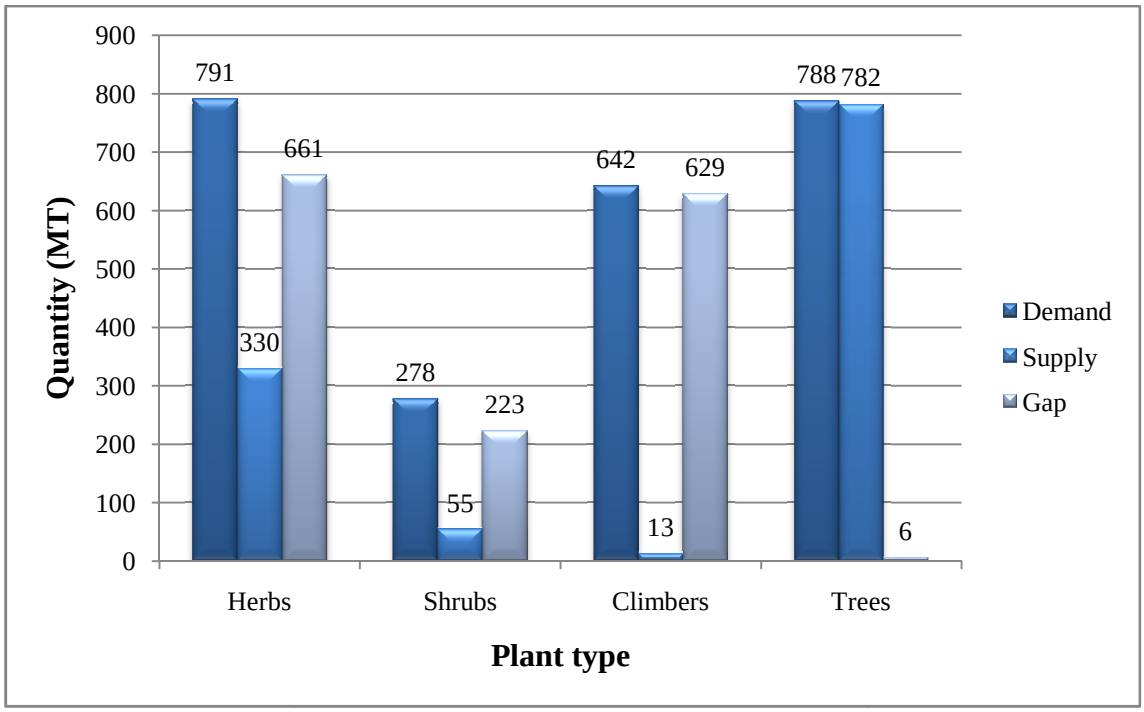

Figure 1: Demand and supply gap of different indigenous medicinal plant in Gujarat (Vibrant Gujarat Report, 2017)

Out of all the medicinal plants used in India, 34% are trees, 34% are shrubs and remaining 32% consists of herbs, grasses and climbers (Maiti, 2004). Climbers are a group of plants which are widely used in all traditional systems of medicines and Chaudhuri (2007) has documented around 265 climber species in India amongst which 125 are woody and the rest are herbaceous of which 100 species are medicinally important. However there is no report on the actual numbers being used for medicinal purpose (Ali et al., 2016). Apart from medicinal uses, climbers also form main components of ecosystems (Gentry, 1991; Jangid and Sharma, 2011). Many families have climbers and one of them is Asclepiadaceae (milkweed), which has around 250 genera and 2000 species which are distributed in tropical and subtropical regions of the world (Sinha and Mondal, 2011). This family contains many important medicinal climbers like *Ceropegia bulbosa*, *Ceropegia thwaitesii*, *Decalepis hamiltonii*, *Gymnema sylvestre*, *Hemidesmus indicus*, *Leptadenia reticulata*, *Pergularia daemia*, *Tylophora india* etc. which are widely used in Indian systems of medicines. The present study was carried out on *H. indicus* which is one of the important medicinal climber of this family.

1.2.1 *Hemidesmus indicus* (L.) R. Br.

Hemidesmus indicus (L.) R. Br. (Fig. 2) is commonly known as anantamula, sariva or Indian sarsaparilla, is a slender, laticiferous, semi-erect twining shrub. The plant is reported to be present in moist part of India, Iran, Pakistan, Bangladesh, Sri Lanka and Moluccas (Anonymous, 1959; Sasidharan, 2004; Siddique et al., 2004; Anonymous, 2005; Nayar et al., 2006). Leaves of this plant are simple, entire, opposite decussate, short petioled, extipulated, apiculate acute or obtuse. Whereas leaves of basal part are to linear to lanceolate often variegated with white above, dark green above but pale green/silvery white and pubescent beneath, and varies in their size. Stems are terete, slender laticiferous, with purplish brown bark which is thickened at nodes, twines anticlockwise and gives a wiry appearance. Flowers are greenish yellow to greenish purple outside, dull yellow to light purplish inside, calyx is deeply five lobed, gamopatelous, stamens five, pistil bicarpellary, ovaries free, many ovuled with distinct styles. Fruits are slender follicles, four inches long, cylindrical, sometimes curved, divaricate. Seeds numerous, black, flattened with a silvery white coma and roots are woody and aromatic (Austin, 2008).

1.2.1.1 Secondary metabolites

Plant contains diverse types of secondary metabolites of some of which are vanillin, rutin, β-sitosterol, isoquercitin (Chatterjee and Bhattacharya, 1955; Subramanian and Nair, 1968; Gupta et al., 1992), saponins and sapogenins (Anonymous, 1965), triterpenoids like lupeol, lupeol acetate, lupanone, α-amyrin, β-amyrin acetate (Padhye et al., 1973; Gupta et al., 1992), pregnane glycosides like desinine, indicine, hemidine, denicunine, heminine (Oberai et al., 1985; Prakash et al., 1991; Sigler et al., 2000), steroids like side-desinine, desminine, emidine, hemidescine, hemidine (Oberai et al., 1985; Prakash et al., 1991; Chandra et al., 1994; Deepak et al., 1997), coumarinolignoids like hemidine-1 and 2, hemidesmine, hemidesmine-1 and 2 (Mandal et al., 1991; Das et al., 1992; Mandal et al., 1995), hemidesmol, tannin, resin (Murthi and Seshadri, 1941), lactone, lupanone, 2-hydroxy-4-methoxy benzenoid/benzaldehyde, 3-hydroxy-4-methoxy benzenoid/benzaldehyde, 4-hydroxy-3-methoxy benzenoid/benzaldehyde, palmitic acid (Gupta et al., 1992; Alam and Gomes, 1998a, b) and oligoglycosides like medidesmine, indicusin, hemisine, desmine (Deepak et al., 1995, 1997).

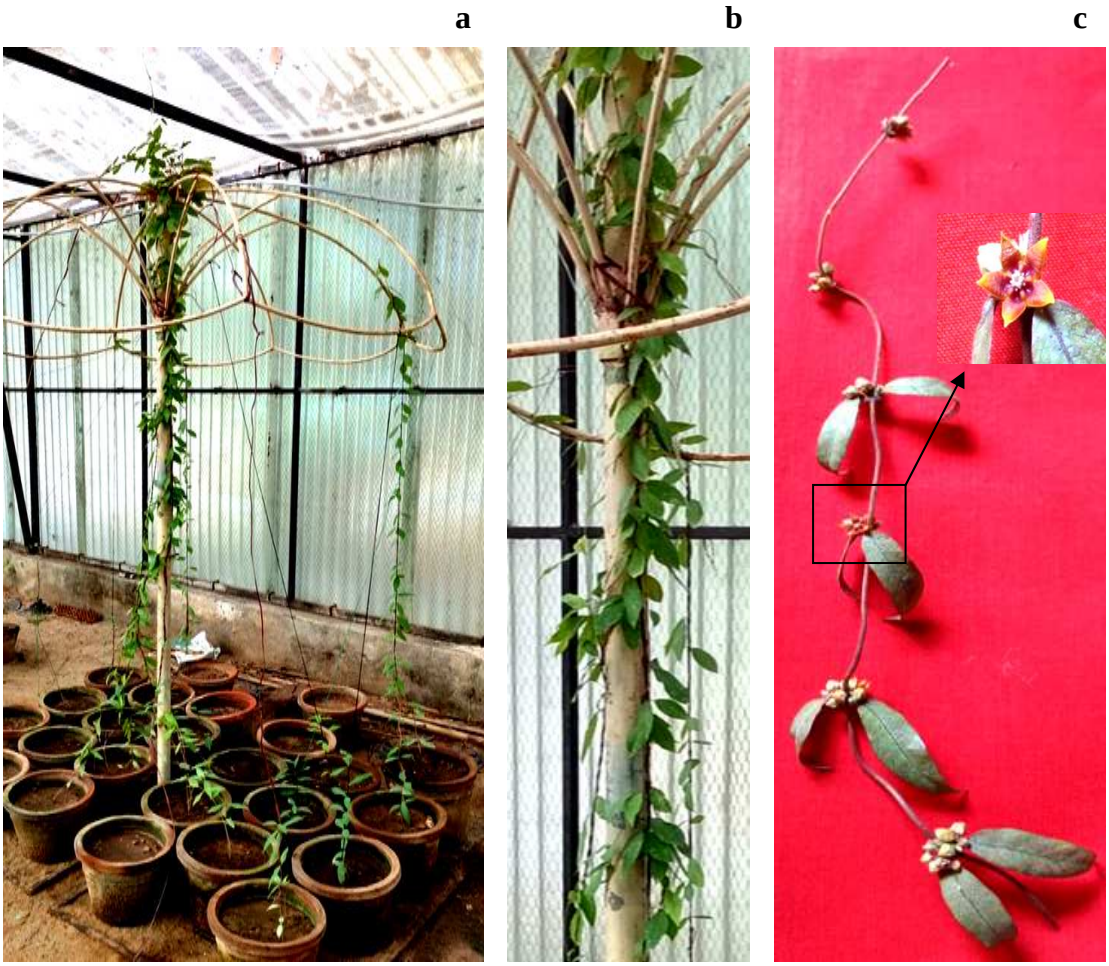

Figure 2: *Hemidesmus indicus*-(a) plants grown in green house, (b) inset and (c) flowering twig.

1.2.1.2 Medicinal properties

H. indicus is used in all three doshas and is also snigdha (oily, unctuous), guru (heavy), rasa (taste)-atikta (bitter), madhura (sweet), sheeta (cooling) and virya (potency) (Sastry and Sawanth, 2008). The karmas (actions) of the plant according to Ayurveda are durgandhanashana (removes odor), amahara (removes undigested food), raktaprasadana (purifies the blood), mutrajanana and mutravirajaniya (diuretic), stanyashodaka (lactus purifying), dahaprashamana (pacifies burning sensations) and sothahara (anti-inflammatory) (Weissner, 2014). The plant is reported as 'Rasayana' drug and is considered to give general well-being along with longevity, rejuvenation and give strength to bones and tissues (Puri, 2003).

Kirthikar and Basu (1935) reported that leaves are useful in vomiting whereas milky latex of stem is used to cure inflamed eyes. It has a high degree of effectiveness against gout dyspepsia, boils, leprosy and is reported to suppress both humoral and cell mediated immunity (Gupta, 1981; Khory and Katrak, 1981; Atal et al., 1986). This plant is widely used in treatment of biliousness, blood diseases, diarrhoea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (Nadkarni, 1989). It is traditionally used in gynaecological complications like leucorrhoea, amenorrhea and also used to gain vitality after child birth and milk secretions (Shukla et al., 2008).

Many workers reported that the plant is used in many other diseases/disorders and has following activities:

- Anti-angiogenic (Turrini et al., 2015)
- Anti-arthritis (Mehta et al., 2012a)
- Anti-cancerous (Das et al., 2012; Statti et al., 2015; Galhena et al., 2017; Turrini et al., 2018a, b)
- Anti-cataractous (Tirumani et al., 2018)
- Anti-diabetic (Sowmia and Kokilavani, 2007; Anuruddhika and Ekanayake, 2013)
- Anti-hepatotoxic (Prabakan et al., 2000; Saravanan and Nalini, 2008)
- Anti-inflammatory (Alam and Gomes, 1998b; Choudhury and Tetali, 2018)
- Anti-microbial (Das and Devaraj, 2006; Rao et al., 2013; Bonvicinia et al., 2018)
- Anti-mutagenic (Shetty et al., 2005; Aqil et al., 2008; Ananthi et al., 2010; Turrini et al., 2018a)

- Anti-nociceptive (Verma et al., 2005)
- Anti-osteoclastic (Di Pompo et al., 2014)
- Anti-osteoporosis (Desai et al., 2017)
- Antioxidant (Ravishankara et al., 2002; Gunathilake and Ranaweera, 2016; Joshi et al., 2018)
- Anti-ulcerogenic (Anoop and Jegadeesan, 2003; Korrapati et al., 2011)
- Antivenom (Alam et al., 1996; Alam and Gomes, 1998b; Das et al., 2016; Mani et al., 2018)
- Cardio protective (Khandelwal et al., 2011; Zarei et al., 2013)
- Hypolipidemic and hypoglycaemic (Venkateshan et al., 2016; Rana et al., 2017; Choudhury and Tetali, 2018)
- Inhibitory activity against acetylcholinesterase (Kundu and Mitra, 2013)
- Inhibitory activity against ischemia induced brain injury (Sivaraman et al., 2012)
- Insecticidal activities against mosquito larvaes (Mallikarjun et al., 2010; Joseph et al., 2011; Arjunan et al., 2012)
- Liver protective (Hazra et al., 2015; Lingaiah and Rao, 2016)
- Otoprotective (Previati et al., 2007)
- Renoprotective (Kotnis et al., 2004)
- Spermatogenic (Desai et al., 2017; Hosmani et al., 2018)

Traditionally this plant is also known as magical spiritual dream herb as the tea prepared from roots gives overall relaxing and calming sensation to the consumer (Fitwe et al., 2013). The root extract is used as a flavouring agent in the preparation of soft drinks and bakery products (Sarasan et al., 1994; Patnaik and Debata, 1996). The methanolic extract of root has tyrosinase inhibitory activity which prevent colour change and thus aid in the market value of the product (Kundu and Mitra, 2014).

1.2.1.3 Selection of this species

H. indicus is widely used in Ayurvedic, Unani and Homoeopathic medicines (Ayurvedic Pharmacopoeia Committee, 1989; Chatterjee and Sastry, 2000). NMPB has listed it amongst 178 species being consumed in volume exceeding 100 MT/year. The demand is around 500-1000 MT and cost of root is around 60-65 Rs/kg (Ved and Goraya, 2007). The plant has also been listed as one of the prioritized plants for cultivation under scheme of NMPB and is an official drug in Indian and British Pharmacopoeia

(Anonymous, 1996, 2003). It is not only an important plant of India but is also gained importance in European medicines since 1831 (Greenish, 1899) and later on in USA (Weissner, 2014).

The plant is conventionally propagated through seeds as well as stem cuttings, but the seed viability is very low and vegetative propagation is not feasible due to rapid degeneration of rootstocks (Austin, 2008). Rooting hormones fail to induce roots in stem cuttings and thus it is not feasible for mass propagation (Philip et al., 1991). Therefore the main source of supply is wild populations and due to indiscriminate harvesting many reports state that it has depleted in Western Ghats and may soon be endangered (Amalraj et al., 1991; Matthew, 1983; Sreekumar et al., 2000; Natarajan et al., 2004; Sukumaran and Raj, 2007). It is a vulnerable plant in Uttar Pradesh and Uttrakhand (Prakash, 2011) and rare in Sadhuragiri hills situated in Southern-Western Ghats (Aadhan and Anand, 2017). The depletion of plant from natural habitats has lead to scarcity of raw materials to the industries and conventional techniques are slow and cumbersome (Sreekumar et al., 2000). Planning commission (2000) reported that this plant is in short supply since many years and hence it has to be imported from other countries like Myanmar, Mexico and Morocco. This gap between demand and supply can be fulfilled by technique like plant tissue culture, an alternative to traditional propagation methods. It helps in conservation of important species and also enables a large quantity of plant material in a short time (George and Sherrington, 1984; Fasolo and Predieri, 1988). This in turn reduces the load on wild population and also fulfils the annual demand of pharmaceutical and herbal industries (Rao, 2004; Bapat et al., 2008; Yadav and Singh, 2012).

Table 1 summarizes some of the important products containing *H. indicus* alone or in combination with other medicinal plants. It suggested that the plant is not only used in Indian companies but also being used by Westerns companies due to its multipurpose medicinal properties.

1.3 PLANT TISSUE CULTURE

Gottlieb Haberlandt-a German botanist and father of plant tissue culture, first cultured the plant cells in Knop’s salt solution and proved the totipotent nature of plant cells under artificial culture conditions (Reinert, 1959; Krikorian and Berquam, 1969). Later Gautheret (1934, 1935) was first to report the culture of *Acer pseudoplatanus* cambial tissue.

Table 1: Products containing *H. indicus* as an ingredient

Products	Uses	Company
Anantamul Powder	Nourishment and rejuvenate the tissues. Cleans and detoxifies the body	Naturevibe Botanicals, New Jersey, USA
Anantmool Ghana	Tonic, diuretic and used in skin and urinary diseases	Chaitanya Pharmaceutical, Nasik, India
Ashwagandhadi Lehyam	Removes blood impurities and nerve tonic, and for weight gaining	Emami Ltd., Kolkata, India
Ashwagandharishta	Treatment of psychiatric, epilepsy, neurological disorder, arthritis and increase digestion	Emami Ltd., Kolkata, India
Brain 2	Boost memory and improve brain function	Meristem remedies, Ahmedabad, India
Dashmularishta	Prevents infectious problems of uterus, bladder, kidneys etc. Detoxifies the body, provides vitality and reduces weakness	Shree Baidyanath Ayurved Bhawan, Kolkata, India Emami Ltd., Kolkata, India
DeTox	Cleans the body and gives healthy balance	Yogi Products, Oregon, USA
Glohills	Nourish and reminerlize the skin	Herbal hills, Navi Mumbai, India
Hemohills	Blood purification and general wellness	Herbal Hills, Mumbai, India
Herboskin Capsules	Corrects burning and oozing of skin and useful in skin allergy, eczema and itches.	Annai Aravindh Herbals, Chennai, India
Imupsora	Psoriasis	Charak Pharma, Mumbai, India
Iobine	Common cold, tonsillitis, skin and other allergies	J. & J. Dechane, Hyderabad, India

Kanchanar Gomutra Ark	Use in goiter, tumors, fibroids and hormonal imbalance	Go Kripa Products, Rajkot, Gujarat
Kesh kanti hair oil	Provides deep nourishment, strengthens hair roots, reduce hair fall and prevents split ends	Patanjali Ayurved, Haridwar, India
Kidney Formula	Cleaning of blood and urinary tract, provide natural support for kidney and adrenal health.	Banyan Botanicals, Albuquerque, New Mexico, USA
Lipomap	Reduce cholesterol level, detoxify blood and increase fat metabolism	Maharishi Ayurveda, Fairfield, Iowa, USA
Mahanarayan Oil	Rejuvenates muscles and helps in joint movements and	Banyan Botanicals, Albuquerque, New Mexico, USA
Male Boost	Relax the anxiety and increase the male hormone	Nature Botanics, Nottingham, UK
Organic Anantamul	Supports genitourinary system, kidneys, urinary tract, detoxifier and promotes healthy skin	Joyful Belly, North Carolina, USA
Purodil	Skin infections like acnes and pimples	Aimil Pharmaceuticals, New Delhi, India
Renalka	Diuretic and relieves painful/burning urination along with maintain urinary pH	The Himalaya Drug Company, Bengaluru, India
Sariva	Urinary tract purification	Gopala Ayurveda, New Delhi, India
Sariva Capsules	Support a natural and balanced healthy lifestyle	Dr Wakde's Natural Health Care, London, UK
Sarsaparilla Powder	Blood purification, antioxidant, lowers hyperacidity and gastric problem	Bixa Botanical, Navi Mumbai, India
Skin Care Cream	Removes acne and psoriasis as well as prevents dark spots, shingles	Sanjeevani Health, Ahmedabad, India

	eczema and	
Sleep Support Blend	Gives relief from insomnia, stress and mental weakness as well as improves memory and immunity	Bipha Drug Laboratories, Kerala, India
Styplon	Helps in healing wounds and control hemorrhage activity	The Himalaya Drug Company, Bengaluru, India
Urtiplex	Antiallergic and anti-itching	Charak Pharma, Mumbai, India
Veria So Hers	Supposed hormonal balance and vitality in women	Vitality Medical, Utah, USA
Winomed	Relaxes tired nerves and improve poor appetite and immunity	JMD Medico, Mumbai, India
Winsoria	Psoriasis and eczema	Kerala Ayurveda, New Delhi, India

The increasing demand of medicinal plants necessitates their large scale propagation and to cope up with it, *in situ* and *ex situ* conservation techniques are used which helps in protecting and conserving important medicinal plants (Paunescu, 2009; Kasagana and Karumuri, 2011; National Medicinal Plants Board, 2015). Vegetative propagation produces genetically uniform plants but there are certain limitations of these techniques arise when the mother plant is infected and only a few disease free plants can be produced. To overcome this, biotechnological approach like plant tissue culture (*ex situ* conservation) can be useful which not only fulfil the demand but also helps in conservation of important plants and in turn prevent possible extinction of the medicinally valuable species (Sridhar and Aswath, 2014). Another advantage is that the rapid production of disease-free plants can be done irrespective of the seasonal constrains using small tissue as an explant in relatively small space (Sharma and Arya, 2016). Tissue culture technique for the plant propagation is essential for conservation as it supplements seed banking as well as other *ex situ* techniques (Thorpe, 2007; Sarasan, 2010). It was reported to be responsible for bringing second green revolution in our country (Singh and Shetty, 2011). This approach is routinely used for propagation of medicinal plants which are used in large quantities from wild (Pence, 2010).

The discovery of cytokinin to auxin ratio which decided the fate of regenerating tissue was the first breakthrough in the field of tissue culture (Skoog and Miller, 1957). Different types of artificial media were used for *in vitro* regeneration, but Murashige and Skoog (1962) developed a medium which is widely used as a nutrient medium in plant tissue culture till date (Thorpe, 2007). Regeneration can be achieved utilizing various plant parts but amongst them leaves and nodes have been frequently used as an explant; leaf explant are reported to be suitable for establishing shoot cultures in medicinal plants like *Tylophora indica* (Thomas and Philip, 2005), *Bacopa monnieri* (Joshi et al., 2010), *Acmella calva* (Amudha and Shanthi, 2011) and *Ajuga bracteosa* (Kaul et al., 2013). Whereas nodes are used in many species and few examples are cited as follows: *Piper longum* (Soniya and Das, 2002), *Eclipta alba* (Baskaran and Jayabalan, 2005) and *Swertia chirata* (Balaraju et al., 2009).

Another application of plant tissue culture is secondary metabolite production as many pharmaceutical industries use plants for the production of drugs (Chand et al., 1997). Extraction of metabolites from plants requires large quantities of plant materials for e.g. 2.5 kg of taxol can be extracted by harvesting around 27,000 tons of *Taxus brevifolia* bark which requires cutting of around 12,000 trees (Rates, 2001). Wild plants are under environmental influence, prone to pathogens, show batch to batch variation due to genetic and/or seasonal variation, association with other plants in their habitat and differences in the soil as well as climatic conditions which affects their medicinal value (Geng et al., 2001). Thus *in vitro* cultures are an alternative way through which important compounds can be produced and extracted without affecting the wild populations of plants (Rates, 2001). These cultures are able to synthesize the same metabolites as mother plant, which ensures that full genetic characteristics of the mother plant is transferred to regenerated plant (Zhou and Wu, 2006). Plants grown through tissue culture techniques are not limited by sources of nutrients or seasonal constraints, and are now commonly used for metabolite production (Collin, 2001). It has been proved to be an effective method for metabolite production as *in vitro* cultures are maintained under controlled conditions and can be manipulated to get desired results (Parr, 1989; Murch et al., 2000; Vanisree and Tsay, 2004; Rani and Kumar, 2017). Another advantage of cultures is the rapid synthesis of secondary metabolites as compared to wild plant (Dornenberg and Knorr, 1995).

1.4 SECONDARY METABOLITES

Plant produces different types of metabolites as a result of metabolic processes; primary metabolites play a role in plant growth whereas secondary metabolites play crucial role in ecophysiology of plants (Briskin, 2000). Plants are non-mobile and hence susceptible to attack by pathogens and predators, and to overcome this enormous number of secondary metabolites are produced by them as part of defence mechanism (Bennett and Wallsgrove, 1994; Oksman-Caldentey and Inzé, 2004). These compounds have other roles to play such as attracting pollinators and symbionts, protecting plants against different abiotic stresses like temperature, water, light, UV and minerals etc. (Kaufman et al., 1999; Wink and Schimmer, 1999). Some of the metabolites take part in activities at cellular level such as plant growth regulators, modulators of gene expression and in signal transduction (Kaufman et al., 1999). These compounds not only help plants to adapt themselves according to their surroundings but are also useful for mankind as pharmaceuticals, agrochemicals, aromatics and food additives (Rao and Ravishankar, 2002; Oksman-Caldentey and Inzé, 2004).

Secondary metabolites are mainly grouped into three classes i.e. terpenes, phenolics and nitrogen containing compounds (Krzyzanowska et al., 2010; Rea et al., 2010) and on the basis of their biosynthetic origins, they are divided into five groups: polyketides, isoprenoids, alkaloids, phenylpropanoids and flavonoids (Oksman-Caldentey and Inzé, 2004). Unlike the primary metabolites, which are common amongst all the plants, secondary metabolites have restricted distribution and differ according to the species (Taiz and Zeiger, 2006). Some of the important compounds which are still extracted from plants are: ajmalicine (*Catharantus roseus*), ajmaline (*Rauwolfia serpentina*), artemisinin (*Artemisia annua*), berberine (*Coptis japonica*), colchicines (*Colchium autumnale*), digoxin (*Digitalis lanata*), ginsenosides (*Panax ginseng*), morphine (*Papaver somniferum*), quinine (*Cinchin ledgeriana*), shikonin (*Lithospermum erythrorhizon*), taxol (*Taxus brevifolia*) and vincristine and vinblastine (*Catharantus roseus*) (Rao and Ravishankar, 2002), which either not have any synthetic substitute or difficult to synthesize.

1.4.1 Production of Secondary Metabolites in Shoot Cultures

Secondary metabolites' synthesis is known to depend on physiological as well as developmental age (Dixon, 2001; Oksman-Caldentey and Inzé, 2004). For e.g. taxol production reaches maximum when *Taxus* trees reach around 60 years (Bedi et al., 1996)

whereas *Panax ginseng* roots are grown for six years before harvesting (Bonfill et al., 2002). In comparison to this, *in vitro* cultures are proved to be a potential alternative as they have high metabolic rate due to fast proliferation and thus synthesis of compounds is relatively faster (Srivastava and Chaturvedi, 2011). *In vitro* production of metabolites can be done via callus/cell suspension cultures or shoot and root cultures (Karuppusamy, 2009). But the production is generally higher and stable in organ cultures like shoot and root due to presence of complex cell/tissues which are metabolically more potent (Dornenberg and Knorr, 1995; Collin, 2001; Biondi et al., 2002). Study on *in vitro* cultures of *Ruscus aculeatus* revealed that organogenesis is required for saponin production (Palazon et al., 2006), whereas only redifferentiated cultures produce picroside in *Picrorhiza kurroa* (Sood and Chauhan, 2009).

Some of the plants in which shoot cultures were used for secondary metabolite production are vasine from *Adhatoda vasica* (Shalaka and Sandhya, 2009), asiaticoside from *Centella asiatica* (Kim et al., 2004), hypericin and hyperforin from *Hypericum perforatum* (Santarem and Astarita, 2003; Karppinen et al., 2007), terpenoid from *Mentha arvensis* (Phatak and Heble, 2002), flavonoid from *Salvia officinalis* (Grzegorczyk and Wysokinska, 2008) and withaferin A from *Withania somnifera* (Ray and Jha, 2001).

1.4.1.1 Qualitative and quantitative analysis of shoot cultures

Plant cells are totipotent in nature and *in vitro* cultures also contain genetic information identical to mother plants, hence they are able to synthesize metabolites like mother plants (Srivastava and Chaturvedi, 2008). However sometimes variation arises through tissue culture which may alter the biosynthetic potential of *in vitro* cultures and needs to be assessed (Natesh, 2001; Mohanty et al., 2011; Pathak and Joshi, 2017). This chemical integrity of cultures can be assessed using different chemical, biochemical, molecular and chromatographic techniques (Gan and Ye, 2006; Xiaoqiang and Gang, 2006; Singh et al., 2011c). High performance thin layer chromatography (HPTLC) has advantages over other chromatography techniques as it is fast and economical, many samples can be analyzed simultaneously, requires relatively less solvent and results are reproducible (Srivastava, 2011; Siddiqui et al., 2017). In this technique, plant extracts are separated on TLC plates followed by band development which later on can be analyzed through densitometry scanning to convert them into respective peaks having specific Rf, peak height and area (Moffat, 2001; Gallo et al., 2008, 2011; Nicoletti, 2011; Ram et al.,

2011). Reports suggest that if standard sample of the compound is unavailable, these fingerprints can become a basis for comparison using number, sequence, position and colour of the bands (Mammen et al., 2011; Kamboj and Saluja, 2013). HPTLC fingerprint have been utilized to assess the chemical integrity of *in vitro* cultures of *Asparagus adscendens* (Mehta and Subramanian, 2005), *Celastrus paniculatus* (Martin et al., 2006), *Bacopa monnieri* (Patni et al., 2010), *Kaempferia galanga* (Mohanty et al., 2011) and *Withania somnifera* (Shetty and Chandra, 2012).

As *in vitro* cultures are able to synthesize metabolites, sometimes the content of metabolite is higher than mother plants (Singh and Chaturvedi, 2013), as observed by Dandin and Murthy (2012) for andrographolide synthesis in shoot cultures of *Andrographis paniculata*. Similarly synthesis of metabolites increased in *in vitro* shoot cultures of *Huernia hystrix* (Amoo and Van Staden, 2013) and *Myrtus communis* (Cioć et al., 2018) in comparison to *in vivo* plants. Quantification of metabolites has been also reported in *in vitro* shoots of *Spilanthes acmella* (Singh and Chaturvedi, 2010) and *Hypericum perforatum* (Savio et al., 2012). However synthesis of metabolites under *in vitro* condition depends on many factors and PGRs is one of the important factor (Jaleel et al., 2009; Coste et al., 2011). Synthesis of metabolites in *in vitro* shoots of *Aloe arborescens* is reported to be affected by the presence of PGRs in medium and type as well as concentration enhanced the metabolite content in comparison to *in vivo* shoot (Amoo et al., 2012).

However secondary metabolites are either produced constitutively or in response to particular stress or pathogenic attack (Morrissey and Osbourn, 1999; Wittstock and Gershenzon, 2002). One of the methods to enhance the metabolite production under *in vitro* condition is to treat the cultures with elicitors (Oksman-Caldentey and Hiltunen, 1996; Karuppusamy, 2009).

1.4.1.2 Elicitation of secondary metabolites in shoot cultures

Treatment of *in vitro* cultures with elicitors is an effective strategy to enhance secondary metabolite production (Verpoorte et al., 1999; Chong et al., 2005; Smetanska, 2008). It can trigger the physiological and/or morphological responses along with accumulation of metabolites (Namdeo, 2007). Earlier this term was used for molecules which induced phytoalexins, but then it is being used for all the compounds which promote various kinds of defence systems (Hahn, 1996; Nürnberger, 1999). They produce an

osmotic pressure which stimulates the defence factors and causes activation of key enzymes of various metabolic pathways (Gagnon and Ibrahim, 1997; Radman et al., 2004). Zhao et al. (2005) later on observed that they act as signal molecules and switch on signal transduction network inside the cell. This cascade further activates transcription factors regulating the expression of secondary metabolite genes. The first study was reported by Keen (1975) who treated *Glycine max* cv. *Harosoy* culture with *Phytophthora megasperma* fluid for phytoalexin production. Elicitation can be done in static or liquid media, however later is beneficial as it provides uniform conditions, support rapid growth and feasible for scalling up at bioreactor level (Zhou and Wu, 2006).

Different types of elicitors are reported to enhance *in vitro* production of different groups of metabolites like alkaloids, flavonoids, terpenoids, coumarins and phenolic compounds (Brader et al., 2001; Wang et al., 2001). They are classified on the basis of their nature i.e. abiotic or biotic, or on the basis their origins i.e. exogenous and endogenous elicitors (Namdeo, 2007). Abiotic elicitors are chemicals like methyl jasmonate and salicylic acid, heavy metals like Cu^{2+}, Cd^{2+} and Ca^{2+} ions and physical factors like UV irradiation and wounding. The biotic elicitors have biological origins like plant (pectin or cellulose) or micro-organisms cell walls (chitin or glucans), glycoproteins, G-protein or intracellular proteins, chitosan, fungal homogenate, yeast extract etc. (Veersham, 2004; Vasconsuelo and Boland, 2007).

One of the commonly used biotic elicitor yeast extract (YE) is used in experiments of plant-microbe interactions (Korsangruang et al., 2010). It has complex composition having amino acids, vitamins, minerals and ions like Zn, Ca and Co due to which it triggers the metabolite synthesis (Ertola and Hours, 1998; El-Nabarawy et al., 2015). A number of findings have documented the use of YE as an elicitor in shoot cultures of *Bacopa monnieri* (Kamonwannasit et al., 2008), *Drosera burmanii* (Putalun et al., 2010), *Ruta graveolens* (Diwan and Malpathak, 2011) and *Psoralea corylifolia* (Ahandani et al., 2013). Plant hormones like salicylic acid (SA) and methyl jasmonate (MJ) are abiotic elicitors, which are known to elicit a wide range of compounds by inducing the expression of genes for various biosynthetic pathways (Baenas et al., 2014). SA is reported to be an essential component in the activation of numerous plant defence responses and induce the synthesis of phytoalexins (Li et al., 2003; Hayat et al., 2010). Shoot cultures of *Hypericum hirsutum* and *H. maculatum* (Coste et al., 2011), *H. perforatum* (Gadzovska et al., 2013) and *B. monnieri* (Largia et al., 2015) increased the metabolite content after treatment with

SA. MJ has been reported to induce wide array of chemical defences and also act as transducers of elicitor signals for the production of metabolites (Farmer et al., 2003; Wolucka et al., 2005). It is reported to enhance the metabolite content in *Nigella sativa* (Scholz et al., 2009), *Bacopa monnieri* (Sharma et al., 2013a), *Gentiana straminea* (Zhao et al., 2013) and *Solenostemon scutellarioides* (Sahu et al., 2013) shoot cultures.

1.4.2 Gene Expression Analysis for Secondary Metabolite Pathway

Higher plants contain large genome size, and are known to have around 20,000 to 60,000 genes of which approximately 15–25% encodes enzymes for secondary metabolism (Bevan et al., 1998; Somerville and Somerville, 1999). Elicitation is reported to increase the expression of genes related to secondary metabolites pathways which in turn increase the production of metabolites (Zhang et al., 2016; Jiao et al., 2018). However many studies have confirmed the effect of elicitors on metabolite accumulation and in depth studies regarding its effect at molecular level is lacking (Yin et al., 2014). Elicitors are known to induce signal transduction pathways which vary according to type and concentration of elicitors as well as culture and plants. Hence identifying the signalling network during elicitor reveals the interactions between signal transducers and genes of secondary metabolite biosynthesis (Xu et al., 2005; Zhao et al., 2005; Goel et al., 2011). Functional genomics approaches are reported to be an important tool to understand the secondary metabolism in plants (Naoumkina et al., 2010). Transcriptional analysis not only helps in investigating the effect of elicitors as well as in elucidating the molecular events involved in the biosynthetic pathway (Fan et al., 2013). Understanding the biosynthetic pathway of metabolites will also help in genetic engineering of plants for enhanced production of metabolites (Moses et al., 2015).

Keeping all these in mind it was thought that a rapid regeneration protocol needs to be developed using leaf and/or nodal explant of *H. indicus*. The shoot cultures developed from nodal explants were analyzed qualitatively and quantitatively for metabolites like lupeol and rutin. Elicitation of shoot culture was also tried using abiotic (YE) and biotic (SA and MJ) elicitors and based on this following hypothesis is proposed.

1.5 HYPOTHESIS

In vitro regeneration of *H. indicus* can be achieved utilizing leaf and nodal explant efficiently. Shoot cultures which are developed from nodal explants are generally reported to be genetically uniform and hence these shoots will be selected for secondary metabolite studies. They will be assessed qualitatively using chemical profiling along with quantification of lupeol and rutin. Elicitation studies will be carried out on shoot cultures utilizing elicitors like YE, SA and MJ to enhance the content of lupeol and rutin in shoots.

1.6 OBJECTIVES

- Development of shoot cultures from leaf and nodal explants
- Isolation and quantification of lupeol and rutin from *in vivo* and *in vitro* shoots
- Elicitation and quantification of lupeol and rutin in shoot cultures
- Identification of expressed genes using molecular tool