ASSOCIATION STUDY OF GENETIC POLYMORPHISM WITH SECONDARY METABOLITES OF ARTEMISIA ANNUA

by

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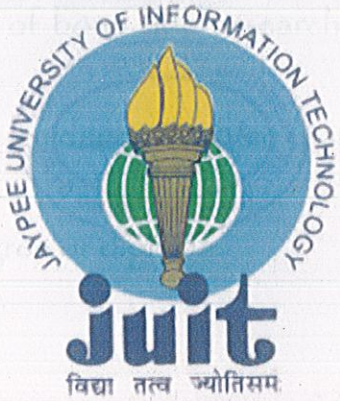
A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Bachelor of Technology

IN

BIOTECHNOLOGY



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WAKNAGHAT, SOLAN 173215, HIMACHAL PRADESH, INDIA

MAY 2010

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CERTIFICATE

This is to certify that the thesis entitled "Association study of Genetic polymorphism with Secondary Metabolites of ARTEMISIA ANNUA" submitted by **Atul Arora** and **Sugandhika Khosa** to the Jaypee University of Information Technology, Waknaghat in fulfillment of the requirement for the award of the degree of **Bachelor of Technology** in **Biotechnology** is a record of bona fide research work carried out by them under my supervision and guidance and no part of this work has been submitted for any other degree or diploma.

(Dr. Harvinder Singh)

DECLARATION

We hereby declare that the work presented in this thesis has been carried out by us under the supervision of Dr. Harvinder Singh, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for a degree or diploma of any other university. All assistance and help received during the course of the investigation has been duly acknowledged.

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ACKNOWLEDGEMENT

The success of our research would never have happened without the support and valuable advice from many people who contributed to our valuable experience.

We would like to express our sincere gratitude and deep appreciation to our advisor, Senior Lecturer, Dr. Harvinder Singh for his guidance, invaluable advice, supervision, kindness and encouragement throughout this study.

Our sincere appreciation is also extended to Dr. Pradeep Naik, our co-advisor for his helpful criticism, suggestions and for his constructive comments.

A special acknowledgment is extended to the Faculty of Biotechnology, Jaypee University of Information Technology (JUIT), Waknaghat for giving us the opportunity to study the Bachelor of Teachnology in Biotechnology, Department of Biotechnology and JUIT for providing us with the best research facilities. Our great appreciation is also extended towards our Head of Department, Dr. R. S. Chauhan.

A special thanks to our Vice-Chancellor, Dr. Y. Medury and Registrar, Brig. (Retired) Balbir Singh, for providing us with the right environment to work.

We would also like to express out our thanks to all our friends for their warm friendship and support and assistant until we finished our study.

Finally, we would like to express our deepest gratitude to our dearest parents, our family for their encouragement, understanding, care, love and continuous support throughout our life.

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ABSTRACT

Artemisia annua is an important medicinal plant; currently being the most potent source in treatment of malaria. The secondary metabolite artemisinin is used for treatment of malaria. Twenty-four primers were designed using gene sequences which have been found to play an important role in production of Artemisinin, the secondary metabolite of *Artemisia annua*. These primers were used to amplify the regions of Artemisian genome. DNA extraction as well as the protocol for PCR amplification was standardized. Standardization was required because of the presence of high amount of polyphenolic compounds present in this plant. Fourteen primers gave amplification. Artemisinin was extracted from the leafs and flowers of the plant which yielded analytical results stating that content of Artemisinin with the stage of the flower, in early stages(budding stage) content was found to be between 0.057-0.064% in 100gm, whereas in late stages, it was 0.078-0.086%. Also, the content found in plants from Leh region (0.065-0.086%) is more than that of Partapur region (0.057-0.079%) at each stage of the flower. Highly significant correlation (>0.9) was observed in artemisinin content (w/w%) obtained by GC-FID and spectrophotometric methods.

CHAPTER 1

Introduction

1.1 Epidemiology of malaria

Malaria is a deadly disease threatening half of the global population. It is caused by protozoan parasites of the phylum Apicomplexa and the genus *Plasmodium*. There are more than 100 *Plasmodium* species that can infect mammals, birds and reptiles. Five *Plasmodium* species can infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. *P. knowlesi*, whose natural host is macaque monkeys, was recently suggested to be the fifth species that can infect humans (White, 2008). It has been shown to be a major cause of malaria in Malaysia (Cox-Singh et. al., 2008). *P. falciparum* and *P. vivax* are the most common human species, while *P. falciparum* causes most severe disease and death. It has been estimated that malaria transmission occurs in 109 countries putting 3.3 billion people at risk. In 2006 there was an estimated 247 million malaria cases, resulting in nearly 900000 deaths. This makes *P. falciparum* one of the leading causes of death worldwide, from a single infectious agent. The malaria burden is greatest in African children as 90% of the deaths occur in sub-Saharan Africa and 85% of the mortality affects children under five years. Although the number of deaths is similar to that of 2004, amelioration of the malaria situation has been achieved in seven African regions, where malaria control measures have resulted in 50% or more reduction in malaria incidence and mortality since 2000, and further 22 countries in the world have reached similar effects (WHO, 2008a-c). Morbidity and mortality are not the only consequences of malaria infection. The disease is estimated to be responsible for an average annual reduction of 1.3% in economic growth for countries with the heaviest malaria burden (Sachs & Malaney, 2002). The great variation in malaria burden between different geographical regions can be driven by several factors. Moreover, the parasite is becoming resistant to commonly used antimalarial drugs.

1.2 Life cycle of *P. falciparum*

For *Plasmodium falciparum,* mosquito is always the vector, and is always a female *Anopheles* mosquito. There are 380 species of *Anopheles* mosquitoes, but only 60 can transmit malaria. With the exception of *P. malariae* (which may affect the higher primates), the other three species of *Plasmodium* that infect man are obligate parasites of human beings. The life cycle of *Plasmodium* parasite is very complex and requires two different hosts, a vertebrate host (man) and an invertebrate host, the female *Anopheles* mosquito (vector) (Figure 1.1). The different stages were named on the basis of their morphology such as

merozoite, trophozoite, gametocyte found in humans and zygote, ookinete and sporozoite found in mosquitoes (Wirth, 2002).

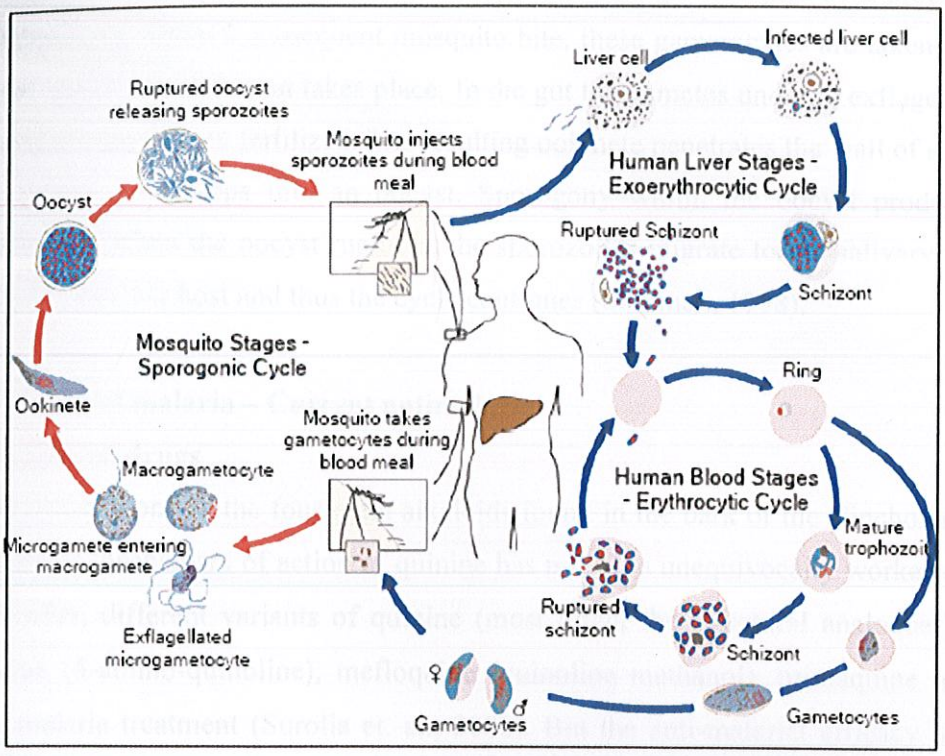


Figure 1.1 Various stages in the life cycle of malaria parasite involving human host and the vector, mosquito (Adapted from http://www.cdc.gov/malaria/lifecycle).

The cycle in man begins with the bite of a female *Anopheles* mosquito harboring sporozoites in its salivary gland during its blood meal. The sporozoites travel through the punctured skin into the blood stream. The sporozoites in the blood stream travel to the liver and invade hepatocytes within 30 minutes of being released by the mosquito. In the liver cells they reside for 9-16 days and then start multiplying asexually within the cells. Asexual reproduction (exoerythrocytic schizogony) in the liver releases thousands of merozoites, which are the first stage of the 48-hour asexual reproduction cycle in the red blood cells (erythrocytic schizogony). The blood stages constituting this cycle, studied by light and electron microscopy (Bannister et. al., 2000) are the merozoite, the ring, the trophozoite and the schizont (Figure 1.1). Schizont ruptures to release around 8-32 merozoites which are ready to invade fresh red blood cells. The erythrocytic schizogony is the time when the human host suffers periodic cycles of clinical symptoms like fever and chills. While the merozoites continue invading fresh RBCs and continue asexual reproduction, some of them exit the

asexual reproduction cycle and mature to male and female gametocytes by a process known as gametogenesis. Upon a subsequent mosquito bite, these gametocytes are taken up into its gut, where sexual reproduction takes place. In the gut the gametes undergo exflagellation and the macrogametocytes are fertilized. The resulting ookinete penetrates the wall of a cell in the midgut, where it develops into an oocyst. Sporogony within the oocyst produces many sporozoites and when the oocyst ruptures, the sporozoites migrate to the salivary gland, for injection into another host and thus the cycle continues (Sherman, 1998).

1.3 Treatment of malaria – Current antimalarials

Quinine related drugs

Quinine is one of the four main alkaloids found in the bark of the Cinchona tree. Till date the actual mechanism of action of quinine has not been unequivocally worked out. Over many decades, different variants of quinine (most often, the structural analogues) such as chloroquine (4-amino-quinoline), mefloquine (quinoline methanol), primaquine have been used for malaria treatment (Surolia et. al., 2002). But the anti-malarial efficacy of each of these has been far from satisfactory due mainly to two major factors: (1) these drugs act on the targets whose biochemical structure/function overlaps with that of the human host (Milhous and Kyle, 1998) (2) evolution of resistant strains of the parasite within the last two decades due to indiscriminate usage of the drugs (Padmanaban and Rangarajan, 2001). Chloroquine is a lysomotropic drug. It is a weak base, uncharged at neutral pH and gets positively charged at acidic pH. Owing to this property, chloroquine is selectively accumulated inside lysosomes. The uncharged compound rapidly diffuses through the plasma and lysosomal membranes, while once charged the compound becomes trapped inside the acidic lysosomal compartment of the parasite (Homewood et. al., 1972). This may lead to the generation of concentration gradient of several orders of magnitude. The intracellular trophozoite feeds on the hemoglobin of the red blood cell that serves as a source of amino acids. Digestion of the globin protein takes place inside the *Plasmodium* lysosome resulting in the generation of free heme (ferriprotoporphyrin IX, FP). The latter is insoluble and precipitates in the form of a black malaria pigment inside the lysosomes. Chloroquine in the lysosome interferes with pigment formation and the FP-chloroquine complex is highly toxic to the parasite.

Drug resistance in *P. falciparum* malaria

Globally the control of malaria is deteriorating. Key factor contributing to the increasing malaria mortality and morbidity is the wide spread resistance of *P. falciparum* to the

conventional antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine (SP) and mefloquine (Figure 1.2) (Ridley, 2002; Ronn, 1996; Sowunmi et. al., 1998; van Agtmael et. al., 1999). Multidrug-resistant *P. falciparum* malaria is prevalent in Southeast Asia and

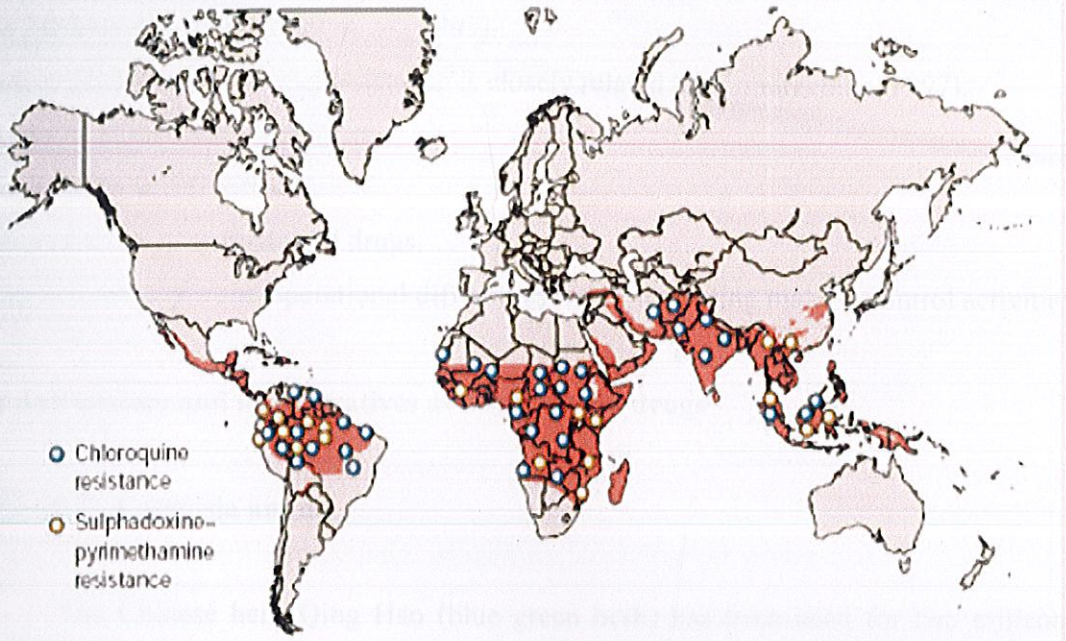


Figure 1.2. Global status of resistance to chloroquine and sulfadoxine/pyrimethamine. Data are from the WHO.

South America. Now Africa, with the highest burden of malaria, is also being affected (Wernsdorfer, 1994; Wesdorfer et. al., 1991). Antimalarial drug resistance is usually a result either of changes in drug accumulation or efflux (chloroquine, quinine, amodiaquine, mefloquine, halofantrine resistance) (White, 1998) or reduced affinity of the drug target resulting from point mutations in the respective genes encoding the target (pyrimethamine, cycloguanil, sulphonamides, atovaquone resistance) (Foote et. al., 1994; Ward et. al., 1995). The resistance occurs when the drug concentrations are sufficient to reduce the susceptible parasite population, but inhibit less or do not inhibit multiplication of the mutants (Chawira et. al., 1987). Resistance causes drug failures when, because of reduced susceptibility, drug levels that would normally eliminate the infection can no longer do so. However, fully drug-sensitive parasites can still cause a recrudescent infection if the plasma concentrations of the drug are insufficient (White, 1999a). Increasing multidrug resistant *P. falciparum* in many parts of the world has aggravated the problem of deciding which antimalarial to use, particularly in countries where *P. falciparum* has developed resistance to chloroquine,

mefloquine primaquine, antifolates such as Fansidar (Sulphadoxine-Pyrimethamine) and, to some extent, quinine which previously was effective in the treatment of severe and complicated malaria (Olliaro et. al., 1995).

Further proliferation of drug resistance is closely related to (Kondrachine, 1997):

- Massive population movements.
- Inadequate health services.
- Improper use of antimalarial drugs.
- Limited resources and operational difficulties in implementing malaria control activities.

1.4 Artemisinin and its derivatives as antimalarial drugs

The plant Artemisia annua

The Chinese herb Qing Hao (blue green herb) has been used for two millennia in Traditional Chinese Medicine (1979; 1992; van Agtmael et. al., 1999). The earliest reference to the plant goes back to "52 prescriptions"' found in the Mawangudi Tomb in an era dating back to 206 BC-AD23. The first prescription of Qing Hao for treatment of related symptoms is found in "The handbook of Prescriptions for Emergencies" by Ge Hong, who lived during AD 281- 340 (Wu and Li, 1995). Active moiety Artemisinin (qinghaosu) was isolated by Chinese scientists in 1972 from the aerial parts of *Artemisia annua* L (Journal Report; Klayman, 1985; Liu, 1979). The compound showed good *in vitro* and *in vivo* antimalarial activity. Several studies showed artemisinin to be an exceptional antimalarial agent with negligible toxicity and high efficacy against human malaria parasites, including those malaria-resistant to conventional antimalarials (Li et. al., 1994).

Chemical structure and metabolism

Artemisinin is structurally different from the previously known antimalarials. The compound is an unusually stable sesquiterpene lactone with an endoperoxide ring (empirical formula C15H22O5) (Figure 1.3). Presence of the endoperoxide moiety is the key to its antimalarial activity (Brossi et. al., 1988; Klayman, 1985; Lee et. al., 1990; Luo et. al., 1987).

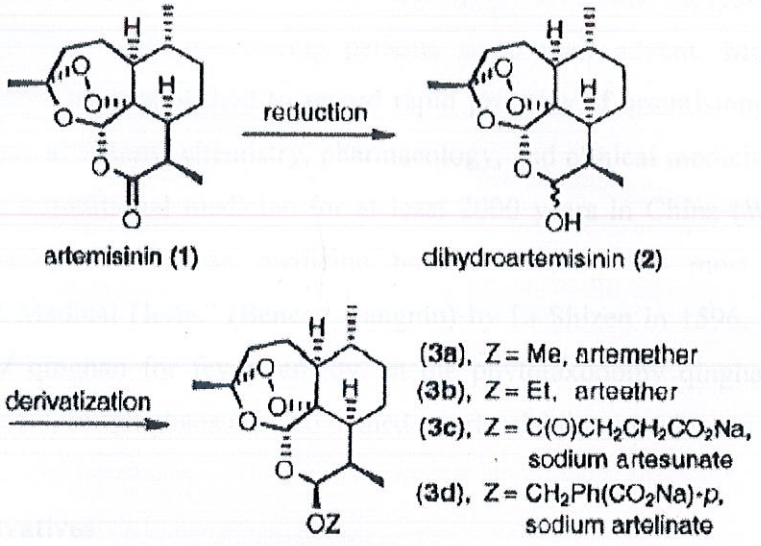


Figure 1.3. Artemisinin and its derivatives.

The white needle crystals of artemisinin are hardly soluble in water or oil therefore formulations other than oral and rectal are not in clinical use. However, since the peroxide bridge is stable under certain chemical reactions, several more soluble artemisinin derivatives, arteether, artemether, sodium artesunate, sodium artelinate and dihydroartemisinin (DHQ) have been synthesized for the treatment of malaria (Figure 1.3). DHQ is the first metabolite of artemether, arteether and artesunate. DHQ is the most effective compound of this class (Janse et. al., 1994. Artesunate can be regarded as a pro-drug of DHQ.

Antimalarial activity

Now artemisinin and its derivatives have been recognized as a new generation of powerful antimalarial drug for combating the most popular infectious disease malaria worldwide. Artemisinin and its derivatives induce more rapid reduction of parasitemia (van Agtmael et. al., 1999), decreasing the number of parasites faster than any other known drug. As a consequence they are of special interest for severe malaria (Hien and White, 1993). The fast decline in the number of parasites is also beneficial in combination therapies. Most of the antimalarials work at late trophozoite and schizont stages of the malaria parasite but artemisinins also act at early trophozoites and ring stages (van Agtmael et. al., 1999). Artemisinins do not affect liver-stage parasites or stages within the mosquito (Price et. al., 1996). Artemether and artesunnate were approved by the Chinese authority and collected in

the "Essential Medicine List" by WHO. These derivatives have been successfully applied to remedy several million malaria-suffering patients since their advent. Meanwhile many research papers have been published to record rapid progress of artemisinin research from different disciplines of botany, chemistry, pharmacology, and clinical medicine etc. Qinghao has been used as a traditional medicine for at least 2000 years in China (Wallaart, 2000). Since then a series of Chinese medicine books including the most famous book "Compendium of Medical Herbs" (Bencao Gangmu) by Li Shizen in 1596, have described the application of qinghao for fever remedy. In the phytotaxonomy qinghao is *Artemisia annua* L. Composites, so Qinghaosu is also named as artemisinin or seldom as arteannuin.

Artemisinin derivatives

From indigenous *Artemisia annua* L., continuous phytochemical studies by Chinese researchers in the early 1980s led to the excavation of another 10 sesquiterpenes including deoxy-artemisinin (Tu et. al., 1981), artemisinin D (Tu et. al., 1981), artemisinin F (Zhu et. al., 1984), artemisinin E (Wu et. al., 1984), artemisinin A (Tu et. al., 1981), epoxyarteannuinic acid (Wu et. al., 1984), artemisinic acid (Deng et. al., 1981; Tu et. al., 1981), artemisinic acid methyl ester (Zhu, 1982) artemisinol (Zhu, 1982) and arteannuin B (Tu et. al., 1981). From biogenetic viewpoint, artemisinic acid or its 11, 13- dihydroanalogue, dihydro-artemisinic acid which was isolated later from *A. annua* is late precursors in the biogenesis of qinghaosu. By the year 1991, 16 closely related sesquiterpenes had been isolated from aerial part of *A. annua* and briefly summarized by Zaman and Sharma (1991). A bisnor-sesquiterpene, norannuic acid was reported in 1993 (Misra et. al., 1993) and three more sesquiterpenes were isolated and reported by Misra et al. (1994). Sy et. al., (1998) isolated seven new sesquiterpenes in 1998. Two amorphane sesquiterpenes, deoxyarteannuin B and dihydrodeoxyarteannuin B were introduced to sesquiterpene family isolated from aerial pars of *A. annua* in 2001. Recently, the first phytochemical investigation of natural products from the seeds on *A. annua* led to discovery of fourteen new sesquiterpenes (Sy et. al., 2001). In addition, two sesquiterpene plant hormones, abscisic acid and its methyl ester were found in an Indian growing *A. annua* (Tewari et. al., 2003). Apart from sesquiterpenes from *A. annua*, essential oils are another active research interest as it could be potentially used in perfumery, cosmetics and aromatherapy. Depending on its geographical origin, the oil yield in *A. annua* ranges between 0.02-0.49% on fresh weight basis and 0.04-1.9% on dry weight basis (Bagchi et. al., 2003; Bhakuni et. al., 2001; Jain et. al., 2002; Rasooli et. al.,

2003; Liu et. al., 1981). Other chemical compounds in *A. annua* includes carbohydrates, traces of glycosides, resins etc.

Pharmacology

Antimalarial drugs derived from natural *Artemisia annua* L. have many advantages: quick reduction of fevers, fast clearing parasites in blood (90% of malaria patients recovered within 48 hrs) and no significant side effects. Experimental and clinical studies reveal that artemisinin, Artemether and artesunnate are not only the potent antimalarial drugs but also the useful agents for other disease, especially as antiparasitic agent. In 1970s, Artemether and artesunnate were confirmed to be more active than artemisinin in animal models (Le et. al., 1980; Le et. al., 1982; Wu et. al., 1995). Some components of *A. annua* Such as qinghaosu, artemisinin B, artemisinic acid, artemisitene, flavonoids and other terpenoids, showed antitumor activities at varying concentrations against L-1210, P-388, A-549, HT-29, MCF-7 and KB *in vitro* (Zheng et. al., 1994; Jung, 1990; Jung, 1997). It was found that dihydroartemisinin can selectively kill cancer cells in presence of holotransferrin, which can increase intracellular iron concentrations, and normal breast cells (HTB 125) and lymphocytes had non-significant changes. It seems the mechanisms of anticancer action and of antimalarial activity are similar (Lai et. al., 1995; Moor, 1995; Singh, 2001). As a response to increasing levels of antimalarial resistance, WHO recommends that all countries experiencing resistance to conventional mono therapies should use combination therapies preferably those containing artemisinin derivatives (ACTs- Artemisinin based Combination Therapies) for *falciparum* malaria. WHO currently recommends the following therapeutic options:

- artemether
- artesunate + maodiaquine
- artesunate + sulphdoxine/ pyremethamine
- artesunate + mefloquine
- amodiquine + sulphaodxine-pyrimethamine

Malaria is a highly treatable disease, and very effective treatments available in the form of Artemisinin based Combination Therapies (ACTs). WHO call on all "Roll Back Malaria (RBM) " partners to unite in a global coalition to enable countries to accelerate access to ACTs and make these lifesaving medicines affordable to the people in need (WHO, 1998).

Artemisinin drugs in the treatment of *Plasmodium falciparum* malaria in India

The National Anti Malaria Drug Policy envisages treatment of uncomplicated *Plasmodium falciparum* (suspected and laboratory diagnosed) with choloroquine and primaquine and in five provinces by the co-administration of artesunate and sulphoxine pyrimethamine (http://namp.govt.in). In this connection, the article on artemisinin-based combination therapy (ACT) by Sushil Kumar and Srivastava is timely (Kumar et. al., 2005). In 2003, the National Vector Borne Disease Control Programme, NVBDCP in short (formerly National Anti Malaria Programme), reported 1.87 million cases of malaria (including 0.86 million *P. falciparum* cases) and 1006 deaths. In 2004, the largest number of malaria cases was reported from Orissa, followed by Gujarat, Chhattisgarh, West Bengal, Jharkhand, Karnataka, Uttar Pradesh and Rajasthan (http://namp.govt.in). The epidemic has been occurring with increasing frequency, killing and demoralizing the affected population; and pushing people below the poverty line. For example, in India, 90% *P. falciparum* cases occur in states below the poverty line (Sharma, 2003). Such epidemics prevent national development in all walks of life and retard the gross domestic product (GDP) (Sachs et. al., 2002). Recent examples of malaria epidemics reported by the NVBDCP include *inter alia* Rajasthan, Haryana (1976), Gujarat, Goa, West Bengal (1997), Goa, Maharashtra (1998), Andhra Pradesh, Assam, Bihar, West Bengal (1999), Uttar Pradesh, Madhya Pradesh, Karnataka (2000), and Rajasthan (2003) (http://w3.whosea.org/EN/Section10/Section21/Section1987.htm). Obviously, the disease burden in the country is hugely under-estimated. This is also reflected by the fact that India's chloroquine consumption in 1976 was 61 metric tons (mt) to treat 6.45 million cases; and in 2005 cases have reduced by 70% but antimalarial usage has increased ten fold (Department of commerce, Government of India, New Delhi, 2004). Based on environment determinants, the World Health Organization (WHO) estimates 100 million cases in the South East Asia Region (SEARO) (WHO, 2004), 70% of these contributed by India (WHO, 2004). *P. falciparum*, the killer parasite accounts for 45–50% malaria cases. Control of *P. falciparum* is important but difficult, as was evident from the failure of the Swedish International Development Agency (SIDA) supported Indian *P. falciparum* Containment Programme (1977–88) (Sharma, 1998). *P. falciparum* parasite is present all over the country, but its distribution is highly uneven. It is the major cause of infection in the Northeast, Orissa, tribal settlements across the country and forests. In the plains of India, *Plasmodium vivax* peak is

followed by *P. falciparum* and in all other endemic areas *P. falciparum* predominates. *P. falciparum* abounds in communities lacking awareness, resources and suffering from endemic poverty (Sharma, 2003; Sharma, 1996; Sharma, 1999; Anon, 1987). With the national antimalaria drug policy of sequential monotherapies and serious compromises in vector control, drug resistance against chloroquine and sulphodoxine pyrimethamine (SP) is on the rise, and more areas are coming under multidrug-resistant malaria1 (Anon, 1987). Drug pressure is selecting for mutations, for example, Pfcrt K76T mutation, an important determinant of chloroquine resistance is present in 95% of the isolates studied (Vathsala et. al., 2004). A direct consequence of drug resistance is the rise in malaria morbidity and mortality, and steep rise in treatment cost by a factor of 40–50 compared to chloroquine (Gupta et. al., 2005).

ACT is a scientific approach to tackle this problem. It is a combination of artemisinin derivative drug with one or more long acting antimalarial drug having different modes of action and different drug targets (Lee, 2002; White, 1999). Artemisinin drugs have a short half-life of 1–4 h or so, but because of their strong anti-plasmodial activity, they reduce biomass of the existing parasites by about 10–4 or say by 95% at each dosage of administration, and also kill the sexual stages of the malarial parasite. Residual parasites, if any, and the recrudescences are eliminated by the long acting antimalarial and the host immunity (International Artemisinin group, 2004; Davis et. al., 2005).

1.5 Mechanism of action

Although artemisinin has been on the market for more than 30 years little is known to date about its biological targets (Wu, 2002). One of the main reasons is that artemisinin does not exert its lethal effect through the whole intact molecule, but rather, through some transient species generated after cleavage of the peroxy bond (Wu, 2002). Artemisinin is hydrophobic and passes biological membranes easily (Augustijns et. al., 1996). *In vitro* studies have suggested an uptake of artemisinin by both healthy and malaria infected red blood cells (Asawamahasakda et. al., 1994). During the blood stage phase of the parasite, more than 70% of the hemeoglobin within the infected erythrocyte is digested (Francis et. al., 1997). Heme is released which is toxic for the parasite and neutralized by polymerization into hemeozoin or "malaria pigment" in the form of a crystalline, insoluble, black-brown pigment. The heme polymerization pathway is specific to the malarial parasite and offers a potential biochemical target for the design of antimalarials. Heme or iron (II) salts triggers reductive

cleavage of the peroxide bond in artemisinin to form oxygen centred radicals. Oxy radicals then form carbon centred radicals (Kamchonwongpaisan et. al., 1996) (Figure 1.4). These radicals cause oxidative stress and damage to the parasite's membrane systems like mitochondria, rough endoplasmic reticulum and plasma membranes (Asawamahasakda et. al., 1994; Cumming et. al., 1997; Maeno et. al., 1993). Recent studies have shown that artemisinin taken up by the malarial parasite growing *in vitro* was selectively concentrated in the parasite food vacuole and was associated with hemozoin (Hong et. al., 1994). Artemisinin also interacts with heme, forming covalent adducts (Hong et. al., 1994; Meshnick et. al., 1991). However, it has also been reported that the artemisinin heme complex does not possess any antimalarial activity (Meshnick et. al., 1991). Further studies related to structural and mechanistic aspects of the interaction of artemisinin with heme may yield important information for the design of better antimalarials.

PfATP6, a target for artemisinin

More recently, an alternative mechanism of action for artemisinins based on inhibition of the malarial parasite's calcium ATPase (sarco endoplasmic reticulum calcium ATPase, SERCA) has been suggested. Only one *P. falciparum* SERCA orthologue, PfATP6, has been identified (Kimura et. al., 1993; Varadi et. al., 2003). It was demonstrated that the SERCA inhibitor thapsigargin could induce calcium release into the cytosol from intracellular stores, probably endoplasmic reticulum (ER), by inhibition of the PfATP6, suggesting that PfATP6 is essential for *P. falciparum* calcium homeostasis (Varadi et. al., 2003) and that PfATP6 is functionally related with higher mammal homologues. The SERCA inhibitor thapsigargin is a sesquiterpene lactone, as are artemisinin. From these structural similarities the hypothesis emerged that ART act by inhibiting PfATP6. This was supported by the demonstration that artemisinin specifically inhibited PfATP6 expressed in *Xenopus laevis*, as thapsigargin. The two drugs showed an antagonistic interaction in *P. falciparum* cultures and similar localization in the parasite. Hence PfATP6 was suggested to be a target of ART (Eckstein-Ludwig et. al., 2003). However the mechanism of interaction as well as the binding affinity of artemisinin with PfATP6 has not yet known.

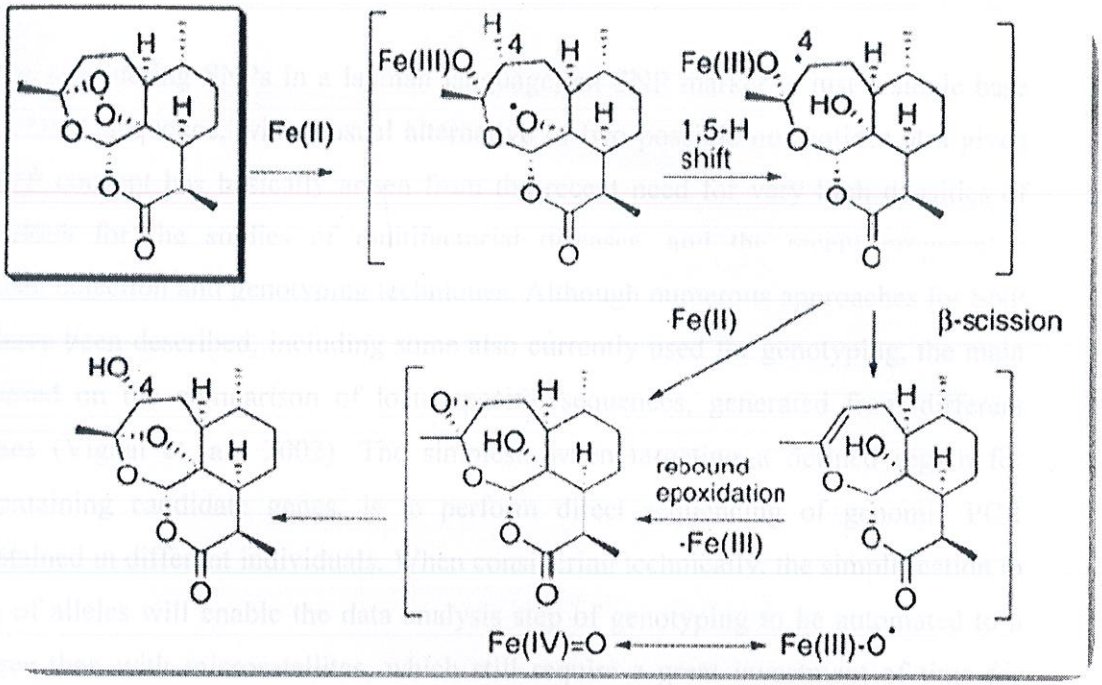


Figure 1. 4. Mechanism of action of artemisinin. Formation of free radicals.

1.6 Single Nucleotide Polymorphism

When considering SNPs in a layman language, an SNP marker is just a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. SNP concept has basically arisen from the recent need for very high densities of genetic markers for the studies of multifactorial diseases, and the recent progress in polymorphism detection and genotyping techniques. Although numerous approaches for SNP discovery have been described, including some also currently used for genotyping, the main ones are based on the comparison of locus-specific sequences, generated from different chromosomes (Vignal et. al., 2002). The simplest, when targeting a defined region for instance containing candidate genes, is to perform direct sequencing of genomic PCR products obtained in different individuals. When considering technically, the simplification in the scoring of alleles will enable the data analysis step of genotyping to be automated to a higher degree than with microsatellites, which still require a great investment of time for reading the data, even with the use of analysis software such as GENOTYPER (Applied Biosystems) or other automated allele analysis methods (Idury et. Al., 1997). SNPs are also being used for the generation of positional candidate gene and for the development of comparative maps. The very high density of SNPs in genomes, usually allows to develop several of them in a single locus of a few hundred base pairs. By reconstructing haplotypes, multi-allelic systems can eventually be defined for analyses, to overcome the limitations due to the low heterozygosity of SNPs. With increasing progress being made in the molecular techniques used to produce SNP data, in the automation of allele scoring and in the development of algorithms for genetic analyses (Abecasis et. al., 2002), the effort needed to produce an equivalent amount of information as with microsatellites may some day be equivalent.

CHAPTER 2

Material and Methods

2.1. InSilico Analysis

BlastClust (http://toolkit.tuebingen.mpg.de/blastclust) was performed on our dataset of 85,282 EST sequences to remove the highly similar sequences using 60% sequence similarity and 100% sequence coverage and ended up with 68974 EST sequences.

2.1.1. Primer Designing

The important enzymes responsible for the biosynthesis of Artemisinin in the DOXP pathway were identified (Figure 2.1). As for Artemisia annua, only ESTs are available. The enzyme sequences were downloaded from NCBI database (www.ncbi.nlm.nih.gov). Now, these sequences were used to find similarity between these sequences and EST sequences available for Artemisia annua using Nucleotide BLAST (Basic Local Alignment Tool). Multiple Sequence Alignment was then performed on sequences which resulted in score bit of greater than 149, this was done using MegAlign (ClustalW method). Conserved region was taken from the resultant MSA conserved sequence and Gene prediction was done from consensus sequence using online gene prediction tool GENSCAN (http://genes.mit.edu/GENSCAN.html). Using online available primer designing tool 'Primer 3'(http://frodo.wi.mit.edu/primer3), we were able to design left and right primers. (Appendix I).

2.2. Plant materials

Twenty genotypes of Artemisia annua were collected from two valleys (Leh and Nubra) with altitude ranging from 9,600 m (Nubra) to 11,500 m (Leh) from the cold arid desert of Trans-Himalayas (Ladakh) (Figure 2.2). Ten genotypes from each valley were obtained from the nursery of Defense Institute of High Altitude Research (DIHAR). The leaves were stored in laboratory at –20°C until further analysis. The interval between samples was 100-200m, the pair wise distance between valley divisions was 50– 250 Km.

2.3 DNA extraction and PCR amplification

Several experiments based on the available protocols (Doyle and Doyle 1990; Stewart and Via 1993; Stange et al 1998) were performed using fresh plant materials for (i) incubation time of buffer and tissue mixture at 65^0C, (ii) buffer to tissue ratio and (iii) extraction with phenol:chloroform:isoamyl alcohol vs. Tris saturated phenol followed by chloroform: isoamyl alcohol extraction in extraction and purification phases. All the experiments were repeated 3-4 times to check reproducibility.

2.3.1 Extraction Buffer

The extraction buffer (pH 8.0) contained 2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 3% PVP and 0.2% β-mercaptoethanol.

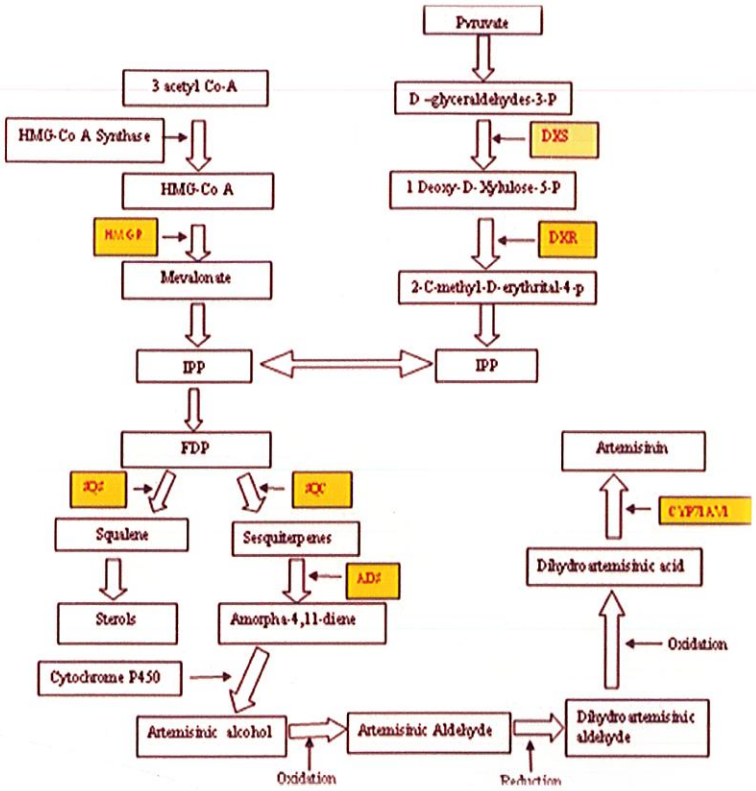


Figure 2.1 DOXP pathway for biosynthesis of Artemisinin

Figure 2.2. Collection sites of 20 *Artemisia annua* genotypes from two valleys (Leh and Nubra) and the two collection sites (Leh and Partapur) located in Ladakh (Jammu and Kashmir, India).

2.3.2 Reagent and Solution:

Tris saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1), 70% and 80% ethanol, 4M NaCl, 3M sodium acetate (pH 5.2) and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Solutions and buffers were autoclaved at 1210C at 15 psi pressure. The stock solution 10 mg/ml of RNase A was prepared as per the user's manual (Sigma USA).

2.3.3 DNA Extraction

Fresh young plant leaves plucked form the nursery were rinsed with distilled water and blotted gently with soft tissue paper, 0.1 g of this tissue, precooled using liquid nitrogen, was ground to a fine powder with a mortar and pestle along with 10 mg (2% of extraction buffer) of PVP (Sigma). The powdered tissue was scraped into a 2.0ml microcentrifuge tubes containing preheated (65^0C) extraction buffer in a 1:5 ratio (0.5 ml). β-Mercaptoethanol was then added to the final concentration of 0.2 M and mixed well. The mixture was incubated in water bath at 65^0C for 90 min and cooled for 5 min. An equal volume of chloroform:isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion for 5 to 10 min to form an uniform emulsion. The mixture was centrifuged at 8000 rpm for 8 min at room temperature. Chloroform: isoamyl alcohol extraction was repeated again. The aqueous phase was pipetted out gently, avoiding the interface. To the above solution, 5 M NaCl (to final concentration 2M) and 0.6 volume of isopropanol of the total solution was added and incubated at room temperature for 1 h. To the above solution, two volumes of 80% ethanol was added and incubated again for 10 min at room temperature for DNA precipitation. After incubation, the mixture was centrifuged at 10,000 rpm for 15 min. The white/translucent pellet was washed with 70% ethanol, dried and resuspended in 200 µL of TE buffer.

2.3.4 Purification phase

The sample was then treated with 20 µL of 10 mg/ml of RNase and incubated at 37^0C for 60 min. After incubation with RNase, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inverting the microcentrifuge tube followed by centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was pipetted out into a fresh tube. The sample was then extracted with equal volumes of chloroform:isoamyl alcohol (24:1) twice. The DNA was precipitated with 0.6 volumes of isopropanol, 2.0 M NaCl (final concentration) and incubated for 10 min. To the above, 20 µL of sodium acetate and 1 volume of 80% ethanol were added, incubated for 30 min and

centrifuged at 5,000 rpm for 3 min to pellet the DNA. The pellet was then washed with 70% ethanol twice; air-dried and finally suspended in 40-50 µL of TE buffer.

2.3.5 Evaluation of resultant DNA

The yield of the extracted DNA and purity was checked by running the samples on 0.8% agarose gels along with standard (non restriction enzyme digested), lamda DNA marker (Biogene, USA).

2.4 PCR Amplification

Twenty-Four primer sets were obtained from 'Applied Biosciences, India' and PCR amplification was performed. Amplification reaction were performed in volumes of 25 µl containing 10 mM Tris- HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 200 µM of each dNTPs, 0.4 µM primer, 20 ng template DNA and 0.5unit of *Taq* polymerase (Sigma-Aldrich, USA). Initial denaturation for 4 min at 94 °C was followed by 40 cycles of 45 second at 94 °C, 30 second at specific annealing temperature (± 5 °C of Tm), 2 min at 72 °C and a 5 min final extension step at 72 °C. PCR products were stored at 4 °C before analysis. Table 2.1

The amplification for each primer was performed twice independently with same procedure in order to ensure the fidelity of markers. Amplification products were electrophoresed on 1.5 % at constant voltage (70 V) in 1X TAE for approximately 2 h, visualized by staining with ethidium bromide (0.5 µg ml-1) and a total of 2.5 µl loading buffer (6X) was added to each reaction before electrophoresis. After electrophoresis, the gels were documented on a gel documentation system (Alpha Innotech, Alphaimager, USA). Molecular size of amplicons was estimated using a 100 bp and 1 Kb DNA ladders ('Bangalore Genei, India).

Table 2.1

Reaction Mixture for PCR Amplification

Components	Stock Concentration	Used Concentration	Amount(in µl)
DNA template	-	-	3.0
Autoclaved Water	-	-	16.7
Forward Primer	50pM	5pM	0.5
Reverse Primer	50pM	5pM	0.5
dNTPs	2mM	.2mM	0.6
$MgCl_2$	25mM	1.8mM	1.0
PCR Buffer	10X	1X	2.5
Taq Polymerase	5U	.5U	0.2
Total	-	-	25 µl

2.5 EXTRACTION OF ARTEMESININ

Weight of each sample was noted and 100 mg of the powdered form of sample was taken. Dried leaves and inflorescences were ground to a powder in a pestle and mortar. Artemisinin was extracted following the procedure of Broomhead et al., (1990). About 5 ml of ethanol was added and heated at 30°C for 2 hours. The extract was filtered through Whattman filter paper No.1. Again 5ml of ethanol was added to the residue and heated at 30°C for 2 hours. Second, third and fourth extractions of the same samples were done with 10 ml ethanol for 10 min. under condition mentioned above. All the extracts were pooled and ethanol evaporated to dryness in a water bath shaker at 60 ^{0}C. The resultant residue was dissolved in 10 ml acetonitrile (HPLC grade) and filtered with 0.22 µm durapore membrane filter (Millipore) for GC analysis.

Figure 2.3 Powdered leaves and inflorescence of Artemisia annua plant

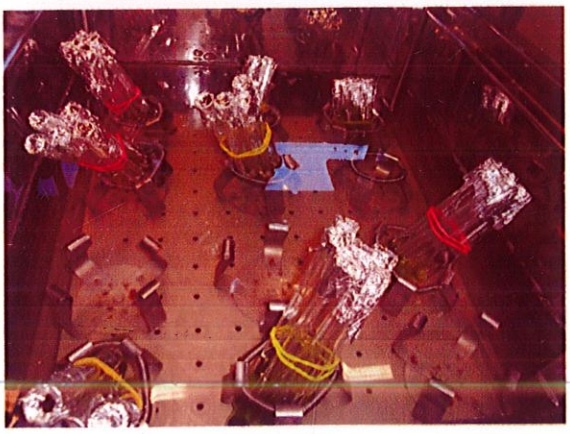


Figure 2.4 Samples kept for heating during extraction of artemisinin

Figure 2.5 Filtered solution in a test-tube.

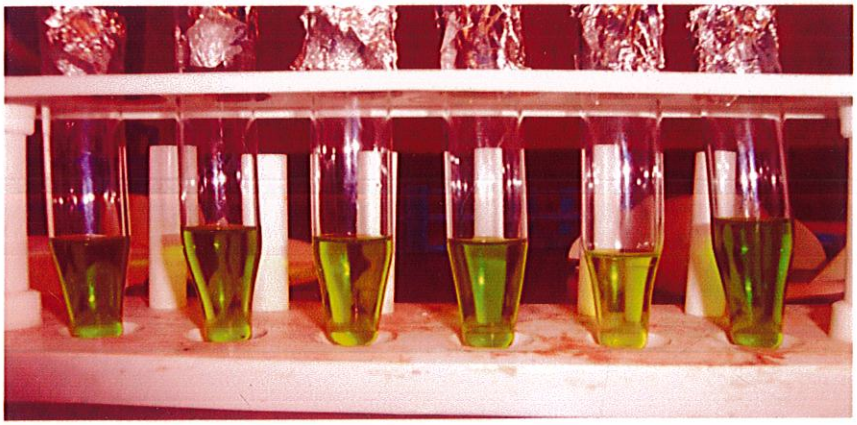
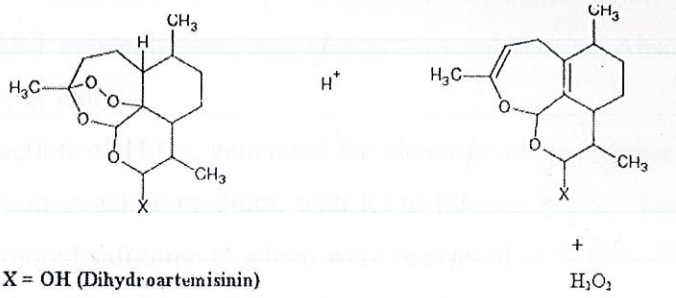


Figure 2.6 Filtered extracts

2.6 Quantification of Artemisinin: analysis of presence of Artemisinin by Spectrophotometric and gas-chromatographic techniques (gc- fid)

2.6.1 Spectrophotometric determination of Artemisinin (ART)

A simple and rapid method for determination of ART is described. The method is based on the reaction of H_2O_2 generated by cleavage of endoperoxide linkage of ART and its reaction with potassium iodide (KI) to liberate iodine (I). Liberated I bleaches red colored safranine O to colorless species and is measured at 521nm. Beer's law is obeyed in the range of 16-112 µg/ml for both ART & DHA. The molar absorptivity, Sandell's sensitivity, detection limit and quantitation limit for ART were found 0.3401×10^4 l/mol/em, 1.43×10^{-2} µg/cm^2, 0.0625 µg/ml and 0.2075 µg/ml. The optimum reaction conditions and other parameters were evaluated. The statistical evaluation of the method was examined by determining intraday and inters day precision.



SCHEME 2. I.1 - REACTION OF ARTEMISININ AND DIHYDROARTEMISININ IN ACIDIC MEDIUM TO FORM HYDROGEN PEROXIDE (H_2O_2)

$$H_2O_2 + 2H^+ + 2I^- \longrightarrow I_2 + 2H_2O$$

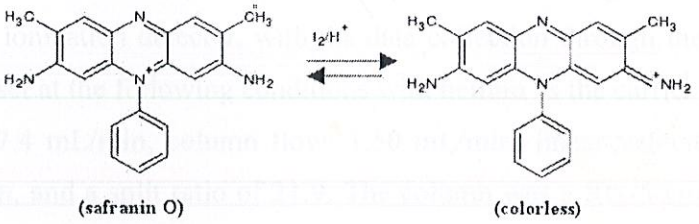


Figure 2.7 REACTION BETWEEN HYDROGEN PEROXIDE –POTASSIUM IODIDE SYSTEM WITH SAFRANIN O

2.6.2 Experimental procedure:

Apparatus used was spectrophotometer. Reagents involved were HCL(5M), 2% Potassium Iodide(KI), Sodium Acetate(CH3COONa)(2M) and Safranin(0.01%). To prepare 0.01% safranin, 0.01gm of safranin was dissolved in 50% ethanol and the volume was raised to 100ml using water. Standard ART solution: stock - 1000µg/ml (0.1g ART was dissolved in

ethanol and the volume was raised to 100ml), working solution – stock solution was diluted appropriately to get the working solution.

2.6.3 Method of determination of Artemisinin:

A 10ml calibrated flask was taken and different aliquots of ART in a series (16.0-112.0µg/ml) were transferred to it. Then 1ml of KI and 1ml of HCl to each flask was added and shaken gently

until a yellow color appeared due to liberation of iodine. The reaction mixture was shaken for 5 minutes. Contents were diluted with distilled water and mixed well.The absorbance of each solution was measured at 521nm against corresponding reagent blank (made by replacing ART by distilled water). Absorbance corresponding to the bleached color, which in turn corresponds to analyte ART concentration, was obtained by subtracting Absorbance of test solution from Absorbance of blank.

The method involves reaction of H_2O_2, generated by cleavage of endoperoxide linkage of corresponding ART solution in acidic medium, with KI to liberate iodine. This liberation of iodine bleaches the red colored safranine O which were measured at 521nm. The decrease in absorbance is proportional to Artemisinin concentration.

2.6.4 GC-FID method for artemisinin estimation

Analysis of artemisinin and precursors was performed in a gas chromatographer equipped with a flame ionization detector, with the data collection through the GC-Solution software. The GC was set at the following conditions with helium as the carrier gas: pressure: 15.6 psi, total flow: 37.4 mL/min, column flow: 1.50 mL/min, linear velocity: 50.5 cm/s, purge flow: 3.0 mL/min, and a split ratio of 21:9. The column was a Rtx-5 crossbond 100% dimethyl polysiloxane, (15m×0.25mm ID, 0.25_m film thickness). Column temperature was set at 195 °C, injector at 240 °C, and FID temperature set at 300 °C, and sampling rate of 40 ms.

CHAPTER 3

Results and Discussion

3.1 DNA Extraction

Many protocols were attempted for extraction of good quality DNA from *Artemisia annua* but were not possible to obtained good quality DNA. The Artemisia species obtained from high altitude (Leh and Partapur) contain high amount of polysaccharides, polyphenols, essential oils & other secondary metabolites that interfere with DNA isolation. These secondary metabolites entangled to nucleic acid during DNA isolation & interfere with subsequent isolation procedure. We developed a simple and efficient method of genomic DNA extraction from leaf of *Artemisia annua* by modifying CTAB protocol (Khanuja et al 1999). Figure 3.1(a-c) shows the stages of the procedure of DNA extraction as well as the purity of DNA sample. By using 3.5 M NaCl in extraction buffer and 80% ethanol with 2.0 M NaCl (final concentration) during precipitation and further purification with Tris saturated phenol during purification phase the quality and quantity (200-400 ng) of DNA was improved significantly without contamination of polysaccharides and secondary metabolites. In the present protocol, the use of 3.5 M NaCl in the extraction buffer reduced 90% of polysaccharides contamination and very little or no jelly like precipitate was found during precipitation of DNA. One of the most significant steps of our protocol was the use of only Tris saturated phenol (pH 8.0), followed by chloroform: isoamyl alcohol extraction. Most of the protocols in the literature used phenol: chloroform: isoamyl alcohol (25:24:1) or chloroform: isoamyl alcohol (24:1) for protein removal (Doyle and Doyle 1990; Dellaporta et al 1983), whereas in our experiments use of either phenol: chloroform: isoamyl alcohol (25:24:1) or chloroform: isoamyl alcohol (14:1) gave yellowish pellet (Figure 3.1b), which confirmed the presence of protein contamination. By using Tris saturated phenol (pH 8.0) followed by chloroform: isoamyl alcohol (24:1) extraction, protein impurities could be successfully removed, without affecting DNA yield. It was also observed that buffer to tissue ratio and incubation time were also important factors for obtaining higher yields of DNA and in case of Artemisia annua 5:1 buffer to tissue ratio and 90 min incubation at 75^0C gave best results. On agarose gel electrophoresis, DNA gave sharp bands (Figure 3.2). Use of high concentration of NaCl in the extraction buffer decreased contamination of polysaccharides (Danshwar P and Sher-ullah 2004). Compared to precipitation at -20 ^{0}C (Khanuja et al 1999), we could achieve the same at room temperature without compromising the quality and quantity of DNA suitable for ligation, PCR amplification and other downstream processes necessary for DNA fingerprinting (Figure 3.3 & 3.4).

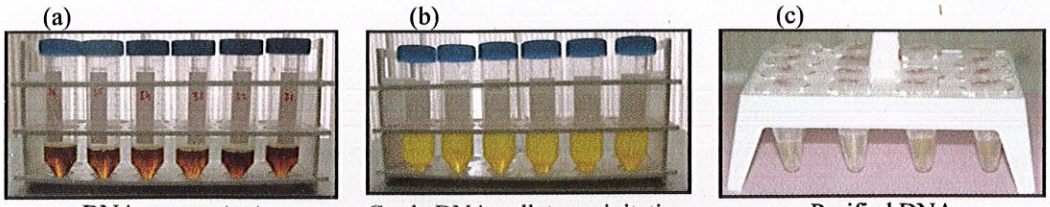


Figure 3.1. The stages of the procedure of DNA extraction as well as the purity of DNA sample.

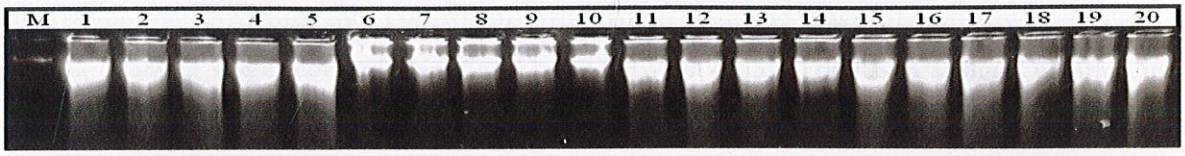


Fig 3.2: Agrose gel electrophoresis showing purified high molecular weight *Artemisia annua* genomic DNA (The genomic DNA used for this study was extracted and purified with modified Doyle and Doyle method).

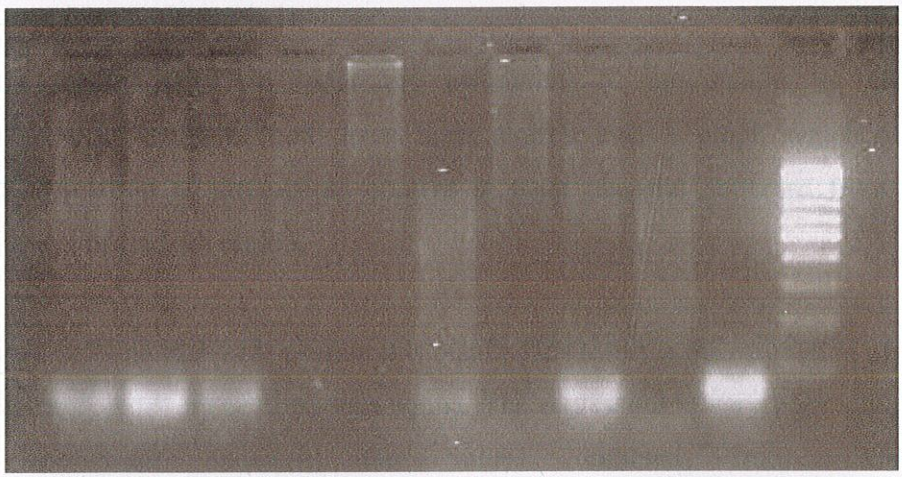
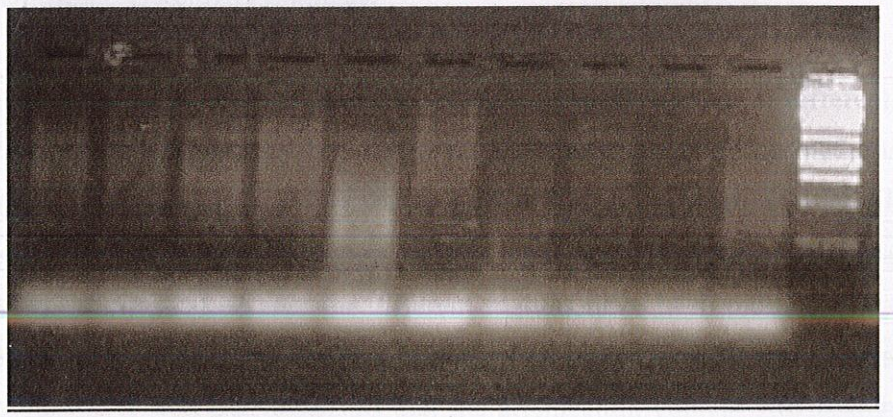


Figure 3.3 and 3.4



3.2 Artemesinin estimation

Artemisinin was extracted from the leafs and flowers of the plant which yielded analytical results stating that content of Artemisinin with the stage of the flower, in early stages(budding stage) content was found to be between 0.057-0.064% in 100gm, whereas in late stages, it was 0.078-0.086%. Also that the content found in plants from Leh region (0.065-0.086%) is more than that of Partapur region (0.057-0.079%) at each stage of the flower. Highly significant correlation(>0.9) was observed in artemisinin content (w/w%) obtained by GC-FID and spectrophotometric methods.

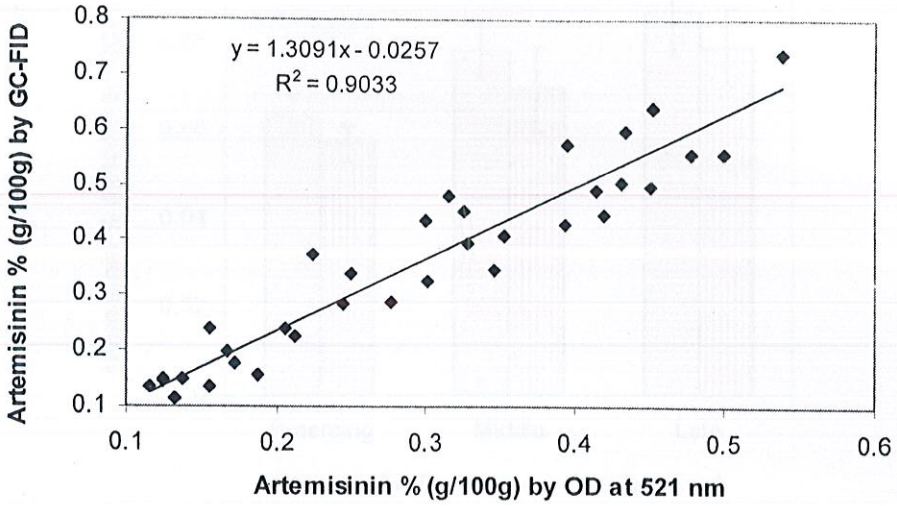
This section tells us about the estimation of artemisinin by GC-FID and spectrophotometry results of both have been plotted over a graph which gives highly significant correlation of artemisinin content in plant material collected from Nubra and Partapur valleys of Ladakh region.

Figure 3.5(a) and 3.5 (b) present this result. This result is the estimation of artemisinin from 35 different cultivars of Artemisia annua. The regression is very high which implies a positive correlation between the values obtained from GC-FID and SPECTROPHOTOMETRIC methods. The graph here shows a linear increase in atremesinin content obtained by using these methods.

Figure 3.6 shows the Artemisinin content present during 3 different stages of inflorescences, when analyzed by both GC-FID and spectrophotometric methods, the three different stages are emerging (budding stage), middle (flowering stage) and late (later stage). The values in the bar graph are means of ten replicates and vertical bars represent the standard deviation. Individual bars for different regions have been plotted. The artemisinin content was found to be more in samples from the Leh region as with those compared to the Partappur samples. It was also observed that the artemesinin content was found to be very high in the later stages (Late) of inflorescence than the emerging and the middle stages.

Table 3.1 shows quantified artemisinin data from both Leh and Partapur region. Mean values have been given from each stage of development of inflorescence. F1 and F2 are the continuous probability distribution between the stages of development and site of sample collection. The F-value between the interaction of sites and stages of inflorescence development is 0.03 and 0.02 respectively using both the estimation methods and are not statistically significant. The statistical test used for the analysis is 2-way AMOVA. The p-value is the observed significance level of a hypothesis test which equals the probability of making a Type I error. As the p-value is very also is nearly all the cases, null hypothesis is rejected. The result in our case is statistically significant because the F-values in all the cases are greater than p-values.

(a) Partappur



(b) Leh

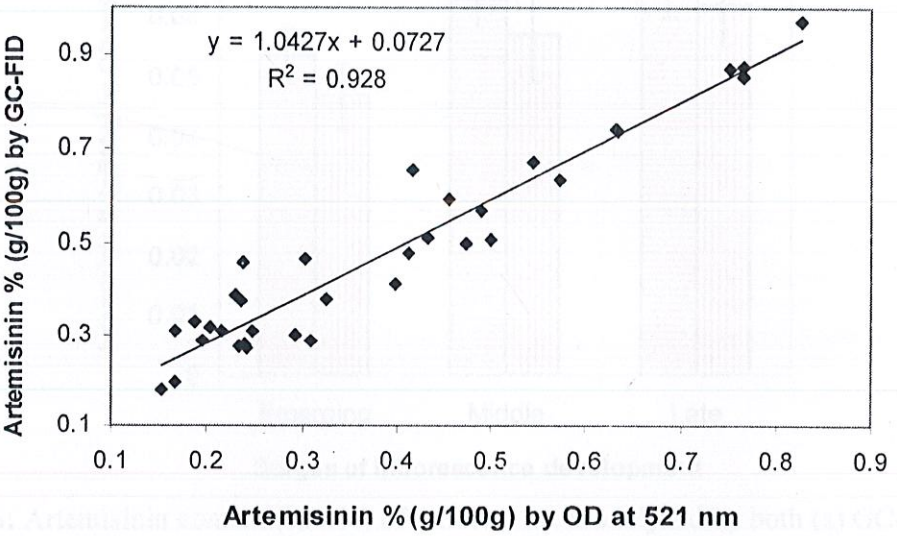
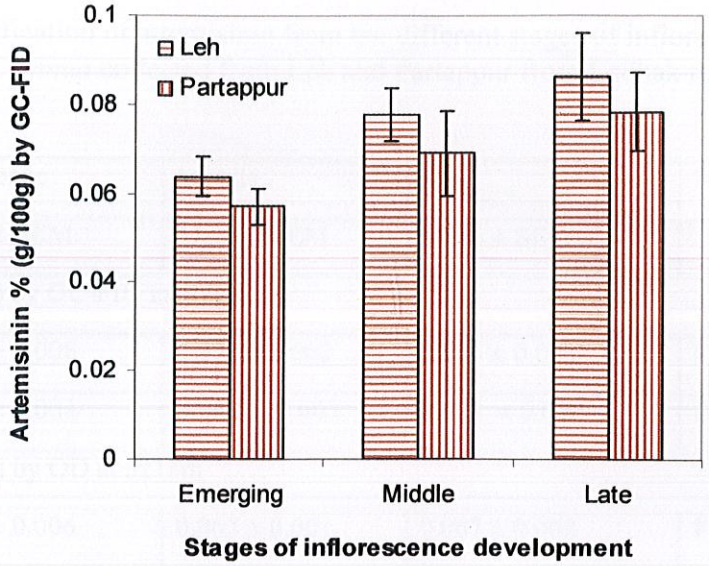


Figure 3.5: Correlation of artemisinin content (w/w%) of 35 plant materials analyzed by both GC-FID and spectrophotometric methods. These plant materials consisted of leaves from different parts of 35 cultivars of *A. annua*. Samples were collected from 2 different sites: (a) Partappur and (b) Leh from Ladhak region.

(a)



(b)

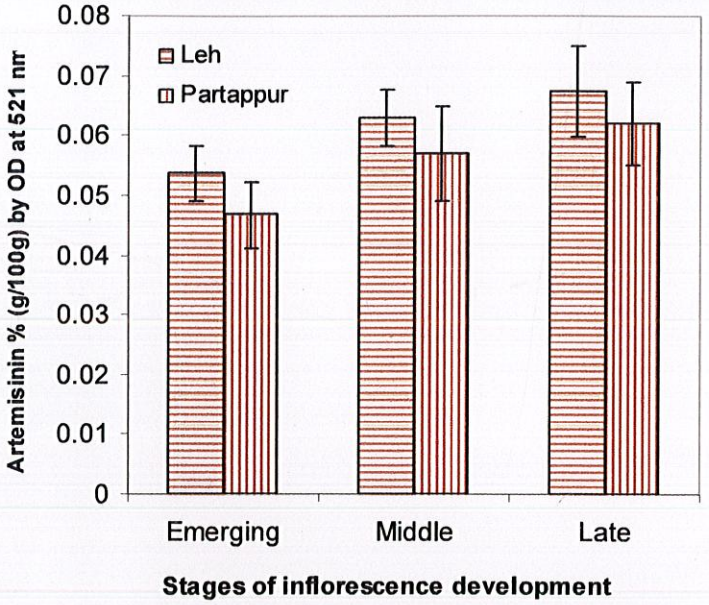


Figure 3.6: Artemisinin content (w/w%) of inflorescences analyzed by both (a) GC-FID and (b) spectrophotometric methods at 3 stages of development of *A. annua*. The values are means of 10 replicates. Vertical bar represent ± standard deviation. Samples were obtained from 2 different sites Partapur and Leh from Ladhak region.

Table 3.1. Quantification of artemisinin from the different stages of inflorescence development of *A. annua* collected from Leh and Partappur from Ladhak region.

	Emergence	Middle	Late	F-value	P-value
	Mean ± SEM	Mean ± SEM	Mean ± SEM		
(a) Artemisinin % (g/100g) by GC-FID method					
Leh	0.064 ± 0.008	0.078 ± 0.002	0.086 ± 0.003	F1 = 13.20	< 0.001
Partappur	0.057 ± 0.004	0.069 ± 0.003	0.078 ± 0.002	F2 = 5.00	0.030
(b) Artemisinin % (g/100g) by OD at 521nm					
	0.054 ± 0.006	0.063 ± 0.001	0.067 ± 0.002	F1 = 8.71	0.001
	0.047 ± 0.004	0.057 ± 0.002	0.062 ± 0.007	F2 = 4.55	0.038

Conclusion

DNA extraction protocol was standardized, which was a must as quality of DNA was very bad because of the presence of polyphnolic compounds, the extracted DNA was used for amplification of regions of Artemisia annua genes which are responsible for production of secondary metabolite, Artemisinin. These genes were of those enzymes which play a key role in the formation of Artemisinin. The primers designed for amplification were designed after a thorough study of DOXP pathway for Artemisinin production.

Artemisinin was also extracted from the samples collected from partapur and leh valley of ladakh region. These samples were analyzed and quantified, results of which state that Artemisinin content increases with the stage of flower, that is, its low in early stage (budding stage) whereas comparatively higher in late stages, also, content of Artemisinin in samples from Leh region were higher than those of Partapur region.

The amplified regions have been sent for sequencing results of which are awaited, the sequenced results would be used in identification of Single Nucleotide Polymorphisms.

Results of this research could be very helpful in selection of genotype of Artemisia annua for commercial production. Also, the SNPs which will be identified can be used to generate comparative maps for regions of Artemisia annua genome.

Appendix I

S.N.	Sequence 5'-------3'	Length	G+C%	Tm
1 L	AAATGTTGGCATTTGTATTCCAC	23	34.78	49
1R	CACATATTTACTCCAGATCTCACGA	25	40	54
2L	ATTTCAGTTTTGGCCTCGTG	20	45	49
2R	CAAAAACAATACCTTTTACATTTCAAG	27	25.93	50
3L	ATCTCGTAAGACTTATGCGGTA	22	40.91	50
3R	ACCACTTAGCAGAGTCCTGTG	21	52.38	54
4L	TAGGAAAGCGGCACATACCAGG	22	54.55	56
4R	AGCCATCAGTGAGATACCACTCTG	24	50	57
5L	TTGGCCCGTACGTCCTGACACAA	23	56.52	58
5R	TCAAACAAACAAACACAGCCCA	22	40.91	50
6L	GGGAATTCAGCACTGTCTTTGAGC	24	50	57
6R	AGGTTAACCGGTCTCCCTGAA	21	52.38	54
7L	TCATTCCAGTCGCTTCGTGAAGAA	24	45.83	55
7R	AGGTAGTGGAGGGTGCAACCTCAA	24	54.17	58
8L	TACCAGACGGGATTGGGGTTCCA	23	56.52	58
8R	CGTACTTGGGCAAACACGAGAGG	23	56.52	58
9L	CTACAGACTGTTAGAAAGACAGC	23	43.48	53
9R	TCTTGCAGCAAATGTAACCGCA	22	45.45	52
10L	ACTCGGTGTTTTGATGTAGGCA	22	45.45	52
10R	CGATACTATGACCATCAACCGGA	23	47.83	55
11L	CGTGGTATGATTAGTGGTTCTG	22	45.45	52
11R	AGCTGAGCTTTTTAACATGGTTGC	24	41.67	53
12L	ACGGAACCCCAATCCCGTTTCCAC	24	58.33	60
12R	GGTCAATTGGAGGGTTCGGCTC	22	59.09	58
13L	AACATGGGAGCCGAACCCTCCA	22	59.09	58
13R	CTGCAGACCAATTAGCCGAAGC	22	54.55	56
14L	GCTTCGGCTAATTGGTCTGCA	21	52.38	54
14R	CGATACTATGACCATCAACCGGA	23	47.83	55
15L	AGCAAGGGGTTATTGAGGTTGC	22	50	54
15R	GCATCAAGCGGAATTGGTGACAGC	24	54.17	58
16L	GGAAGGCGCTGTCACCAATTCC	22	59.09	58
16R	CTGCACAACCAACTATGCCCGTG	23	56.52	58
17L	AGCAAGGGGTTATTGAGGTTGC	22	50	54
17R	CATACACTCGATGGTTGAGACAC	23	47.83	55
18L	AGCTGCATACTCTCGAGCCCACAA	24	54.17	58
18R	TTCAACCGGCCAATCCCTGAAAGC	24	54.17	58
19L	CATCGATGAACATCTCAACAGC	22	45.45	52
19R	TAGCAGAATGTTCTGAGTCGGCTG	24	50	57
20L	TCAACGGCTTTCTCGTTAGCTGCA	24	50	57
20R	AAGATCACGGTTGACTCTGCCA	22	50	54
21L	ACCATGGCTACACGAGATGTC	21	52.38	54
21R	TACGAGAAGTGAGCATAGAGC	21	47.62	52
22L	TGTACCCGTCTTGACACCAACG	22	54.55	56

22R	AAACCGTCTCCAGCCAATGAC	21	52.38	54
23L	TCGTGTCAGTGAAAAGAACCTGG	23	47.83	55
23R	GACATCTCGTGTAGCCATGGTG	22	54.55	56
24L	ACCATGGCTACACGAGATGTC	21	52.38	54
24R	CAGTGCCCAAAGAACGTTGACCCA	24	54.17	58

List of Abbreviations

ACT	Artemisinin Combination Therapies
AMOVA	Analysis of Molecular Variance
ART	Artemisinin
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl Trimethyl Ammonium Bromide
DHQ	Dihydroartemisinin
DNA	Deoxyribonucleic acid
EST	Expressed Sequence Tag
GC-FID	Gas Chromatography- Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
RBCs	Red Blood Cells
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-pyrimethamine
TCD	Thermal Conductivity Detector
WHO	World Health Organization

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