CHAPTER-5

SUMMARY

In 21\textsuperscript{st} century, Malaria continues to be one of the major public health problems in Africa, Asia and Latin America. Approximately 40\% of world population is at risk of Malarial parasite infection (Simooya, 2005). Each year, more than one million people around the globe die of malaria and more than two billion people in over 100 countries and regions are threatened by the disease (WHO 2008). The WHO Roll Back Malaria department has reported that recent estimates of the global malaria burden have shown increasing levels of malaria morbidity and mortality, reflecting the deterioration of the malaria situation in many developing countries, especially those in Africa, the morbidity and mortality from malaria are still very high. Africa is the most affected continent, about 90\% of all malaria deaths occur in the areas south of the Sahara, and the great majority of these are in children under the age of five years.

The \textit{World Malaria Report 2012} summarizes data received from 104 malaria-endemic countries and territories for 2011 and it was reported that 99 of these countries had on-going malaria transmission. According to the latest WHO estimates, there were about 219 million cases of malaria in 2010 and an estimated 660 000 deaths (WHO, 2012). Malaria is not just a disease commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development. Malaria has had serious impact on the social and economic development of mankind. The disease has been associated with major negative economic effects on regions where it is widespread. Global fund disbursements for malaria control rose sharply during the past eight years.
and were estimated to be US$ 1.66 billion in 2011 and US$ 1.84 billion in 2012. National
government funding for malaria program has also been increasing in recent years and

In India, Malaria is a major public health problem and one which contributes
noticeably to the overall malaria burden in Southeast Asia. The National Vector Borne
Disease Control Program of India reported ~1.6 million cases and ~1100 malaria deaths
in 2009. Several Researcher argue that this is a serious underestimation and actual
number of malaria cases per year is more than above figures, (between 9 to 50 times
greater) with an approximate 13-fold underestimation of malaria-related mortality (Das et
al., 2012). Since 20th Century, for the prevention and treatment of malaria various
medicinal methods have been available, but increasing resistance to formally effective
treatment has again made malaria therapy a major problem. Artemisinin new antimalarial
drug is a Sesquiterpene lactone endoperoxide isolated from the Chinese herb Artemisia
annua. L (Asteraceae) and currently it is only hope to cure malaria. The herb Artemisia
annua has been used for many centuries in Chinese traditional medicine as a treatment
for fever and malaria. In 1971, Chinese chemists isolated from the leafy portions of the
plant the substance responsible for its reputed medicinal action (Klayman, 1985). In order
to effectively curb the spread of malaria in the world, WHO has recommended the use of
artemisinin-based combination therapies (ACT) for the treatment of this disease (WHO,
2005). Artemisinin is a sesquiterpene, contain 1, 2, 4-trioxane ring structure and this ring
structure is responsible for the antimalarial activity of this natural product.
Artemisinin is the most efficacious antimalarial drug in the world to date (Abdin et al., 2003) and it is only produced in *Artemisia annua* L. plants in very low amounts. Chinese scientist first isolated artemisinin from *A. annua* plants and the structure was later characterized by others as a sesquiterpene lactone with an endoperoxide linkage (Abdin et al., 2003). This endoperoxide bridge rarely exists in natural products but is essential for the medical function of artemisinin (Woerdenbag et al., 1990; Balint, 2001). Artemisinin based drugs are the only antimalarials recommended by the World Health Organization (WHO) because of their safety and efficacy against all kind of malaria including cerebral malaria. Antitumor and antimicrobial functions have also been reported (Meshnick et al., 1996; Singh and Lai, 2004; WHO, 2005a). The drug Artemisinin is one of the most promising next generation antimalarial because of its effectiveness against all strains of *Plasmodium*, now resistant to frontline drugs (Ferreira et al., 2005). Artemisinin and its derivatives are proven antimalarial compounds against *Plasmodium falciparum*. Artemether, arteether, artesunate, dihydroartemisinin are some of the antimalarial semisynthesised from artemisinin. Combination therapies (CTs) with formulation containing an artemisinin compound (ACTs) have emerged as more reliable treatment option (Kumar and Srivastava, 2005). At Present, the best solution for malaria problem is to use Artemisinin based Combination Therapies (ACTs).

Plants have played an important role as a source of effective anti-cancer agents, and it is significant over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Newman and Cragg, 2012) The search for anti-cancer agents from plant sources started...
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in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins.

Unfortunately, the level of the production of artemisinin in A. annua plants is relatively low, only about 0.01 to 0.8% (DW) (Abdin et al., 2003). A minimum of six months is required for cultivating A. annua (WHO, 2004). Due to its unique and complex structure, it is not economically practical to chemically synthesize artemisinin (Abdin et al., 2003). To meet the therapeutic demand, enhanced production of artemisinin is highly desirable. To achieve above mention goal, there is need to facilitate cultivation of plant and in vitro production of artemisinin is also required with reliable quality, both ways will ensure a sustainable supply to meet market demand. In vitro cultures may potentially constitute useful and easily manipulated systems for producing valuable biologically active compound in plants that do not require labor-intensive methods (McCabe et al., 1997).

Although artemisinin production can be increased through larger scale field cultivation of A. annua, the length of cultivation and manufacturing time, the need for a large amount of land and labor, and the expense of extraction are still problems. Hence, alternative approaches are being studied to enhance artemisinin production using in vitro methods. In view of the low artemisinin detected in plant, tissue culture system has been used with great interest as alternative method for production of this drug. Simon et al. (1990) reported concentration of artemisinin ranging from 0.03% - 0.05% on dry weight basis (Simon et al., 1990). Results from experiments with undifferentiated callus and cell
suspension culture of \textit{A. annua} are disappointing with respect to artemisinin production (Jha \textit{et al.}, 1988; Tawfik \textit{et al.}, 1989; Fulzele \textit{et al.}, 1991).

Martinez and Staba (1988) have reported that roots originating from leaf segments contained artemisinin, if they were grown on MS or B5 medium supplemented with IBA or NAA at 0.05-0.02 mg/L. Also they reported that artemisinin content increased when roots developed into plants with a properly developed root system. (Martinez and Staba, 1988). The hairy roots, results of genetic transformation by \textit{A. rhizogenes} have attractive properties of secondary metabolite production. Hairy root cultures provide a promising alternative to the biotechnological exploitation of plant cell culture. (Liu \textit{et al.}, 2006). Transformed roots of \textit{A. annua} have shown their capacity to produce artemisinin in significant quantity (Jazari \textit{et al.}, 1995, Weathers \textit{et al.}, 1994; 2003; 2004). Weathers and group (1994) at Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester have also demonstrated the capacity of these roots to grow in variety of bioreactor and production of artemisinin. They have developed gas phase nutrient mist bioreactor and liquid phase bubble column bioreactor for scale up of artemisinin in hairy roots of \textit{A. annua} (Kim \textit{et al.}, 2001; 2002).

Transformed hairy roots of \textit{A. annua} have been studied for improving artemisinin production. Compared to suspension cultures, hairy roots are more stable, grow faster, and may be easier to scale-up. Many different culture conditions including light, elicitors, and culture in bioreactors have been investigated (Towler \textit{et al.}, 2006; Weathers \textit{et al.}, 2006a, 2006b). However, artemisinin yields in hairy roots are not yet high enough to be economically attractive. Alternatively, shoot cultures of \textit{A. annua} are also being studied.
Different culture conditions such as carbon sources, sugar concentration, $\text{NH}_4^+/\text{NO}_3^-$ ratio, phosphate concentration, phytohormones (Basile et al., 1993; Woerdenbag et al., 1993; Liu et al., 1998), addition of precursors such as mevalonic acid and elicitors or addition of metabolic inhibitors have been studied (Abdin et al., 2003).

Shoots cultured in bioreactors (Liu et al., 1998) have also been studied. Unfortunately, artemisinin production in shoot cultures, although greater than in hairy roots, is also still much less than in whole plants (Abdin et al., 2003). More recently, Martin et al. (2003) have introduced a portion of the artemisinin pathway into E. coli. If this effort succeeds, then E. coli may be used to produce high-yield terpenoid-based drugs including artemisinin in large-scale fermentations with expected costs of extraction also largely decreased (Martin et al., 2003). The relative low yield (0.01-0.8 %) of artemisinin in A. annua is a serious limitation to the commercialization of drug. Therefore enhanced production of artemisinin either in cell/ tissue culture or in the whole plant is highly desirable (Abdin, 2003).

Recently CIMAP has developed a new variety of A. annua, ‘Jeevanraksha’ containing high level of artemisinin. (Kumar et al., 2000). CIMAP also developed processes for isolation and derivatization of artemisinin and holding several patents. CIMAP and CDRI developed antimalarial drug α arteether which is available in market as brand name E-mal. Sharma and Dixit (2006) studied the transformation of A. annua using A. rhizogenes and they reported that cell suspension culture of A. annua produces five-fold more artemisinin than control.
In view of low concentration of artemisinin detected in the plants, biotechnological approaches such as root organ technology, bioreactor technology and downstream technology is essential for enhancement of production of artemisinin in *A. annua* plant. Biotechnological production of artemisinin is promising one. ACTs are much more expensive than other drugs because of relatively low yields of artemisinin in *A. annua*. Therefore, there is need of many efforts to enhance the production of artemisinin *in vivo* and *in vitro* by biotechnology. To fulfill the world demand of artemisinin, it is obvious that there is need for an additional source of artemisinin which supply will be consistent, reliable and inexpensive.

The main emphasis of our work was to study *in vitro* production of artemisinin, that can be exploited as a large scale production process technology. Root organ culture technology and solid phase extraction system was exploited for production of artemisinin. Root organ culture system is used for the production of secondary metabolite; it has several advantages such as rapid growth of roots, are quite easy to prepare and maintain, show a less variation and can be easily cloned to produce a large supply. The said work was carried out to screen the different probable biological activities of artemisinin. The artemisinin obtained from crude extract was used for screening of anticancerous potential because the mode of action of artemisinin on malaria parasite shows that it could be used as an anticancer drug. The study is carried out on cancerous cell line. The isolated artemisinin also tested for its biological activities like antibacterial and antioxidant activity.
Objectives:

To Establish root organ culture of *A. annua*.

Optimization of culture condition for maximum growth of roots *in vitro*.

Development of solid phase extraction system for recovery of artemisinin.
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Plan of Work:

Procurement of seeds and germination

Seeds of *A. annua* have been obtained from CIMAP Lucknow and germinated to seedling on hormone free Gamborg's B5 media.

Establishment of root organ culture

Leaves of *A. annua* seedling were used as a explants for the establishment of untransformed root organ culture and it was maintained on half strength MS media containing IAA and IBA hormone.

Growth and product kinetics

The parameters such as doubling time, specific growth rate, Yx/s (growth yield), Yp/s (product yield) were calculated at shake flask level.

Optimization of culture condition and extraction of Artemisinin

The scale of the process, *in vitro* culture conditions was optimized and biomass was harvested. Reverse phase extraction system were used for rapid extraction of artemisinin.

Testing of biological activities

Biological activities such anti-cancerous activity were tested by using cell lines, antioxidant scavenging potential were calculated by DPPH assay and antibacterial activity were by using disc diffusion method tested.
Results and Discussion

CIMAP, Lucknow has developed a new variety of *A. annua* ‘Jeevanraksha’ containing high levels of artemisinin (Tondon *et al.*, 2003). Two gram Seeds of *A. annua* ‘Jeevanraksha’ have been procured from CIMAP for *in vitro* cultivation of plants. Seeds were germinated on Gamborg’s B₅ medium without any plant hormone and > 95% germination was observed after 5-6 days of incubation at 18°C. During the experiments, shoot tips/axillary buds were used as explants and inoculated on MS medium with BA and NAA, shoots were well developed along with initiation of callus. This callus was transferred on MS medium with same hormonal combination and concentration. Axillary buds obtained from shoot culture were used as explants for multiplication of shoot on MS medium. It was found that full strength solid MS medium with hormone BA -0.5 mg/L and NAA-0.05 mg/L showed better shoot development with initiation of callus. These cultures are maintained as live source of inoculums under laboratory condition, since it is difficult to grow this plant under the climatic conditions of Central India.

Establishment and maintenance of root organ culture:

For initiation of root organ culture, leaves were used as explants. The leaves were cut horizontally into two halves. These explants were inoculated in two media, half strength MS and B₅ for initiation of root organ culture with different concentrations of hormones namely, IBA and IAA. The flasks were incubated on gyratory shaker at 25± 2 °C at 80 rpm in dark. We have tested two media half strength MS and B₅ for initiation of root organ culture with hormone IBA and IAA. It was observed that lateral root system
was fully developed in both the medium. The regular subculture was done by inoculating growing root tips (1.0-1.5cm) in optimal medium at three week interval.

It was observed that half strength liquid MS medium show better results than B5 in terms of root growth, branching, biomass production, while the concentration of the hormone IAA and IBA were kept same in both medium. Hormone concentration of IBA 0.2mg/L & IAA 0.5mg/L was found to be best for root initiation and growth of roots in liquid culture in both half strength MS as well as B5. Half strength liquid MS medium showed better results than B5 in terms of root growth, branching, biomass production.

**Growth Kinetics of roots in shake flask**

Growth kinetics experiment was designed and carried out to find the doubling time of the roots as well as the exact period for next sub-culturing. M ½ medium with IAA and IBA hormones without phytagel was sterilized and 50ml was carefully added in to pre-sterilized Erlenmeyer flasks (250 ml) in aseptic condition. Root tips (10 mg fresh weight) were inoculated and flasks were transferred to orbital shaker at 100 rpm and at 27°C in dark. Flasks (3No. each) were removed every week and roots were harvested, blotted dry and weighed. They were then oven dried at 60-70°C till the weight became constant. Wet weight and dry weight were noted.

During this period, reducing sugars namely glucose and sucrose and phosphate were monitored in the growth medium. The specific growth rate of roots in shake flask was 0.099/day with doubling time was 7.0 days which is reasonably good. The growth yield in terms of sucrose utilization was found to be (Yx/s) 0.33g/g.
Antiproliferative activity:

The anticancer activity of methanolic extracts of shoots, callus and roots of *Artemisia annua* plant was analyzed by cytotoxicity assays (MTT assay). The cytotoxicity of all the extracts were tested on breast cancer cells (MCF7) along with cervical cancer cells (HeLa). The shoot extract exhibited the IC$_{50}$ value 160.3±48.72 µg/ml for MCF7 cells whereas 405.0 ± 41.77 µg/ml for HeLa. The Root extract exhibited 126.4±24.12µg/ml of IC50 for HeLa cells and 155.0±21.85 µg/ml IC$_{50}$ values for MCF7. However, the callus extract showed the IC$_{50}$values 295.0 ± 12.91 µg/ml, 155.0±21.85 µg/ml for HeLa and MCF7 cells respectively. Among the three methanolic extracts, the callus extract was found to be highly effective with the lowest IC$_{50}$ values whereas the shoot extract was least effective with higher IC$_{50}$ values.

Antioxident Potential Screening by DPPH assay:

The *Artemisia annua* is known for its sesquiterpenes having prominent antimalarial activity. The laboratory cultivated plant of *A. annua* were tested for their potential as antioxidants. The methanolic extract of shoot, callus, root tested by using many DPPH standard assays. The DPPH radical scavenging potential of the methanolic extract of *A. annua* shoot/leaves was found to significantly higher than all other extracts. In vitro grown culture of *A. annua* i.e. shoot, callus, root extracts showed 50% inhibition (IC$_{50}$) at 150.02±4.8µg/ml (n=3) and 186.0±5.89 µg/ml (n=3) and 239.0±4.8 µg/ml (n=3) respectively. The glutathione and β-carotene, which were used as reference antioxidants, showed the IC$_{50}$ at 65.5 ± 2.12 µg/ml and 527.5 ± 10.6 µg/ml respectively.
Antibacterial activity:

To determine the antibacterial activity, the methanolic extracts of in vitro grown shoot, callus and root of *A. annua* were tested against some selected human pathogenic bacteria by disc diffusion method. Both Gram-negative (*P. aeruginosa, S. typhi* and *E. coli*) bacteria as well as one Gram-positive (*S. aureus*) bacteria were used for antibacterial activity screening. Only the alcoholic extract was tested as alcohol was found to be better solvent for extraction of antimicrobially active substances compared to water and hexane (Ahmad et al., 1998).

The methanol extracts (60μg/disc) of all three sample of *A. annua* showed excellent antibacterial activity with the average zone of inhibition of 10-16 mm against the tested bacteria by disc diffusion method. Among the tested bacteria, the growth of *S. typhi* and *S. aureus* was highly inhibited. Ampicillin (30 μg/disc) were used as reference antibiotics for comparison.

Conclusion

The main emphasis of our study was to design and develop process to increase the production of antimalarial compound artemisinin *in vitro*. We have used biotechnological approaches such as micropropagation, root organ culture technology, bioreactor technology, which seems to be promising and economical for enhancement of artemisinin production in *A. annua* culture with production cycle of taking less time compare to six month for field cultivation. At present, the only source of drug is extraction of field grown crops of *Artemisia annua*, which is subjected to certain limitation such as low temperature requirement, seasonal and somatic variation and geographical limitation, and
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high production cost. Here we can show that in vitro technology could be used a resource of artemisinin. Stable and consistence supply of artemisinin will reduce the cost of drug so that drug will available at affordable price to poor peoples.

Antioxidants are substances that protect other chemicals of the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body, hence hindering the process of oxidation. *In vitro* grown *Artemisia annua* plant extract shown good radical scavenging activity. These antioxidant elements of the extract may further be exploited as health improving agents and anti-aging factors of future drug molecules. Antimicrobial activity of the plant extract has shown very promising results as far as infectious disease prevention is concerned by bioactive agents from plants.

Antiproliferative activity of the plant extract is evaluated by MTT assay against two cell lines. The experiment has shown significant inhibitory response that reveals use of Artemisinin as drug for cancer.