



# A Review of *Artemisia annua* L.: Its Genetics, Biochemical Characteristics, and Anti-Malarial Efficacy

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## ABSTRACT

This paper presents a review on the genetics of *A. annua* as an important plant species for the treatment of malaria. A short history on the discovery of the plant is discussed. Furthermore, a clear description of the characteristics of *A. annua* is presented as the plant's chemistry, mechanism of action and efficacy are thoroughly discussed. Many cytogenetic studies on *A. annua* focusing on the production of the antimalarial drug, artemisinin are also presented. New technologies on artemisinin production are also discussed focusing on the use of microorganisms. The last part of the review discusses issues and future prospects of *A. annua* and artemisinin production for malaria therapy.

**Keywords:** artemisinin, biochemical property, anti-malarial efficacy, mechanism of action, essential oil

## 1. INTRODUCTION

Caused by blood parasites of the genus *Plasmodium* including *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*, malaria is a global predominant disease in the tropics (Odugbemi, et al., 2007). According to World Malarial Report Malaria and WHO, malaria has a greater morbidity and mortality than any other infectious diseases of the world. In 2010, there were an estimated 216 million cases of malaria and 655,000 deaths worldwide, with children under five years and pregnant women the most vulnerable (WHO, 2011). Over 81% of cases and 91% of deaths were in Africa, with the majority of the remaining being in India, Southeast Asia and South America (Cruz, et al., 2013).

Within the context of traditional practice, malaria is commonly treated by decoctions or infusions from bitter plants (**Randrianarivojosia, et al., 2003**). The use of medicinal plants has been a central component of health care in many cultures for centuries, dating as far back as 5,000 years. It is estimated that up to 80 percent of the world now relies on medicinal plants as their main source of health care (WHO, 2010). As currently observed in the market, more than 120 pharmaceutical drugs contain extracts from medicinal plants (Botsaris, 2007). Many of these drugs are antimalarial in action and origin which have been derived from medicinal plants traditionally used to treat malaria. Odugbemi, et al. (2007) identified 50 plant species including *Morinda lucida* (Oruwo), *Enantia chlorantha* (Awopa), *Alstonia boonei* (Ahun), *Azadirachta indica* (Dongoyaro) and *Khaya grandifoliola* (Oganwo) which were found to be useful for malaria therapy at Okeigbo, Southwest, Nigeria. Similarly, Pascaline et al. (2011) identified a total of 44 plant species in 40 genera distributed in 27 families used to treat malaria in South Nandi District, Kenya. In Brazil, Botsaris (2007) also identified 40 plant species that were used to treat malaria in which eight species, namely, *Bathysa cuspidata*, *Cosmos sulphureus*, *Cecropia hololeuca*, *Erisma calcaratum*, *Gomphrena arborescens*, *Musa paradisiaca*, *Ocotea odorifera*, and *Pradosia lactescens*, are reported as antimalarial for the first time in ethnobotanical studies. He also reported that some species, including *Mikania glomerata*, *Melampodium divaricatum*, *Galipea multiflora*, *Aspidosperma polyneuron*, and *Coutarea hexandra*, were active against malaria in some patients under clinical observation.

*A. annua* was known in 2005 as Youyou evaluated 380 extracts of different plants among which the plant species was included. For centuries, *A. annua* has been known for its efficacy for treating recurrent fever. Youyou first checked on the safety of the extract of *A. annua*, and then she tested it on 21 patients with malaria of whom 90% was cured. In 1972, Youyou isolated artemisinin which is the active element in *A. annua*.

Having extracted artemisinin from *A. annua*, semi-synthetic derivatives such as arthemeter, artesunate, are commonly produced. However, the use of semi-synthetic derivatives is not routinely available in remote rural areas. Moreover, semi-synthetic derivatives are expensive and are not affordable for poor families. Houzé, et al. (2007) added that the shelf life of artemisinin and derivatives is short, not exceeding a few weeks, particularly when stored at ambient temperature over 35°.

Low yields of artemisinin result in relatively high costs for its isolation and purification. *Artemisia annua* is relatively easy to grow in temperate climates. The time for the agricultural cultivation is relatively long and this results in wide swings in supplies and prices. The breeding of *A. annua* depends on the trichome, a fine outgrowth of the plant and efforts have been made for breeding cultivars of the plant for higher trichome densities and for increased artemisinin production (Grove et al. 2007). Also being pursued are transgenic production schemes for artemisinin production (Ferreira, 2005). Thus, the requirement for a more effective treatment of malaria has increased the demand for an affordable, high-quality, robust supply of artemisinin. However, there is still a worldwide shortage of the drug for treating malaria alone, let alone any other diseases against which artemisinin holds such promise (De Ridder et al., 2008). This implies that more low cost production and delivery of artemisinin is needed as WHO has recommended Artemisinin Combination Therapy (ACT) for treatment of malaria.

Graham et al. (2010) reported that the production of artemisinin is challenging because *A. annua* remains relatively undeveloped as a crop. According to the authors, there is the development of an alternative microbial-based system that synthesizes an artemisinin precursor for chemical conversion to supplement but not replace agricultural production, which will continue to be an essential source of supply. Therefore, there is a need for improved varieties of *A. annua* for farmers in developing countries because it would bring

immediate benefits to the existing artemisinin supply chain. The benefits include reducing production costs, stabilizing supplies, and improving grower confidence in the plant species. Thus, cytogenetic studies on *A. annua* are important to identify genes and markers for its fast-track propagation and breeding.

This review aims to answer the following questions:

1. What plant species are used as potential treatment for malaria?
2. What makes *A. annua* as one important plant species for malaria therapy?
3. What is the chemical composition of *A. annua*? What is its mode of action? How effective is *A. annua* as a source of drug therapy for malaria?
4. What are some studies on *A. annua* in terms of the production of artemisinin?
5. What are some issues in using *A. annua* as source of artemisinin for malaria treatment?

What are the prospects for the future using *A. annua* as a source of drug therapy for malaria?

## 2. CHARACTERISTICS OF *A. ANNUA*

### 2.1 Chemistry of the Plant

*A. annua* is commonly known as annual wormwood, sweet wormwood, sweet annie which is a highly aromatic annual herb of Asiatic and eastern European origin widely dispersed throughout the temperate region (Simon, 1990). *Artemisia*, one of the largest genera of the Asteraceae family belongs to a useful group of aromatic and medicinal plants comprising about 300 species which are distributed throughout the world (Bertea et al., 2005).

*A. annua* otherwise known as Qinghao by Chinese, has been used for many centuries in the treatment of fever and malaria (Brown et al., 2003). The plant species is a source of both essential oil (1.4 – 4.0 %) and other substances such as sesquiterpene lactones, flavonoids, polyalkynes and coumarins (Botsaris, 2007). Verdian-Rizi (2008) reported that the essential oil composition has been studied thoroughly and about 60 components have been identified in which camphor, artemisia ketone, germacrene D and 1,8-cineole are the main components. One important component is artemisinin, a potent antimalarial drug that is also effective in treating other parasitic diseases, some viral infections and various neoplasms (Weathers, et al. 2011). The drug can also inhibit the growth of other plants. Thus, it is an allelopathic herbicide.

Haynes (2006) reported that although the total chemical synthesis of artemisinin has been achieved, it is not cost effective. For artemisinin production, the current technology is based on cultivated *A. annua* with best cultivars giving yields of artemisinin of ca. 1.5% of dry plant material and 70 kg/ha (Kumar et al. 2004). Artemisinin is solvent-extracted from the leaves of the plant which is crystallized and typically used for semi-synthesis of important derivatives.

The extraction of the essential oils of *A. annua* is done by steam distillation similar to the commercial distillation of peppermint and spearmint. Simon et al. (1990) extracted the essential oils of *A. annua* via hydrodistillation. From the analysis, the essential oils of *A. annua* were found to contain many constituents including alpha-pinene (0.032%), camphene (0.047%), beta-pinene (0.882%), myrcene (3.8%), 1,8-cineole (5.5%), artemisia ketone (66.7%), linalool (3.4%), camphor (0.6%), borneol (0.2%), and beta-caryophyllene (1.2%).



**Figure 1. Growing *A. annua* plant**  
(Actual plant taken in Bogor, Indonesia on November 8, 2014)

### 2.2 Mechanism of Action of Artemisinin

Artemisinin is a highly energetic molecule containing an endoperoxide bridge (bridge joining two oxygen atoms), which is unstable, and rapid to react and release its energy. Obtained from the molecule are semi-synthetic derivatives, namely, dihydroartemisinin, artemether, artesunate and arteether which are not noticeably more efficient than artemisinin, and are quite rapidly ineffective compared to artemisinin.

Artemisinin is effective on early trophozoite stages of malaria and this action prevents evolution to later stages, during which there can be adhesions of the parasite to the vascular endothelium. A trophozoite is the active, motile feeding stage of a sporozoan parasite. The penetration of artemisinin into the body stops the maturation of schizonts. A schizont is a malaria parasite which has matured and contains many merozoites. A merozoite is a parasite stage that infects red blood cells. Artemisinin produces a higher gametocytocidal effect than standard antimalarial drugs and this decreases the risk of transmission from human to mosquitoes (Odugbemi et al., 2007). This gametocytocidal effect is very important as gametocytes may latently persist in the blood.

Golenser et al. (2006) proposed mechanisms of action of *A. annua*. The specific mechanisms include interference with the protein metabolism of the parasite and interference with the mitochondrial activity of the parasite. Other mechanisms are not specific. Eckstein-Ludwig et al. (2003) proposed another mechanism involving the inhibition of the Ca<sup>++</sup> ATPase. Ca<sup>++</sup>ATPase, is an important enzyme for the synthesis of cellular membrane proteins present in the parasite to ensure the maintenance of calcium ions concentration. The action involves the binding of artemisinin to the enzyme while leaving the peroxide bridge exposed. This allows the bridge to be open as iron attracts the electron of oxygen to activate oxygen atoms attracting the hydrogen ions. This binding generates carbon-centered radicals which leads to inactivation of the Ca<sup>++</sup>-ATPase and eventually death of the parasite.

Both sexual and asexual stages of *Plasmodium falciparum* need mitochondrial activity for respiratory chain. The iron

contained in the mitochondria activates artemisinin. The oxygen atoms reactively interfere with the parasite's electron transport chain leading to the depolarization of the mitochondrial membrane. This action prevents the biosynthesis of pyrimidine which causes the death of the parasite similar to the action of the inactivated Ca<sup>++</sup>-ATPase (Weathers et al., 2011).

One important role of artemisinin when activated is its ability to inhibit inflammation along with the aggression of the parasite as it adheres to the vascular endothelium in the capillaries (Golenser et al., 2006). The efficacy of artemisinin is on edema and hemorrhages due to inflammation.

### 2.3 Yield of Artemisinin from Different Parts of *A. annua*

Many investigations have been made on the treatment of malaria with extracted artemisinin or its semi-synthetic derivatives as well as the efficacy of tea preparation with leaves of *A. annua*. For one, Onimus, et al. (2013) reported that the leaf concentration in artemisinin depends on the time of harvesting, the way of drying, the way and the duration of preserving the leaves, as well as the variety of *A. annua*. From this finding, it was reported that the concentrations spread from 0.10% to 1%. It was 0.50% when the plant was studied in Madagascar. In West Africa, different varieties were studied, including Brasileau, Anamed and Mediplant and the artemisinin content was found to be over 0.8%, up to 1.3%. Mueller et al. (2000) found the concentration to be 0.63%. Ogwang et al. (2011) confirmed that the upper leaves of the plant contain more artemisinin than the middle or lower leaves and the artemisinin concentration is increased through hybridization of the plant. In another study, Jiang et al. (2011) found out that in natural *A. annua*, the concentration was 0.90% while in transgenic *A. annua*, it was 1.45%.

Different methods have been identified for the utilization of artemisinin from the leaves of the plant. Tonk et al. (2011) studied the leaves of *A. annua* which were extracted in petroleum ether by cold extraction, reflux extraction and soxhlet extraction. The third instar larvae of *Anopheles stephensi* was applied with the extract. Based on the larval mortality using the three methods of extraction, it was found out that both soxhlet and reflux extraction methods showed 100 % mortality at 200 ppm after 48 hr treatment of *A. annua*. Another important finding is that LC<sub>50</sub> (20 parts per million value of crude extract using soxhlet extraction showed better results than using reflux extraction (35 parts per million method after 72 hr. Without extraction from the leaves of *A. annua*, Elfawal et al. (2012) found conclusive evidence that orally ingested, powdered dried leaves of the whole plant kill malaria parasites more effectively than a comparable dose of pure drug. The results also revealed that dead parasites were observed in mice which was treated with the whole plant for only 24 hr after treatment.

There is another method of utilizing artemisinin from *A. annua* for malaria therapy. This is tea preparation with the standard

recommended amount of 9-10 grams of dried leaves. As tea, the dried leaves must be infused for 10-15 minutes in one liter of boiling water. Mueller, et al. (2000) found out that pouring boiling water onto the leaves is more effective than adding dried leaves to water. The authors noted that continuous boiling after the addition of leaves should be avoided because boiling reduces the yield of artemisinin after boiling from 5 to 30 minutes. Moreover, the authors pointed out that plastic or glass container should be used for infusing and never use an iron container because iron reacts with artemisinin.

The People's Republic of China has a traditional method of utilizing artemisinin from *A. annua*. The method involved taking the dried herb of the plant as a remedy for fever and malaria. The Chinese specified the amount of leaves which should be from 4.5 to 9 grams of dried herb prepared as a tea infusion with boiling water. The Chinese believe that 25-30 grams of the dried herb should be boiled for 30 minutes to provide an analgesic and antipyretic decoction to be taken once daily for seven days. This practice has been observed among the people for at least 2000 years without the report of serious adverse events. Since many people have been cured, the practice can be considered as a safe and established malaria treatment.



Figure 2. The leaves and flowers of *A. annua* (Actual plant taken in Bogor, Indonesia on November 8, 2014)

## 3. THE GENETIC BASIS OF ARTEMISININ PRODUCTION

### 3.1 Past Studies on the Genetics of *A. annua* and artemisinin production

Ferreira and Janick (1995b) found out that artemisinin production is controlled mainly by genetic factors. The authors investigated on broad sense heritability as compared with the artemisinin content of 24 clones of *A. annua* grown simultaneously under tissue culture, greenhouse and field conditions. The findings indicated that *Artemisia* plants can be grown in two mechanisms; first, it can be kept in a vegetative growth phase under long photoperiods, and second, it can be induced to flower under short days in a greenhouse. Such growth mechanisms imply that genetic gain can be achieved,



and maintained, from intercrossing high artemisinin clones selected in the field, and, induced to flower in a greenhouse. The findings also revealed that under field conditions, flowering of different lines often is mismatched. A study of Delaybas et al. (1997) reported that *A. annua* plants are naturally wind-pollinated and favour outcrossing over selfing. An important finding involved crossing of a late-flowering clone of Chinese origin (rich in artemisinin) with European plants by Mediplant in Switzerland that yielded progenies containing between 0.64% and 0.95% artemisinin, with dry leaf yields between 14 and 21t/ha.

Delabays et al. (2001) elaborated from their study that numerous other hybridizations between Chinese and Vietnamese clones generated hybrids of Artemis with 1.4% artemisinin. This allowed a 38kg/ha potential artemisinin production with some of the improvement of the hybrid populations to produce progenies with 2% artemisinin. The determination of the hybrids involved the cross between two high-artemisinin parental clones although neither was back-crossed to the homozygous stage and cannot be called 'true hybrids'. The maintenance and scaling of the hybrids were through vegetative propagation under long photoperiod involving 14 or more hours of light per day. Hirt (2001) noted that a stock of progenitor plants needs maintenance for hybrid seed production. This is because only a few will germinate if second-generation seeds are taken from the hybrid plants leading to weaker plants of approximately 30% less artemisinin. Ferreira et al. (1995a) added that though the presence of artemisinin is in plant tissues, the yield of artemisinin is not solely dependent on the artemisinin genetic potential, but also on the total biomass production of the plant which is based only on the plant's leaves and flowers because artemisinin is not produced in the roots and less produced in the branches.

Ferreira et al. (1995b) noted that though a uniform population in artemisinin content cannot be achieved by sexual propagation, the original parents should be used whenever fresh seeds are needed. If  $F_1$  plants are outcrossed in order to produce seeds (or  $F_2$  plants), the result is loss of hybrid vigour in subsequent populations ( $F_3$ , etc.). The selection of plants that reaches the peak in artemisinin before, or at the onset of, allows flowering so that harvesting should be done 3–4 months after planting. This procedure allows for at least two crops a year in tropical climates and plants with a high leaf-to-stem ratio would also be desirable. However, there is one problem that has to be considered and this is the synchronization of flowering. Different requirements for photoinductive cycle are expected in plants from different origins. Photoinductive cycle is the number of short days the plant has to be exposed to before flowering. As an example, a Chinese *A. annua* is a short-day plant that flowered 2 weeks after exposure to the inductive photoperiod of 13.3h of light under greenhouse and field conditions in Indiana, USA. When the same line was compared to a Vietnamese line of *A. annua* for the inductive photoperiod in southern Brazil (Marchese et al., 2002), it was confirmed that the requirement is 14 days (or cycles) of short days (13–15h) for the Chinese line to flower under growth chamber and field conditions. However, the Vietnamese line

required an average of 33 short days before flowering. Moreover, 100% of the Chinese plants flowered from the range of 378°C to 198°C, compared to ca 33% of the Vietnamese plants. It was suggested that to have a 100% flowering potential of the Vietnamese plants, the range of temperature should be from 298°C to 138°C and there should be either 7 or 9h of light per day photoperiod. The results also indicated that the flowering of Vietnamese plants decreased to 83.3% with an 11-hour photoperiod. On the other hand, the Chinese plants flowered 100% with photoperiods of 7, 9, 11 and 13h of light per day.

Wallaart et al. (1999) studied on tetraploid *A. annua* ( $2n \frac{1}{4} 36$ ) which was obtained with the mitotic inhibitor colchicine with an efficiency of approximately 20% and found out that the content of artemisinin (0.46% dry wt) during one vegetation period was ca 39% higher than in the diploid parental clone of *A. annua* (0.33% artemisinin). One important finding is that the average essential oil production was ca 32% lower than in the diploid parental clone. This indicates a possible inverse correlation between artemisinin and essential oil production. The results also revealed that the achievement of a higher artemisinin production was at the expense of the essential oil level. Based on this finding, the *A. annua* tetraploid did not achieve the higher levels of secondary metabolites achieved by the other tetraploid plants studied such as *Atropa beladonna* (68% higher), *Datura stramonium* (105% higher) and *Cinchona succirubra* (110% higher). There was also a lower biomass accumulation of the tetraploid *A. annua* compared to the one obtained by the diploid parental clone resulting to a decreased (by 25%) total yield of artemisinin. Such findings are significant for further studies on the glandular trichomes of tetraploid *A. annua* in terms of density and size.

In another study, De Jesus-Gonzalez and Weathers (2003) obtained stable tetraploid *A. annua* root cultures with colchicine and an efficiency of 10% resulting to slower growth in tetraploid root cultures than in diploid cultures. In addition, root diameter of tetraploid roots was larger than in diploids. The results also indicated that though artemisinin production of tetraploid hairy-root clones was from three to six times higher than the production achieved by diploid clones, there was no commercial potential in the levels of artemisinin in those cultures (mg/g dry wt). Moreover, there was a decline and instability in artemisinin production in the isolated diploid clones which was previously reported in the differentiated plant cultures and kept in tissue culture for 2 years. This implies loss of apical dominance which was attributed to epigenetic changes induced by tissue culture abnormal growth conditions indicating that field-selected clones are better maintained under greenhouse than under tissue culture conditions.

Delabays et al. (2001) reported that artemisinin is very difficult to synthesize and its production by means of cell, tissue or organ cultures is very low. With only its extraction from cultivated plants as presently viable, the authors studied and observed a large variation in artemisinin content in the leaves of plants from different origins. Assessment on the genetic basis of this variation provided an evidence for a quantitative



inheritance of the artemisinin concentration and it was found out that additive genetic components were predominant, resulting in a high narrow-sense heritability estimate. Such findings indicated good results from mass selection for the breeding of lines of *A. annua* rich in artemisinin. The presence of dominance variance in the total genetic variability indicates that crosses between selected genotypes should generate progenies with particularly high artemisinin content. Moreover, in wild populations of genotypes with high artemisinin concentration, selection and crossing resulted in hybrid lines with up to 1.4 percent artemisinin from dried leaves of *A. annua*.

### 3.2 Recent Cytogenetic Studies on Artemisinin Production

A lot of cytogenetic studies have been done about artemisinin production. Aimed at answering the demand for an affordable, high-quality, robust supply of artemisinin to eradicate malaria, Graham et al. (2010) performed deep sequencing on the transcriptome of *A. annua* to identify genes and markers for fast-track breeding of the plant species. The extensive genetic variation performed was able to build a detailed genetic map with nine linkage groups. A quantitative trait loci (QTL) map resulted from replicated field trials that accounts for a significant amount of the variation in key traits controlling artemisinin yield. With this finding, enrichment for positive QTLs in parents of new high-yielding hybrids confirms that there are sufficient knowledge and tools to convert *A. annua* into a robust crop. The use of a genetic map is important to accelerate plant breeding of *Artemisia* and rapidly develop the species into a high-yielding crop. There is a demand for increasing artemisinin production but yields are low, making production expensive. As mentioned earlier, *A. annua* favors outcrossing over selfing and the artemisinin content of plants from different origins varies considerably and is highly heritable. Artemis, an F<sub>1</sub> hybrid (population) variety is currently the market leader for artemisinin production. The production of Artemis seeds involved a cross between two heterozygous and genetically different parental genotypes, (C<sub>4</sub> and C<sub>1</sub>), that are themselves maintained vegetatively.

As artemisinin content is highly heritable, there is a strong genetic component contributing to the variation in the cultivated crop. The interaction between the genetic components and the non-genetic components can be exploited for breeding purposes resulting to the production of improved hybrid lines. Townsend et al. (2013) determined the combining ability analysis of a diallel cross to identify robust parental lines for hybrid breeding. The basis for the selection of parental lines was the range of phenotypic traits to encourage heterosis.

For the diallel parental lines, the general combining ability (GCA) values correlated to the positive alleles of quantitative trait loci (QTL) in the same parents. Such a finding indicated the presence of beneficial alleles that contribute to parental performance. There was also the identification of hybrids generated from crossing specific parental lines with good GCA showing an increase in both artemisinin concentration and biomass. Further, there was comparison on the identified

hybrids when grown either in glasshouse or experimental field trials with hybrids grown in the control. This study is of importance because it demonstrates that combining ability via a diallel cross can be useful for the identification of elite parents which are responsible for the production of improved *A. annua* hybrids.

Zhang et al. (2013) cloned abscisic acid (ABA) receptor orthologue, AaPYL9 (highly expressed in leaf and flower) from *A. annua* L.. This overexpression increases artemisinin content after ABA treatment as well as significant enhancement of the expression of key genes in the biosynthesis of artemisinin. Aimed at providing a way to develop *A. annua* with high-yielding artemisinin, the study examined whether constitutive overexpression of *AaPYL9* could enhance artemisinin production when sprayed with exogenous ABA. The study also compared the overexpression of several artemisinin biosynthetic key genes, namely, *HMGR*, *ADS*, *FPS*, and *CYP71AV1* with that of wild-type, before and after ABA-treatment for 6 hours. Results revealed that in both wild-type and transgenic lines, except for *HMGR*, the expression of *ADS*, *FPS* and *CYP71AV1* was enhanced after ABA-treatment. However, there was a significant higher induction in the transgenic lines for the expression of *ADS*, *FPS* and *CYP71AV1* upon ABA treatment than wild-type plants. Further, the artemisinin content of wild-type *Artemisia* as well as 35S::AaPYL9 transgenic lines was approximately 600 (µg/g) before the treatment with ABA. However, during the analysis of the ABA treated leaves, it was found out that there was an increase by 33% in the artemisinin content of wild-type *Artemisia* while there was an increase by 74%~95% in the artemisinin content of 35S::AaPYL9. These findings are significant to conclude that overexpression of *AaPYL9* in *Artemisia* enhanced the ABA sensitivity upon ABA treatment and improved the content of artemisinin.

Another study by Nair et al. (2013) was done by strategizing a microarray chip in order to shortlist the differentially expressing genes at a stage of plant producing highest artemisinin compared to the stage with no artemisinin. Initially, the study analyzed differential gene expression associated with contrasting artemisinin content *in planta* and a genotype having zero/negligible artemisinin content. Since there was no available genotype with zero artemisinin, the study instead compared the different stages of the same genotype with contrasting artemisinin content (seedling - negligible artemisinin, mature leaf - high artemisinin). In order to determine optimal plant stage and leaf ontogenic level for artemisinin content, the SCAR-marked artemisinin-rich (~1.2%) Indian variety 'CIM-Arogya' was used. The study was able to establish a representative EST dataset from leaf trichome at the stage of maximal artemisinin biosynthesis. For comparison of the two stages, genetic parameters were used, namely, high utility small scale custom microarray chip of *A. annua* having all the significant artemisinin biosynthesis-related genes, established EST dataset and gene sequences isolated in-house and strategically selected candidates from the *A. annua* Unigene database (NCBI). Using semiquantitative and quantitative RT-PCR followed by putative annotations through bioinformatics-based approaches, the data was



validated. Based on the study, there was an identification of the probable role in artemisinin metabolism of many candidates as well as important parameters for further functional characterization.

In a related study, Wu et al (2012) investigated the glandular secreting trichomes (GST) expressed proteins in *A. annua* using a comparative proteomics approach, aiming for a better understanding of the trichome proteome and artemisinin metabolism. As the most effective drug against malaria, artemisinin is derived from GSTs of *A. annua* although its low artemisinin content (0.001%~1.54% of dry weight) has hindered its wide application. By using 2D-electrophoresis to compare the protein profiles of GSTs and leaves, results revealed that more than 700 spots were resolved for GSTs, of which ~93 non-redundant proteins were confidently identified by searching NCBI and Artemisia EST databases. In addition, over 70% of these proteins were highly expressed in GSTs and it was found out that functional classification of these GSTs enriched proteins participate in electron transport, transcription and translation. The proteins were annotated based on predominant function even if they may play various roles in different subcellular compartments or at different developmental stages. According to previous transcriptome study on the GSTs extracted from flower buds, there are many high abundant transcript expression genes which also belong to these functional categories. These findings imply association of proteins with artemisinin production.

#### 4. ISSUES AND TRENDS ON ARTEMISININ PRODUCTION

Malaria is a major disease in the world causing in every year 1-2 million deaths which is due to Plasmodium species. The transmission of the disease involves the transit through the mammalian host's liver and blood. In South America, Africa and South-East Asia, there has been a spread of resistant strains of the parasite to the actual therapeutics. This is the reason why a large number of collaborations are developing a vaccine against malaria by targeting either the blood stage or the liver stage of the parasite.

*Artemisia annua* is relatively undeveloped as a crop. For this reason, many studies have been conducted for improving artemisinin concentration and biomass in order to produce an affordable high quality artemisinin. Townsend et al. (2013) has documented the artemisinin content (ranging from <0.01 to >1.0%) in the dry leaf of varieties from different geographical origins. According to the authors, the factors that cause this variation are differences in farming practices, periods of harvest and also environmental factors such as temperature and nutrient availability.

The issue on the increased production of artemisinin supply calls for the development of reforms and technologies. De Vries and Dien (1996) used the extraction and purification technology of artemisinin through the hexane method. The hexane method is a new technique for recovering artemisinin from *A. annua* by high-pressure extraction with CO<sub>2</sub> at 60-85

bar and 20-50°C. The method involved some recovery steps such as (a) grinding the ground parts of artemisinin to powder; (b) subjecting to high-pressure extraction with CO<sub>2</sub> for recovery of artemisinin; (c) isolating artemisinin from the purified extract mixture; and (d) preparing a solution for use in preparation of liquid dosage forms.

Another issue is on the very short half-life of *A. annua*. Sharma (2008) reported that due to this characteristic, artemisinins are unlikely to be affected by the emergence of drug resistance which means that they must be given for at least 5 days for acceptable cure rates to be achieved. According to WHO, the reduction of the risk of drug resistance involves the use of a combination that contains derivatives of artemisinin along with another effective antimalarial drug. These combinations are called artemisinin-based combination therapies (ACTs). These are currently the most effective treatment for malaria with a 95% cure rate against Plasmodium falciparum. However, the manufacture of ACTs involve high cost (Mutabingwa, 2005). The commercialization of the semi-synthetic artemisinin involves the use of genetically engineered bacteria which aims for a much cheaper production of the drug than the plant-derived drug available today. This semi-synthetic drug is a key ingredient in ACTs. The commercialization of artemisinin enhances the security of artemisinin supply and ensures affordable treatment for many malaria infected people (more than 500 million) every year. An important advantage of using the semisynthetic artemisinin is a high quality source of non-seasonal and affordable anti-malarial drug that can alleviate shortages and meet future demand of growing *A. annua* in many parts of the world. It is believed that the semisynthetic artemisinin production processes will improve the availability of high-quality artemisinin derivatives to drug manufacturers and enable millions of people infected with malaria to gain access to lifesaving treatments (University of Berkeley, 2010).

Examples of genetically engineered bacteria are *Saccharomyces cerevisiae* and *Escherichia coli*. In a study of Zeng and Yuan (2008), cloning and expression of the artemisinin biosynthetic genes in these bacteria have led to large-scale microbial production of the artemisinin precursors such as amorpha-4,11-diene and artemisinic acid. Important technologies have been introduced for the production of the precursors. One technology involves engineering the microbial terpene pathway by chemical or biotransformational methods. By this procedure, there will be a novel approach to the scaleable and cost-effective production of artemisinin. If processing steps for purification are simplified, microbe-based artemisinin production can also avoid contamination by other plant-made terpenes. Another technology involves the use of synthetic biology in order to develop strains of

*Saccharomyces cerevisiae* for high-yielding biological production of artemisinic acid, a precursor of artemisinin (Paddon et al., 2013). The conversion of artemisinic acid to artemisinin involves the use of a chemical source of singlet oxygen which is a practical, efficient and scalable chemical process. The strains and processes described here formed the basis of a viable industrial process for the production of semisynthetic artemisinin.



There are requirements to follow regarding the use of artemisinin drug combinations. One requirement for public health service staff is to undertake, in a responsible way, the use of artemisinin drug combinations. This means that the health staff should be properly trained. Another requirement is the availability of the drugs based on the required information supplied in a locally appropriate form. There should also be more specific diagnosis to ensure correct drug treatment. The drugs should be free or affordable to the patient populations. Lastly, if such drug combinations will be introduced, there should be proper monitoring of their impact on mortality and incidence of severe disease.

Since *A. annua* is the sole commercial source for artemisinin production due to its non-viable chemical syntheses, it is economically and practically needed to find ways on how artemisinin could be produced. Thus, selection and breeding of the plant will be possible if there is a comprehension of chemical and genetic variability and suitable selection(s) of elites from within the available population. This means that apart from synthetic biology, a continuous use of molecular tools should be used. Specifically, in a study by Sangwan et al. (2003), Random Amplified Polymorphic DNA (RAPD) analyses of selected chemotypes from a decade old introduced population were carried out using arbitrary primers. The use of RAPD provided the distinction among the *A. annua* plants allowing the detection of highly polymorphic profiles (97 polymorphic markers out of a total of 101 markers). Such finding implies the existence of very high levels of genetic variation that opens out a strong possibility of further genetic improvement for superior artemisinin content. The wide phytochemical diversity was found to be included within the genetic diversity using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analyses of RAPD. These results further support the prospects for selection and breeding of superior artemisinin containing lines. Thus, RAPD can be employed to overexpress gene(s) coding for enzyme(s) associated with the rate limiting step(s) of artemisinin biosynthesis or to inhibit the enzyme(s) of other pathway competing for its precursors. Abdin et al. (1999) added that enhanced production of artemisinin can be either in cell/tissue culture or in the whole plant of *A. annua*.

## 5. CONCLUSION

Malaria, an insect-borne disease, is a critical health issue which is currently endemic in over 100 countries. Scientists, medical doctors and even concerned people have been trying to develop new classes of medicines to combat malaria as a result of infection by *Plasmodium falciparum* and *Plasmodium vivax*. As a matter of fact, some practical efforts have been done to eradicate the disease, from a conventional type of treatment to the use of a variety of new brands of medicines commercially available in the market. Some people traditionally use medicinal plants as nature's gift to make a disease-free healthy life as well as to preserve one's health. Many townspeople would say that medicinal plants are much safer to use and are proven effective in the treatment of various ailments especially malaria. Among the known plant species used as herbal

medicine, it is *A. annua* which has been found to be the best treatment for malaria due to artemisinin, the wonder compound it produces. Having been consumed by humans and used as a herbal therapy for malaria, *A. annua* is considered "generally regarded as safe" (GRAS) herb.

The genus *Artemisia* (Astraceae) consists of about 500 species, occurring throughout the world. Though available worldwide, artemisinin is expensive to produce and is frequently in short supply. It is costly particularly when combined with other antimalarial medications to make it less prone to resistance. Due to the problem on production and yield of artemisinin, various efforts have been made to establish the molecular basis of this medicinal plant. Efforts of genetics researchers include the use of effective molecular tools in order to ensure a good production of the artemisinin. Artemisinin from *A. annua* is the key component in the ACT treatment of malaria. This means that as malaria becomes a global health problem, the demand for ACTs is expected to increase in the future. Therefore, there is the target in all parts of the world to develop new high yielding varieties of *A. annua* which is optimized for production of artemisinin. Local efforts can also be considered where farmers would grow the high-producing cultivars of *Artemisia*. The economy aspect involves giving local farmers and business people the opportunity to earn a living from growing the plant and producing artemisinin. Generally, while it is true that *A. annua* will become an effective therapy for malaria which is commercially available at an affordable price, the production of artemisinin from *A. annua* using specific technology and molecular procedures will help stimulate the economies of developing nations.

With the knowledge on the costly production of artemisinin, science researches should keep on developing and finding the newest technologies in order to meet the demand for this drug. As presented in the paper, one of the newest technologies documented involves the use of genetically-modified or transgenic microbes which will lead to a cost-effective and large-scale microbial production of a derivative of artemisinin. Collaborations with research institutions in the country are, thus, needed in order to conceptualize the commercialization of artemisinin. Moreover, proposals on artemisinin production may be coordinated with local health officials so that proper budget may be allotted for local production of the drug. Activities in one proposal may include a dialogue with local farmers in order to impart awareness generation, motivation and training for nursery raising and cultivation of *A. annua* using organic farming techniques. The cultivation of the plant involves land preparation, organic manuring, plantation, plant protection, weeding, irrigation and harvesting. The second activity may be a dialogue with a group of experts from the Health Department or Phytopharmaceutical industries to work on the semi-processing and preservation, quality-control packaging and marketing of artemisinin. The last activity may be networking and collaboration among various stakeholders dealing with herbal plants.

In this way, local malaria patients may avail of the drug for free or for a very affordable cost. The proposals may be either made by researches or concerned people from a community in the locality.

Not only the local community, but there is also a challenge for the international community regarding the treatment of malaria as well as preservation of the effectiveness of artemisinin. Thus, all of us have the equal and fair responsibility to eliminate and eradicate malaria in order to have a world that is healthy and safe.



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