1. INTRODUCTION

1.1 Malaria a deadly disease

Malaria is a major scourge of humankind which continues to defy science and technology. It is a major health problem in many developing countries, mostly in Africa and Southeast Asia (Snow *et al*., 2005). According to WHO report on malaria (2007& 2012), 40% world's population is living with risk of malaria and over 1.5 million death occur per year, the cost of malaria treatment is $1,800 million US dollar. Most victims of malaria are the children from sub- Saharan Africa, but victims are also counted all over the tropical world. Fifty eight percent of malaria deaths occur in the poorest 20% of the population (Barat, 2002).With a rapidly growing population in regions with high malaria transmission, it has been estimated that in the absence of effective intervention strategies, the number of malaria cases will double over the next twenty years (Bremen, 2001). The parasite responsible for the vast majority of fatal malaria infection is *Plasmodium falciparum*. The global malaria situation is deteriorating faster today than at any time in the past century. Children are especially vulnerable, as more children die from malaria than any other single disease. Pregnant women and especially primigravidae are the next highest risk group for malaria in malaria endemic areas. It is stated that malaria causes 0.96 million deaths of children per year in Africa alone (WHO, 2014). Global pattern of malaria distribution is presented in Fig. 1. 1.

According to the World Malaria Report 2014, 22% of India's population live in high transmission (> 1 case per 1000 population) areas, 67% live in low transmission (0–1 cases per 1000 population) areas and 11% live in malaria-free (0 cases) areas (WHO, 2014). In 2013, 0.88 million cases have been recorded, with 128 million tests being conducted on the suspected cases, with *P. falciparum* causing 53% and *P. vivax* causing 47% of the infections. The incidence of malaria in India accounted for 58% of cases in the South East Asia Region of WHO (WHO, 2014).

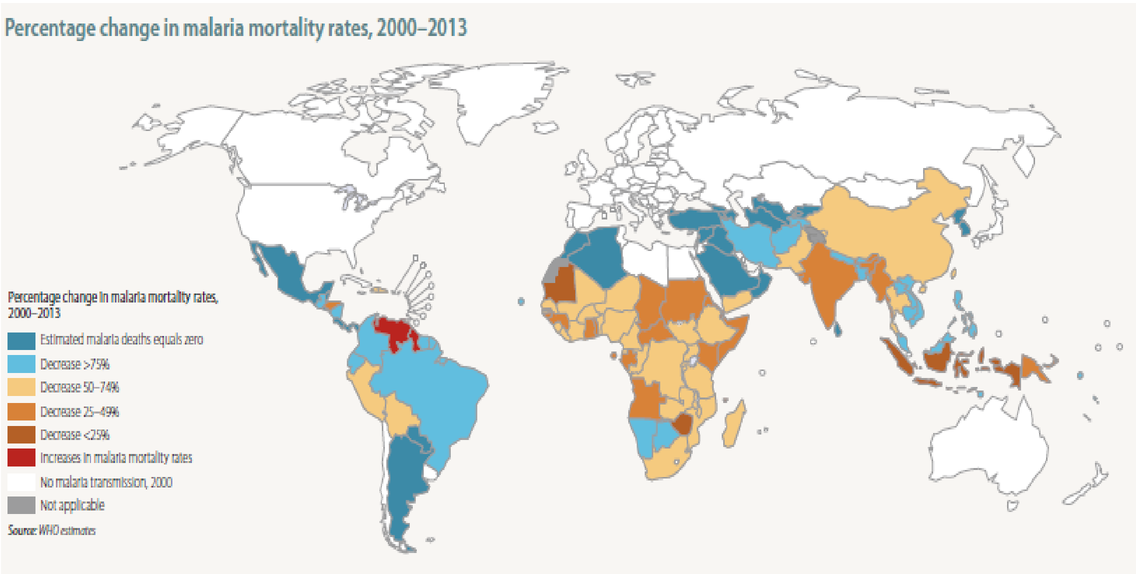

Fig. 1.1: Global pattern of malaria distribution. (Source: World Health Organization, Geneva, 2014).

The biggest burden of malaria in India is borne by the most backward, poor and remote parts of the country, with >90-95% cases reported from rural areas and <5-10% from urban areas; however, the low malaria incidence in urban areas may be due to almost non-existing surveillance.

Unbridled urbanization, drought, migration of workers, and lax control efforts are all contributing to the resurgence of malaria in India and the problem is expected to exacerbate in the years to come. With increasing global warming, it is projected that in 2050s, malaria is likely to persist in Orissa, West Bengal and southern parts of Assam, bordering north of West Bengal, but may shift from the central Indian region to the south western coastal states of Maharashtra, Karnataka and Kerala. Also the northern states, including Himachal Pradesh and Arunachal Pradesh, Nagaland, Manipur and Mizoram in the northeast may become malaria prone (Bhattacharya, 2006).

1.2 Drug history of Malaria

The first effective antimalarial drug was quinine, which was isolated from the bark of Cinchona. The discovery of quinine was followed by an era of synthetic organic chemistry that led to the development of synthetic antimalarial drugs, using the molecular frame work of quinine as a template. A number of useful aminoquinoline-based antimalarials synthesized include pamaquine, chloroquine, amodiaquine, pentaquine, primaquine and meflaquine (WHO, 1986), but unfortunately, *P. falciparum* was shown to have developed resistance to most of these antimalarials (Ridley, 2002). Since then, the incidence of drug resistant *P. falciparum* has been increasing at a faster rate than that of the efforts for development of new drugs. The search for a vaccine has been plagued by a number of shortcomings. Many of the short comings are related to antigenic variation, antigenic diversity, and immune evasion mechanism exhibited in various stages of the complex life cycle of malaria parasites. Thus with the problem of resistance at one hand and unavailability of vaccine on the other, it becomes inevitable to look for an alternative drug that would cure this deadly disease.

A number of control measures employed against malaria have their weakness. For instance, some strains of the malaria parasite have developed resistance to traditional treatments using quinine and chloroquine, which were previously effective (Snow *et al*., 2005). There is therefore an urgent need for affordable and effective treatment alternatives. The World Health Organization (WHO) recommended use of Artemisinin-Based Combination treatments such as artemether-lumefantrine, sulfadoxine/pyrimethamine as the first line treatments for multidrug – resistance strains of malaria (artesunate-mefloquine, artesunate-amodiaquine, and artesunate-sulfadoxine) (WHO, 2004).

Artemisinin a sesquiterpene-lactone with an endoperoxide bridge, isolated from the aerial parts of *Artemisia annua* L. has shown strong potential as the antimalarial drug (Li *et al*., 1982; Wernsdorfer, 1994; Abdin *et al*., 2003). Unlike quinine related drugs and antifolate drugs, artemisinin and its derivatives are the most potent and rapidly acting antimalarial drug, the parasite biomass is reduced by 10,000 fold per asexual life cycle compared to 100 to 1000 fold for other antimalarials. They also decrease gametocyte carriage by 90%, thus reducing transmission of malaria (White *et al*., 1999). Artemisinin-derived drugs have low toxicity because they target protozoan cells that are loaded with iron, acquired through the *Plasmodium* feeding on hemoglobin (Ferreira, 2004; Krishna *et al*., 2004).

More recently, it has also been shown to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon cancer and small cell-lung carcinomas (Efferth *et al*., 2001; Singh and Lai, 2001). Due to its current use in artemisinin based-combination therapy (ACT), its global demand is increasing continuously. Due to this discovery, *A. annua* L. is now rated as one of the top ten industrial crops of the modern world.

1.3 *Artemisia annua* L.

1.3.1 Range and distribution

The plant is native to China but is currently found in many countries. *A. annua* occurs naturally as part of vegetation in the northern parts of Chahar and Suiyuan provinces (40N, 109E) in Northern China, at 1000–1500m above sea level (Wang, 1961). The plant grows in many countries, such as Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, USA and former Yugoslavia (Klayman, 1993). The crop is grown in China and Vietnam as a source of artemisinin and cultivated on small scale in the USA as source of aromatic wreaths (Klayman, 1993).

The geographic range of *A. annua* is paramount in determining areas for potential cultivation. Although *A. annua* originated in relatively temperate latitudes it appears it can grow well at much lower tropical latitudes with lines which are either found in these areas or which have been adapted by breeding (Magalhaes, *et al*., 1996). The current availability of late-flowering clones makes it possible to cultivate *A. annua* in areas, which were previously considered unsuitable due to their proximity to the equator, and short photoperiod. The high artemisinin concentrations (0.5– 1.5%) in the leaves of some of these clones could allow high artemisinin yields in tropical latitudes, such as Vietnam, Madagascar and sub-Saharan Africa, even though the leaf biomass may not be as high as some strains of *A. annua* grown in temperate latitudes (Delabays *et al.*,1993). The influence that higher altitudes have on the production of *A. annua* at tropical latitudes is a principle that could be applied to parts of tropical Africa and elsewhere. Currently, with the opportunity offered by the availability of late-flowering clones and the world demand for artemisinin, several international agencies are carefully analyzing the possibility of cultivating *A. annua* in tropical countries including Kenya and Tanzania (Technoserve, 2004).

Artemisia annua plant was introduced by CIMAP at Kashmir in 1986. The first variety was developed in 1990 which was named Asha (0.1% AMS). Jeevan Raksha (0.7-1.0%) variety was the second to be developed in 1998 by CIMAP

CIMAP (Cental Institute of Medicinal and Aromatic Plants, Lucknow, Indiia) successfully developed and released a variety named 'Jeevan Raksha' from an isolated population containing high artemisinin in the foliage (0.5–1.0 %) (Kumar *et al*. 1999). This plant 'Jeevan Raksha' not only produces high artemisinin but also maintains the synchronized conversion to higher level of artemisinin during May to October. (Tandon *et al*., 2003).

CIMAP is using molecular breeding techniques with *Agrobacterium tumefaciens* to enhance the production of artemisinin. *Agrobacterium* tumefaciens-mediated system of high efficiency of genetic transformation and regeneration of *A. annua* has been established (Delabays *et al*., 1993). The process of identifying a few more genes in *A. annua* that, if transplanted into *Escherichia coli* could enable the bacterium to go a few extra steps in the chemical process and produce artemisinic acid, a precursor of artemisinin is being investigated (Srivastava, 2002).

1.3.2 Taxonomy, morphology and phenology of *A. annua* L.

Kingdom: Plantae

Division: Dicotyledons

Class: Gamopetalae

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *annua*

(Bentham and Hooker, 1862-1883)

Artemisia annua (*A. annua* L.) also known as sweet wormwood, originated from China and is the most important and popular herbaceous plant in the daisy family Asteraceae (McVaugh, 1984). The crop grows to a height of 1 – 3 m and 1m in width and it is an annual plant with a growth cycle of about 190 days (80 days in the nursery and 110 days on the field) (Ferreira and Janick, 1995). The plants are generally longer lived, more hardy and aromatic when grown in poor dry soil. *A. annua* is a large shrub with a single-stem and alternate branches and the leaves are aromatic; fern-like and deeply dissected (Fig. 1.2) and ranges from 2.5cm to 5cm in length (Whipley *et al.*, 1992).

The plant has a short tap root and aggressive fibrous root (Laughlin, 1994). Mitchell (1975) noted that *A. annua* has flowers which are greenish-yellow and about 2 to 3 mm in diameter. He further found out that the pollen has no spines but is extremely allergic.

The most valuable parts are the leaves and the flowers where artemisinin is concentrated (Ferreira and Janick, 1995). Glandular trichomes are more prominent in the corolla and receptacle florets. There is strong evidence that artemisinin is sequestered in the glandular trichomes (Duke and Paul, 1993). Artemisinin is the most important active pharmaceutical compound.

1.3.3 Phytochemistry

The main constituent of the plant is artemisinin, which is obtained from the aerial parts of the plant and varies from 0.05-0.17% with an average of 0.1%. It is a promising anti-malarial drug effective against *Plasmodium vivax* and *P.falciparum*. the distillation of aerial parts of plant also yield essential oil (0.2-0.4%) which comprised of many chemical constituents with the major compounds including myrcene (3.8%), 1,8- cineole (5.5%), *A. annua* ketone (66.7), linalool (3.4%), camphor (0.6%), alpha-pinene (0.032), camphene (0.04), β- pinene (0.882%), borneol (0.2%) and β-caryophyllene (1.2%). The essential oil is used in perfumery, cosmetics, dermatology and also have fungicidal properties (Brown, 2010).

It contains many different classes of compounds: at least 28 monoterpenes, 30 sesquiterpenes, 12 triterpenoids and steroids, 36 flavonoids, 7 coumarins and 4 aromatic and 9 aliphatic compounds (Bhakuni *et al*., 2002; Tang and Eisenbrand, 1992).

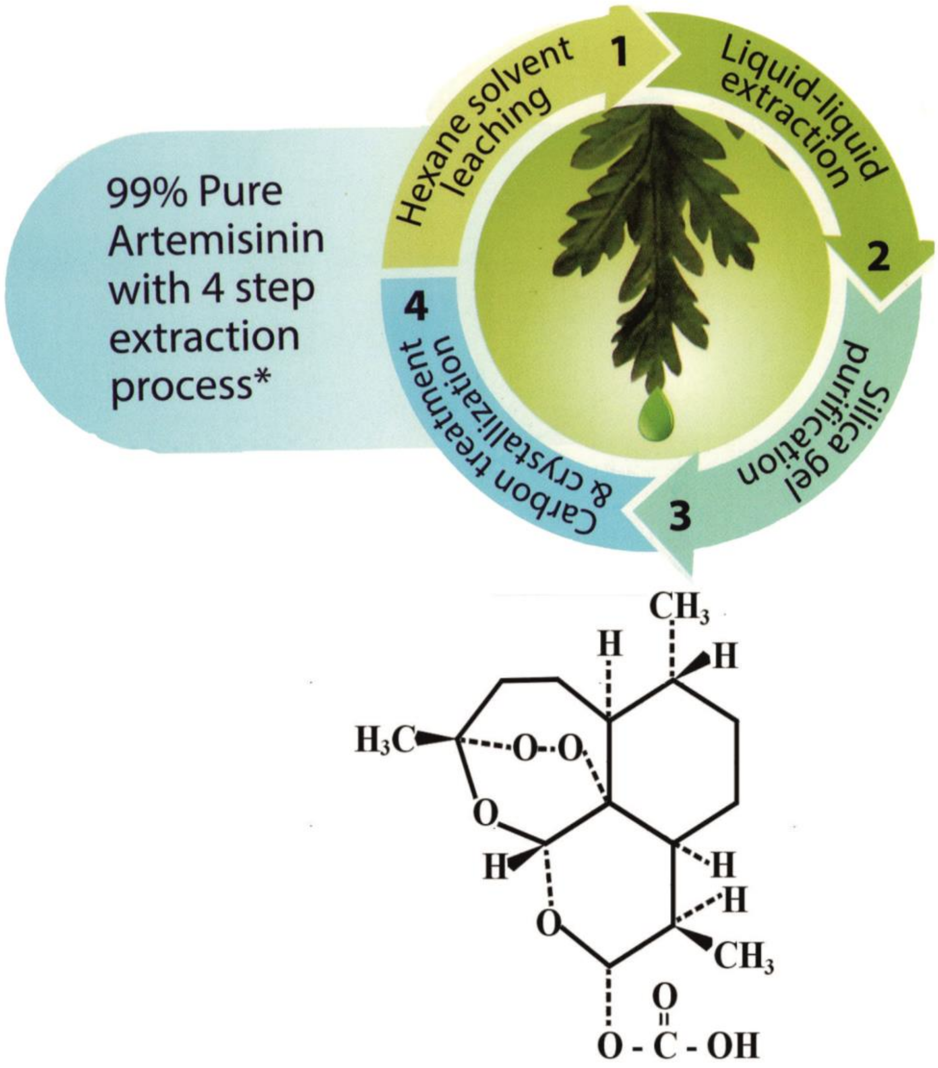

Fig 1.2: Extraction process of Artemisinin

1.3.4 Artemisinin structure

Artemisinin, a sesquiterpenes lactone formerly known as Qinghaosu was isolated in 1971 from the leafy portions of the *A. annua* plant (Klayman, 1985) and its structure was defined by X-ray analysis in 1979 (Anonymous, 1979). The empirical formula for artemisinin, $C_{15}H_{22}O_5$, arrived through elemental analysis and high- resolution mass spectroscopy (Liu *et al.,* 1979). Chemically, artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge, which is essential for its activity (Fig.1.2). Unlike most other antimalarials, artemisinin lacks a nitrogen-containing heterocyclic ring system (Klayman, 1985). The compound has been used successfull in treating several thousand malaria patients, including those with both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* (Abdin *et al.,* 2003). Derivatives of artemisinin, such as, dihydroartemisinin, artemether, and the water- soluble sodium artesunate are more potent than artemisinin itself and are effective against multidrug-resistant *Plasmodium falciparum* strains mainly in Southeast Asia and more recently in Africa, without any reported cases of resistance (Krishna *et al.,* 2004).

Artemisinin is an odourless, non-volatile compound, which is purified as white crystals with a melting point of 156–157°C (Lin *et al*., 1985). Its molecular weight is 282.1742 kg/mol, with an empirical formula of $C_{15}H_{22}O_5$ (Fig 1.2).The chemical name is 3R, 5aS, 6R, 8aS, 9R, 12S, 12aR-Octahydro-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano (4.3-j)-1, 2-benzodioxepin-10(3H)-one (Lin *et al*., 1985),

The structure of artemisinin is represented below:

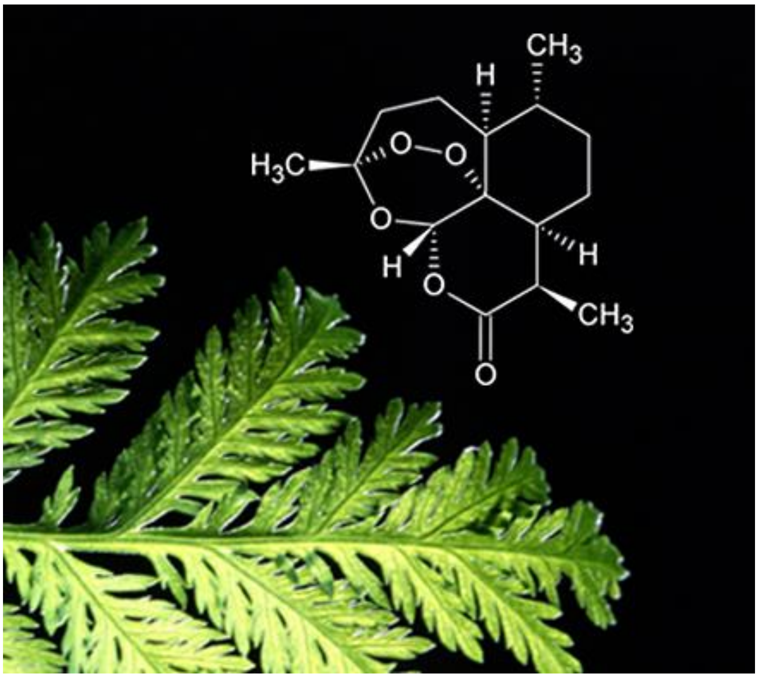

Figure 1.3: Chemical structure of artemisinin

1.3.5 Artemisinin distribution in *A.annua* plant parts

Artemisinin has been reported to accumulate in main stem, side stems, leaves, and inflorescence (Acton *et al.,* 1985; Ferreira *et. al.,* 1995; Liersch *et.al.,* 1986; Abdin *et.al.,* 2003). Its content was found to be more in leaves and inflorescence, but neither artemisinin nor its precursors were detected in roots and pollen (Trigg, 1989; Charles *et.al.,* 1991; Ferreira, 2004). Artemisinin content (% dw) was shown to be 4 to 11 times higher in the inflorescences as compared to leaves (Ferreira *et al.,* 1995) (Table 1.1). But various reports are contradictory to this report and have shown higher artemisinin content in leaves compared to other plant parts (Woerdenbag *et al.,* 1994; Ram and Kumar, 1997; Gupta *et al.,* 2002; Baraldi *et al.,* 2008). The occurrence of artemisinin in the achene (seed) is due to the presence of floral remnants. Although artemisinin immunolocalization has not been achieved, there is strong circumstantial evidence that the compound is sequestered in the glandular trichomes (Duke and Paul, 1993; Duke *et al.,* 1994). Factors affecting trichome initiation are important. Maximum artemisinin content % and trichome index (0.128) was found when methyl jasmonate was applied (Dangash *et al*., 2014).

Organ/ structure	Artemisinin (% DW x 1000)	
	Greenhouse	Field
Leaves	3-30	6-60
Main stems	0-3	0.4-7
Side stems	0	0.4-14
Roots	0	0
Flowers	12-42	104-264
Pollen	0	ND^{z}
Seed husks	ND	116
Seeds^{y}	36	81

Table 1.1 Artemisinin content in different organs of *A. annua* grown in greenhouse and field (Ferreira *et al*. 1995).

^{z}Not determined, ^{y}Containing floral debris.

1.3.6 Phenological stage specific distribution

Although some authors reported artemisinin being highest during pre-flowering stages (Acton *et al*., 1985; Liersch *et al*., 1986; El-Sohly, 1990; Woerdenbag *et al*., 1991 & 1994), others reported artemisinin reaching its peak during flowering (Singh *et al*., 1988;

Pras *et al*., 1991; Morales *et al*., 1993; Ferreira *et al*., 1995; Laughlin, 1995). The artemisinin yield estimated at different steps of development reveals a positive correlation between plant age and artemisinin content. This is assumed to be due to both an increase in leaf yield and artemisinin content with the progressive increase in plant growth (Singh *et al*., 1988). There are mainly two suggestions on the stage of the highest content of artemisinin in plant development: one is that the highest content of artemisinin is reached before plant flowering (Gupta *et al*., 2002), while in other studies it is reported that it reached in the full flowering period (Baraldi *et al*., 2008). Wang *et al*. (2004) studied on the effects of fpf1 gene (flowering promoting factor1) on *A.annua* flowering time and the linkage between flowering and artemisinin biosynthesis. They found that flowering was not a necessary factor for increasing the artemisinin content, and the best harvest time was the period between the later vegetative growth stage and the emergence of flowering bud.

Artemisinin, used in the semi-synthesis of related compounds in Artemisinin-Based Combination therapies (ACTs), are found mainly in the leaves and flowers of *A. annua*, little artemisinin is found in the stems, and none is found in seeds or roots(Acton *et al*.,1985). Not all shrubs of this species contain artemisinin. Apparently, it is only produced when the plant is subjected to certain conditions (Charles *et al*., 1990). The leaves from the same plant have different artemisinin contents according to their localization along the stem with upper leaves containing significantly more artemisinin than middle and lower ones (Charles *et al*., 1990; Laughlin, 1995). The plant content of artemisinin also varies during the season (Delabays *et al*., 2001).

Artemisinin and its precursor, artemisinic acid, have been shown to be localised in the glandular trichomes on the leaf surface. Many vascular plants invest considerable resources in building, maintaining and filling glandular trichomes on aerial surfaces

(Levin 1973; Wagner 1991; Aagren and Schemske 1993). Glandular trichomes have several secondary functions and in A. annua they are thought to contribute to plant defence (Duke and Paul 1993; Hu *et al*. 1993; Duke 1994). The biseriate capitate GT consist of 10 cells stacked in pairs (Duke and Paul 1993). The four lower cells function primarily as a stalk for the six topmost cells.

The glandular trichomes are more prominent in the corolla and receptacle florets than in leaves, stems, or bracts (Mehrotra *et al*., 1990). Although these glands are present since the early stage of development on both leaves and inflorescences, artemisinin increases at anthesis, suggesting that it accumulates as the glands reach physiological maturity, a stage, which coincides with the end of cell expansion in floret development (El-Sohly *et al*., 1990).

As glands approach maturity, there appears to be a cellular discharge into the subcuticular space around the apical cells and the contents are spread over the epidermis when the glands burst. After anthesis, artemisinin decreases and so does the number of intact glands. The association of artemisinin with glandular trichomes sequestration explains why artemisinin was not detected in parts of the plant that do not bear glands such as pollen or roots (Ferreira *et al*., 1995a).

Glandular trichomes are observed in leaves and stems of differentiated shoot cultures and artemisinin content of in-vitro shoot cultures was similar to artemisinin content in vegetative clones grown in the greenhouse (Ferreira *et al*., 1995, Dangash *et al*., 2014). Nicely grown-up plants may be devoid of artemisinin due to the rupturing of the glandular trichomes (Duke *et al*., 1994). In order that this product is synthesized by the plant, special agricultural conditions must be respected (Ferreira *et al*., 1995). As plant can grow in many places but it may not contain artemisinin (Duke *et al*., 1994).

1.3.7 Mechanism of action of artemisinin

Artemisinin contains two oxygen atoms linked together in what is known as an 'endoperoxide bridge', which react with iron atoms to form free radicals and the artemisinin becomes toxic to malaria parasites. When it reacts with the high iron content of the parasites, it generates free radicals, which leads to damage to the parasite (Luo and Shen, 1987). By this same mechanism, artemisinin becomes toxic to cancer cells, which sequester relatively large amounts of iron compared to normal, healthy human cells. Tests conducted show that artemisinin causes rapid and extensive damage and death in cancer cells and yet has relatively low toxicity to normal cells (Luo and Shen, 1987).

1.3.8 Other uses of artemisinin and its derivatives

Artemisinin derivatives are active against *Schistosoma mansoni* and *Schistosoma japonicum in vitro* and in experimental animals (Sano *et al.*, 1993; Xiao and Catto, 1989). This is of mechanistic interest, since schistosomes, like malaria parasites, degrade hemoglobin and produce hemozoin (Homewood *et al.*, 1972). Activity has also been demonstrated against *Leishmania major* (Yang and Liew, 1993), *Toxoplasma gondii* (Chang and Pechere, 1988; Holfels *et al.*, 1994; Ke *et al.*, 1990; Yang *et al.*, 1990), and *Pneumocystis carinii* (Merali and Meshinick, 1991) *in vitro* and against *P. carinii in vivo* (Chen and Maibach, 1994). Artemisinin derivatives have immunosuppressive activity (Chen *et al.*, 1994; Tawfik *et al.*, 1990) and also, potentially, anticancer activity (Lai and Singh, 1995; Woerdenbag *et al.*, 1993). The concentrations or doses of artemisinin derivatives which are necessary for these alternate activities *in vitro* and *in vivo* are substantially higher than those required for antimalarial activity. The cytotoxic effect of artemisinin is specific to cancer cells because most cancer cells express a high concentration of transferrin receptors on cell surface and have higher iron ion influx than normal cells *via* transferrin mechanism. Artemisinin tagged to transferrin *via* carbohydrate chain has also been shown to have high potency and specificity against cancer cells. The conjugation enables targeted delivery of artemisinin into cancer cells (Nakase *et al.*, 2008)

Artemisinin from the genus *A. Annua* has been shown to regulate plant growth by inhibiting lateral root growth (Duke *et al*., 1988). Artemisinin has been more effective than glyphosate, when tested as a herbicide in the mung bean (*Vigna radiata*) (Chen *et al*., 1991). Purohit and Pandya (2011), observed the inhibitory effect of artemisinin on *Amaranthus spinosus*, while the effect of artemisinin was stimulatory in case of *Brassica*. However, its herbicidal mode of action has not been elucidated.

1.4 Need of Artemisinin production

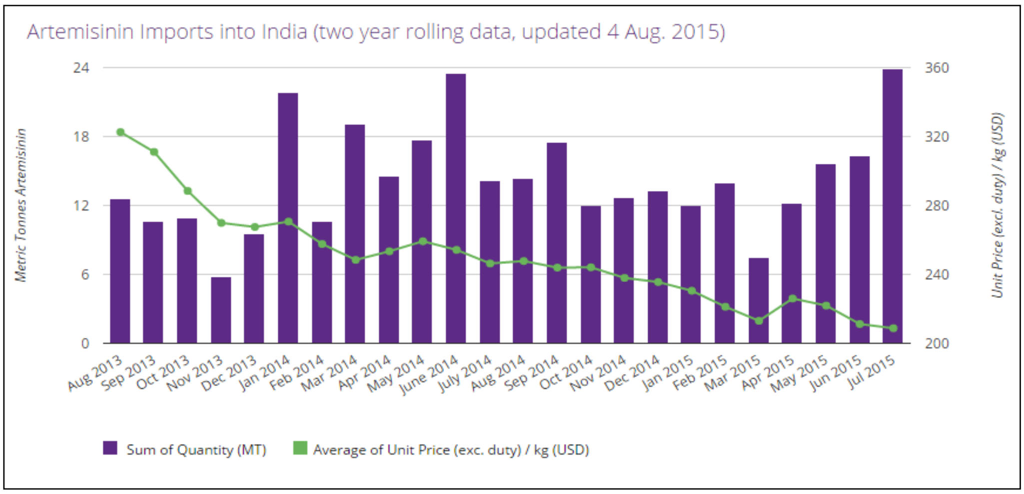

Figure 1.4: Artemisinin imports into India

The concentration of artemisinin in *A. annua* L. is very low in the range of less than 0.1 to 1%, of the plant dry weight, depending on the geographical origin of the plant (Woerdenbag *et al*., 1993; Haynes *et al*., 1994). The relatively low yield of artemisinin is, therefore, a serious limitation to the commercialization of the drug (Van Agtmael *et al*., 1999; Laughlin *et al*., 1994 and Abdin *et al*., 2003). Its chemical synthesis is possible (Schmid and Hofheinz, 1983; Xu *et al*., 1986; Roth and Acton, 1989; Avery *et al*., 1992), but is complicated and economically unviable (Ravindranathan *et al*., 1990; Avery *et al*., 1992). Hence, the enhanced production of artemisinin is highly desirable and can be achieved by conventional breeding, biochemical, physiological, molecular approaches and hairy root culture techniques (Smith *et al*., 1997; Liu *et al*., 1999; Chang *et al*., 2000; Wallaart *et al*., 2001; Wang *et al*., 2002; Abdin *et al*., 2003; Martin *et al*., 2003; Picaud *et al*., 2005; Weathers *et al*., 2005; Ro *et al*., 2006 and Newman *et al*., 2006). In India also, the demand of *A. Annua* is increasing and mostly is fulfilled by import of the drug (Fig 1.4).

There is an urgent need for a new strategy to develop this medicinal plant as commercial crop. This could be both as an alternative crop for large commercial farmers as well as for small scale farmers. The production and trade of *A. Annua* have gained prominence in many developed and under developed countries in recent times. There is a gap in between production and demand of artemisinin (Fig 1.5). The crop is currently processed by pharmaceutical firms for the production of Artemisinin and its derivatives for Artemisinin based combination therapies (ACTs) in the treatment of malaria (Ferreira *et al*., 2005).

Artemisinin accounts for less than 1% in the international anti-malaria medicine market due to the shortage of artemisinin raw materials (Simon *et al*., 1990). This is because the promising anti-malaria compound remains expensive and hardly available on the global scale. However, the demand for artemisinin based therapies is high since the parasite is rapidly developing resistance to all other drugs. In addition, medicinal herbal tea can be prepared from dried leaves of A. annua for the treatment of malaria without negative side effect (Hirt and Lindsey, 2000). Not only malaria but also some other medicinal problems such as hemorrhoids, bronchitis, billharzis and certain forms of cancer can be treated with *A. annua* tea which showed good results (Hirt and Lindsey ,2000) moreover , it is widely used as antibacterial agent and natural pesticides. Apart from Artemisinin, essential oil is another active research interests from *A. annua* as it could be potentially used in perfumery, cosmetics and aromatherapy (Muzemil, 2008). The oil has been also reported to possess antimycotic and antimicrobial activities (Woerdenbag et.al., 1993). The essential oil also has repellent effect on certain beetles (WHO, 2006). Inspite of its multiple uses the supply of artemisinin against its demand is petite and much effort is needed to increase the production and availability of artemisinin.

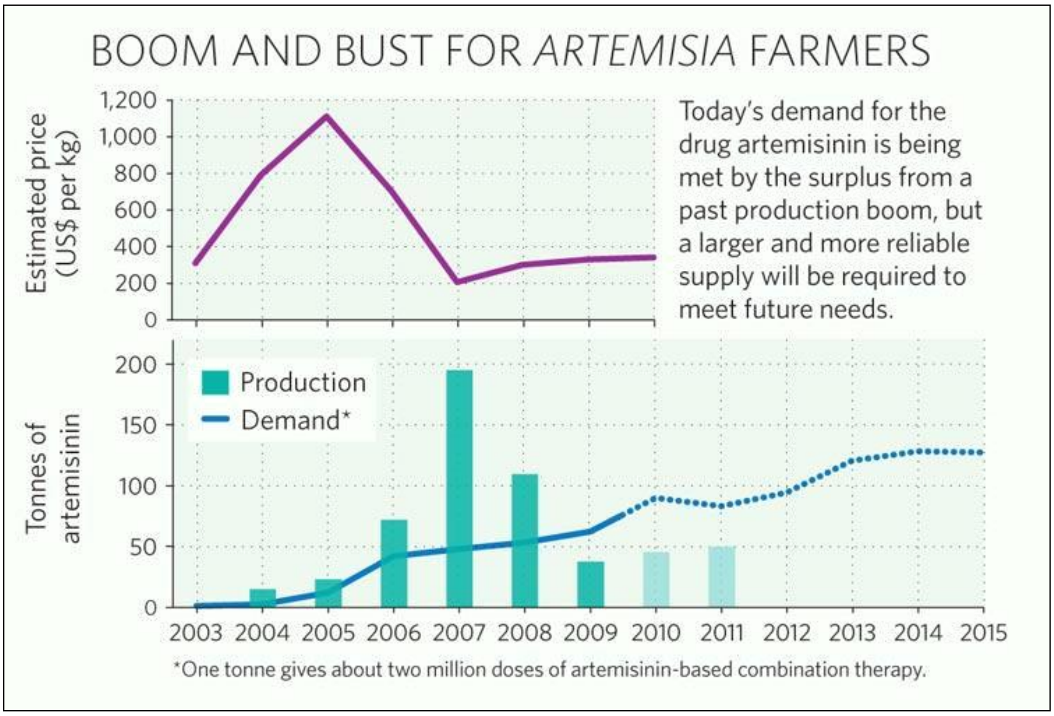

Fig 1.5: Demand and Production of *A.annua* (Nature, 2010)

Field production of *A. annua* is therefore; recommended as the only commercially viable methods to produce Artemisinin since the total chemical synthesis of the molecule is complex and uneconomical (Yadav *et al.*, 2003)

1.5 Constraints to *A. annua* cultivation

In many countries where *A. annua* is grown, cultivation has been affected by numerous factors (biological, physical, climatic and socio-economic) which have led to decreased yields (Technoserve, 2004). In the sub-tropics and the tropics, rainfall is the most important factor. Drought at the early stages of growth induces flowering (Ferreira and Janick, 1995). However, the step to maximize artemisinin yields is to achieve high biomass before the onset of flowering (Laughlin, 1994).

Another constraint to *Artemisia* cultivation is unavailability of enough agro-technology. Laughlin *et al*. (2002) indicated that technical information on ideal planting dates, seed density, harvesting system, post-harvesting and optimum fertilizer application rates under different climatic conditions required for higher yields is not enough. The challenge is to develop a composite variety with stable high artemisinin content. What is most significant to producers is that the artemisinin and essential oil contents should be high (Charles *et al*., 1991). A major problem to *A. annua* cultivation is seed availability and quality. The most productive *A. annua*seed (Mediplant hybrid, 44CQ App4) is expensive and in short supply (Technoserve, 2004).

Hence, F2 seeds that are cheap and easy to obtain is preferred even though it has lower yield potential. Planting from cuttings is also an option but it is difficult to apply on a large scale (Technoserve, 2004).

Post harvest studies

In agriculture, postharvest handling is the stage of crop production immediately following harvest, including cooling, cleaning, sorting and packing. The instant a crop is removed from the plant, it begins to deteriorate. Post harvest treatment largely determines final quality. Post harvest management practices that reduce product loss to spilage or shrinkage will reduce microbial risks. Both quantitative and qualitative losses occur in horticultural commodities between harvest and consumption. Qualitative losses, such as loss in edibility, nutritional quality, caloric value and consumer acceptability of fresh produce, consumer preferences vary greatly across countries and cultures and these differences influence marketability and the magnitude of post harvest losses. Strategies for loss prevention include use of genotypes that have longer post harvest life, use of integrated crop management systems and good agricultural practices that result in good quality of the commodity and use of proper post harvest handling practices in order to maintain the quality and safety of produce (Kader & Rolle FAO, 2004).

1.6 *In vivo* cultivation of *A. annua* L.

A. annua is a xeromorphic temperate plant. An examination of the growth and flowering behavior of *A.annua* in the subtropical climate region of India demonstrated the plant grew normally and flowered profusely in the winter cropping season, late October to late April, at Lucknow, India (Bhakuni *et al*., 2002). Considerable inter-plant variation was observed, however, in growth habit and flowering time. Plants could be grouped into four classes: early-maturing dwarf, early-maturing tall, late-maturing dwarf, and late-maturing tall. Early-maturing plants which flowered in February and March produced fertile achenes, completing the life cycle in 7–8 months. Late-flowering plants that flowered in May and June, when the maximum day temperature was over 40˚ C, produced florets without seeds. The high-temperature conditions to which the late-flowering plants were exposed appeared to prematurely dry the stigma. Late-flowering plants sprouted branches from the vegetative and flowering parts of the plant during the rainy season. *A. annua* appears to be the only Artemisia species that contain appreciable amounts of artemisinin. In India, (Balachandran *et al*., 1987) also did not find artemisinin in various *Artemisia* species of Indian origin. Considering the importance of artemisinin which is tedious and difficult to synthesize chemically, study was undertaken to develop *A.annua* plant varieties with high artemisinin content starting with development of agrotechnology for increase yield of these compounds. More particularly, the study was focused on agrotechnology involving method of optimizing the planting time, transplanting scheduling, population density, number of harvests and harvesting schedule leading to enhanced yields of artemisinin and related metabolites which have pharmaceutical value of anti-infectives, particularly as antimalarial drug. As the content of artemisinin fluctuates from zero level at the time of planting to more than 0.4–1.00 % during May and June with subsequent functions of increase till October, it was necessary to scientifically develop cultivation methodology for the crop to maximize the vigor of the foliage and biosynthesis of artemisinin by systematic scheduling. For this purpose, the present study was carried out planned experiments with variation in planting times, population density, and harvest from the crop to increase the yield from limited area within optimum span of time. Until now, artemisinin production has depended on extraction from *A. annua* L. plants grown outdoors. There are two methods to enhance artemisinin production in intact *A. annua* plants. One method is to define the appropriate developmental stage at which to harvest the leaves of the plants. At this developmental stage, both the highest artemisinin content and leaves yielding can be obtained. The other method is to breed high-artemisinin-yielding strains

Efforts have been done to establish a proper protocol for *in vitro* propagation of *Artemisia*. Cultivation of this plant is a viable alternative and can fulfill the demand of this important therapeutic agent.

1.6.1 Factors influencing artemisinin production

As for any other commercial crop the yield of product is affected by the prevailing conditions which include all micro-climatological and physical factors such as water, radiation, temperature, evaporation, soil conditions, human management and economic and political considerations. Laughlin (1993) indicated that genetic improvement of the crop plant alone would not meet the world demand for artemisinin. For crop productivity to be increased, the planting of high-yielding cultivars must be combined with improved practices of irrigation, fertilization, pests and disease control. *A. annua* is grown in temperate and sub-tropical climates. The plant is not adapted to the tropics because flowering will be induced when the plants are very small but grows easily in temperate areas and tropical areas at higher altitude (Klayman, 1993). On the other hand, it has also been reported that the crop can be grown in the tropics at 1000 – 1500 metres about sea level (Duke and Paul, 1993). It grows well on well-drained sandy loam soils and prefers soils with pH 5.0 – 8.0 with good water holding capacity (Laughlin, 1993). Once established, the plants are drought tolerant. It thrives in temperate to sub-tropical climates but not very well in the tropics (Ferreira *et al*., 1997). Marchese *et al*., (2000) indicated that depending on genotype and geographical origin, *A. annua* shows variations in the flowering behaviour under the same photoperiod and temperature condition.

Water is required at the start of the planting season for good establishment of seedlings but dry weather conditions are needed at harvest for drying. Moisture stress also induces early flowering and reduce yield. The plant requires an average rainfall between 1000-1500mm with a minimum rainfall of 600mm during the growth period (Marchese *et al*., 2000). Irrigation is needed to avoid the negative effect of drought (Technoserve, 2004).

Temperature has an important bearing on the productivity of *Artemisia*. A study has revealed that suitable temperature for *A. annua* cultivation and production ranges between 10 – 17ºC with the optimum temperature between 13 – 29ºC (Liu *et al*., 2003). The plant grows well between longitude 105º-115ºE and latitude 25º-35º N by producing high biomass and artemisinin content (Duke and Paul, 1993). Similar findings have been reported from Vietnam, where the artemisinin content was high in the high-altitude north than in the low-altitude south (WHO, 2003). *A. annua* is a short day plant with a photoperiod requirement of 13.5 hr (Ferreira *et al*., 1995) and a chromosome number of 2n = 2x= 18 (Bennett *et al*., 1982). The plant is naturally cross–pollinated by insects and wind (McVaugh, 1984). Ferreira *et al*., (1997) reported that self-pollination is not only rare but difficult to achieve which infers the presence of self-incompatibility. The plant at present does not seem to have any particular insect or disease problems.

Soil amendments such as fertilizers are reported to increase the total leaf biomass and thereby increased total essential oil and artemisinin obtained from *A. annua* (Simon *et al*., 1990), but these have not been evaluated in India. In view of this, soil fertility is now a problem in most farmers' fields because they do not use appropriate fertilizers. Plants usually respond positively to N application, but this is not always true depending on the agro-ecology and the cropping history of the field (GGDP, 1996). Adequate and balanced nutrients are necessary to obtain high yields. WHO (1988) conducted a study on the vegetative growth response of *A. annua* to specific micronutrients; nitrogen, phosphorus and potassium. They reported that significant increase of total plant and leaf dry matter was obtained where a complete fertilizer mixture containing 100 kg N, 100 kg P and 100 kg K /ha was applied. Dry leaf yields of *A. annua* between 6 – 12 t/ha were obtained in a mixed fertilizer containing 60 kg

N, 60 kg P and 50 kg K/ha (Laughlin, 1994). Muchow (1988) reported that nitrogen supply to plants increase leaf area development and delays leaf senescence and increases the photosynthetic capacity of the leaf canopy

Plant population density can have a strong impact on growth, development, yield productivity, chemical composition and the practicability of both weed control and harvesting of plant. According to Simon *et al*., (1990) from the tested plant population density of 69,930, 109,890, and 30,303 plants / ha. , the highest biomass yield of *A. annua* was obtained at highest density. In another study by the same author, plant population density of 111,111, 55,555 and 27,777 plants /ha. were tested and the higher biomass weight per plant was obtained at the lowest density, where as the higher essential oil and biomass yield per hectare was obtained at the higher density. It was also observed that plant from the most densely populated treatments was slightly taller produced less side stems and had longer internodes.

Ball *et al*., (2000), described plant spacing and population densities as a powerful management tools that can strongly influence early season light interception, crop physiology, growth yield and yield components. According to Donald (1963), as the number of plants per unit area increased, competition for growth resources such as nutrients, water and light also increased. Variation in plant density influences the various growth promoters of plants. Plant height, number of side shoots, branch number leaf area leaf area index, and dry matter accumulation are some of the crop growth parameters which are highly affected by spacing and population density. Loss *et al*. (1998) pointed out high population density results in significantly earlier canopy closer, large green area index and more radiation adsorption, more dry matter accumulation, particularly during the early vegetative growth and resulted in greater yield than at low plant population density. On the other hand, Sangoli (2000) stated that reduction of yield components at higher population is observed due to inter plant competition for incident light, soil nutrition and water.

Plant population density and its components of inter and intra row spacing is most important in determining yield and the practicability of both weed control and harvesting (Ratkowsky, 1983). *A. annua* was planted under different population densities and low density of 1 plant m^2 gave yields of 1 to 4 t/ ha of dried leaf (Maynard, 1985; WHO, 1988).

A. annua can be grown using different population density in different countries. According to Wright (2002), if inter-row cultivation were to be used to control weeds before the row close, then inter-row spacing of 0.5-1.0 meters may be appropriate. Similarly, wide inter-row spacing may also be appropriate.

1.6.2 Mode of harvesting of *A. annua*

The concept of harvesting for both essential oils and artemisinin and its derivatives needs exploration. The optimum time of harvest must be taken into consideration; to achieve maximum yields of artemisinin per unit area and balance against production and extraction cost (Technoserve, 2004).

Woerdenbag *et al*. (1994) compared yield response of *A. annua* to different harvesting stages and noted that harvesting at pre-flowering stages promotes the production of high artemisinin contents. Laughlin *et al*. (2002) also confirmed that the optimum time to harvest *A. annua* is just before flowering; this is also important because of the allergic reaction of the pollen. It was further observed that time of harvesting at different ecological zones for high artemisinin content remains a agrotechnological problem.

On the other hand, it has been reported that artemisinin reaches its peak during flowering. The association of peak artemisinin with flowering is related to the abundance of glandular trichomes in the inflorescence particularly florets and receptacle (Ferreira *et al*., 1995). Morales *et al*. (1993) also confirmed that peak artemisinin was achieved during full bloom in a number of greenhouses and field trials. This is confirmed by Ferreira *et al*.(1995) who found that artemisinin reaches its peak during full flowering in a Chinese clone for both greenhouse and field conditions. EABL (2005) also reports that when half to three quarters of the plants shows signs of bud initiation, artemisinin content will be at maximum.

The optimum time of harvest must be taken into consideration for maximum artemisinin content and biomass yield. Wright (2002) however, observed that the optimum time of harvest depends on the target compound desired and on the variety grown. All these have a strong genotype and environment interaction and the optimum time of harvest will have to be established locally (Laughlin *et al*., 2002).

1.7 In vitro production of artemisinin

In view of low concentrations of artemisinin detected in the plants, tissue culture systems have also been used with great interest as alternative method for the production of this drug. However, the results so far are not very encouraging. The artemisinin content that has been detected so far in shoot culture of *A. annua* is lower than in intact plants. The first trial of using tissue culture technique for artemisinin production came from He *et al*. (1983). The yield was 0.008% in shoots from the callus of *A. annua* plants of Chinese origin. Martinez and Staba (1988) did extensive monitoring with shoot cultures and were able to isolate high (0.3mg/g dw) and low (0.03mg/g dw) artemisinin synthesizing lines. Whereas, Simon *et al*. (1990) reported concentrations ranging from 0.03% to 0.05% on dw basis. Presence of artemisinin in roots, unrooted shoots and callus was shown by Jha *et al*. (1988). They also induced shoot regeneration from callus that could be transplanted to soil. Yields as high as 0.4

to 0.7 mg/g dw have been reported by Whipkey *et al*. (1992). Results from experiments with undifferentiated callus and cell suspension cultures of *A. annua* L. plants are disappointing with respect to the artemisinin production (Kudakasseril *et al*., 1987; Jha *et al*., 1988; Martinez and Staba, 1988; Tawfiq *et al*., 1989; Woerdenbag *et al*., 1991). Gulati *et al*. (1996) obtained *in vitro* flowering from various explants by supplementing the medium with GA_3, where artemisinin content reached up to 0.1%.

There have been contradictory reports indicating both the presence and absence of trace amounts of artemisinin in callus and cell cultures. No artemisinin or related sesquiterpenes could be found in callus cultures initiated from the leaves of selected high artemisinin producing clones (Elhag *et al*., 1992). No artemisinin was detected in suspension grown cells and in the spent medium. When root initiation occurred from shoot cultures, active synthesis of arteannuin B and artemisinin was observed, suggesting that a certain degree of differentiation is required for the production of artemisinin (Martinez and Staba, 1988; Fulzele *et al*., 1991). The reason for obtaining only trace amounts of artemisinin in cell cultures has been attributed to high peroxidase activity in undifferentiated cultures (Woerdenbag *et al*., 1993). Paniego and Giuletti (1994) reported the establishment of dedifferentiated and differentiated cultures of *A. annua*. In the primary callus, 1.13 and 0.78 mg artemisinin/g dw were obtained. They failed to detect artemisinin in cell suspension cultures and only trace amounts were found in the multiple shoot cultures. Woerdenbag *et al*. (1993) have reported that the shoot cultures show better growth and produce more artemisinin on 2% sucrose. Kumar *et al*. (1997) reported that substitution of sucrose with other carbohydrates or combination of sucrose and other sugars resulted in differences in growth and artemisinin production. Best growth could be observed in the medium supplemented with sucrose or a combination of sucrose and maltose. Least growth was observed when lactose was used instead of sucrose. Use of maltose alone as the carbohydrate source resulted in lower biomass production (Kumar *et al*., 1997). Woerdenbag *et al*. (1993) reported a high percentage of artemisinin content (0.16% of dry weight) from shoot cultures using MS medium supplemented with 0.05 mg /l NAA, 0.2 mg/l BAP and 1% sucrose. Most workers (Martinez and Staba, 1988; Tawfiq *et al*., 1989; Kim and Kim, 1992; Gulati *et al*., 1996 a; Ferreira and Janick, 1996) did not detect artemisinin in roots. Roots do not contain artemisinin, but evidently enhance its production in cultured shoots. Removal of roots from shoots cultured in hormone free liquid medium reduced artemisinin content in shoot by 53% (Ferreira and Janick, 1996).

Attempts have been made to improve the artemisinin production by admittance or addition of medium components like plant growth regulators, casein hydrolysate, by inclusion of precursors of artemisinin biosynthetic pathway and elicitors and by addition of sterol synthesis inhibitors as well as mutagens. Casein hydrolysate, a source of aminoacids and oligopeptides, in low concentration enhances the artemisinin production, but its prolonged exposure negatively affects the biomass production and growth of shoots. Kumar (1997) reported that incorporation of boron; casein hydrolysate and gibberillic acid enhances artemisinin production by 25%, 36% and 65%, respectively. No effect on growth was observed. Fulzele *et al*. (1995) reported the stimulation of terpenoid synthesis in plantlet cultures from *A. annua* by addition of GA_3. However, other scientists found only a slight effect on biomass production of plants treated with 40 and 80 mg/l GA_3 (Martinez and Staba, 1988; Charles *et al*., 1990; Fulzele *et al*., 1995). Smith *et al*. (1997) reported that GA_3 (0.01mg/l) increased the growth rate of hairy roots of *A. annua* by 25% with a slight increase in artemisinin levels as compared to control. A combination of benzylaminopurine (BAP, 1mg/l) and kinetin (KN, 10mg/l) increased the yields of artemisinin *in vitro* by 3.6 and 2.6 fold, respectively due to increase in dry matter production which can overcome a concurrent decrease in the artemisinin content (Whipkey *et al*., 1992).

Rationale of study

Field production of *Artemisia annua* L is presently the only commercially viable method to produce artemisinin because the synthesis of the complex molecule is uneconomical. The plant has to be grown each year starting from seeds. The plant is peculiar in its behavior. Nicely grown up plants may be devoid of artemisinin. In order that this product is synthesized by the plant special agricultural and climatic conditions must be respected. The plant grows at many places but it may not contain artemisinin. *Artemisia annua* L is a plant with a strong aroma, containing camphor and essential oil. A robust plant grows in many areas of the world. However, only plants grown in special agricultural and geographic conditions contain artemisinin. The quantity and quality of artemisinin are very sensitive to various conditions such as, cultivation method, harvesting time, the pre-manufacturing processing, transportation, extracting, purification processes, manufacturing, storage conditions, etc. Even in optimal conditions, *Artemisia annua* L yields very low artemisinin. Analysis of artemisinin is difficult because the compound is unstable, concentration in the plant is low, and the intact molecule stains poorly and other compound in the crude extracts interfere in its detection.

The stereo-specificity of this molecule makes total synthesis very difficult and uneconomical which is not adaptable to industrial production at acceptable price level.

Therefore, there is urgent need for cultivated, extracted, and formulated production for commercial purposes to bridge the demand and supply gap.

Considering the above information, the proposed study aims to develop and fine tune the protocol of cultivation which not only suits the local need but also improve the content of artemisinin and herbage or biomass.

The broad objectives of the study were:

1. **Agronomical approach (*in vivo*)**

   Experiment 1:- Effect of different fertilizer treatments on plant height on *A.annua*.

   Experiment 2:- Effect of plant population density on *A.annua* Growth

   Experiment. 3:-Effect of growth regulators and stress on *A. annua* growth.

   Micromorphological studies

   Experiment 4:-Effect of planting time on growth, biomass and artemisinin yield.

2. **Post harvest studies**

   Experiment 5: Effect of shade drying and oven drying on artemisinin content of *A.annua*

   Experiment 6: GC-MS analysis data from different samples of *Artemisia* leaves

   Experiment 7. Studies on utilizing waste (leaves generated after extraction)

   i. Briquette making

   ii. Estimation of Scopoletin

3. **Biotechnological approach (in vitro- Tissue culture)**

   i. Regeneration of *A. annua* L. plants

   ii. Callus induction

   iii. Shoot regeneration

   iv. Shoot multiplication

v. Shoot elongation and root regeneration

vi. Hardening of tissue culture raised plantlets of *A. annua* L. plants.

vii. Artemisinin content in leaves of tissue culture raised plants.