Review

Building a golden triangle for the production and use of artemisinin derivatives against falciparum malaria in Africa

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Use of the conventional quinoline- and sulphanamide-based drugs for the symptomatic treatment of malaria is gradually being replaced by artemisinin-based combination therapies (ACTs) due to increasing resistance by the Plasmodium parasite. This development has drastically increased artemisinin demand worldwide, and Artemisia annua L. is currently the only commercial source for the supply of this vital antimalarial drug to the international market. Recent advances, however, demonstrate that the production of isoprenoid precursors in microorganisms is a feasible complementary strategy that would help reduce artemisinin cost in the future. The key genes encoding for enzymes regulating the biosynthesis of artemisinin in planta are fully understood to enable metabolic engineering of the pathway, and results from pilot genetic engineering studies in microbial strains thus far are very inspiring. This review, therefore, explores the current status of artemisinin derived drugs against malaria and highlights some implications of crop agronomy, biotechnology and solvent extraction strategies in enhancing the total yield of artemisinin for the production of ACTs, which are responsible for saving the lives of countless numbers of patients in malaria-stricken societies and are currently in very high demand, especially in Africa.

Key words: Artemisia annua, ACTs, biotechnology, malaria, Plasmodium spp.

INTRODUCTION

Malaria appears to be one of the greatest sources of misery on earth today. It is caused by protozoan parasites, notably Plasmodium falciparum, which is spread following the bite of infected female Anopheles mosquitoes. Infection in humans starts when parasitic sporozoites invade hepatocytes and replicate as merozoites before erupting into the blood stream and infecting erythrocytes where they multiply, as shown in Figure 1, causing malaise, jaundice, cyclical burning fevers and severe body pains in complicated cases.

Worldwide prevalence of the disease causes an estimated 500 million clinical episodes each year, resulting in over one million deaths annually (Snow et al., 2005). Unfortunately, about 90% of malaria-related mortality usually occurs amongst children less than five years of age and pregnant women in sub-Saharan Africa (Rinaldi, 2004), where a large part of the population has no access to health services or relies on herbal remedies which are often not effective. In addition, malaria also has substantial negative impact on the economic development of African nations where the disease is endemic (Mueller et al., 2004). The drain on these economies, for example, is...
estimated to be about US$12 billion each year (WHO, 2002) and the threat of malaria can be a serious deterrent to tourism and internal trade, further constituting a serious obstacle to socio-economic development of the continent.

For decades, quinoline-based drugs were the main choice for the prevention and treatment of malaria. Unfortunately, the emergence through mutation, of multi-drug resistant *Plasmodium* species, which has recently sparked a global dissemination of resistance, has rendered traditional and affordable antimalarial drugs such as chloroquine and sulphadoxine-pyrimethamine (SP) ineffective. Recent studies have also shown that artemisinin has therapeutic potential against *Toxoplasma gondii* (Jones-Brando et al., 2006), *Trypanosoma* and *Schistosoma* species (Mishina et al., 2007; Xiao et al., 2001; Utzinger et al., 2001), which cause toxoplasmosis that is associated with behavioural abnormalities in patients, human trypanosomiasis or ‘sleeping sickness’ and schistosomiasis, respectively, as well as other pathogens responsible for cryptosporidiosis, amoebiasis, giardiasis, leishmaniasis (Ma et al., 2004) and clonorchiasis. Collectively, these are common diseases in developing countries that appear to afflict over 800 million people each year. However, more compelling and of even greater pharmacological significance is the fact that artemisinin or its derivatives have been demonstrated to be novel anti-tumour agents for some of the deadliest cancers known to man. For example, artemisinin derivatives have been shown to be very effective against radiation-resistant breast cancer cells *in vitro* (Singh and Lai, 2001), drug-resistant small cell lung carcinoma cells (Sadava et al., 2002), human leukemia cell lines (Lai and Singh, 1995) and colon cancer and active melanomas (Efferth et al., 2001). Due to these discoveries *A. annua* has been rated as one of the top ten industrial crops of the modern world (Sangwan et al., 1998).

Artemisinin destroys parasitic organisms and cancer cells through the generation of highly reactive oxygen-based free radicals or electrophilic intermediates, by alkylating and oxidizing proteins and lipids of parasite membranes (Figure 2) as well as inactivation of channel proteins (Ridley and Hudson, 1998). It has been demonstrated that the effect of artemisinin is equally mediated through disruption of membrane potential by interacting with the electron transport chain in the mitochondrial membrane, resulting in free radical damage and dysfunctional mitochondria (Li et al., 2005). However, more recently an...
alternative mechanism of action based on inhibition of sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) of \textit{Plasmodium} has been suggested (Eckstein-Ludwig et al., 2003), which has reconciled some intriguing observations on the actions of artemisinins against the parasite (Woodrow et al., 2005).

Artemisinin and its derivatives kill the \textit{Plasmodium} parasite faster than other antimalarial agents and are toxic to the parasite at very low concentrations (WHO, 2002). Thus far no \textit{in vivo} resistance has been described, making them the most effective antimalarials currently in the arsenal for the treatment of multi-drug resistant malaria (Meshnick, 2002; van Agtmael et al., 1999). Moreover, clinical studies have equally shown that the side effects from their use also appear to be limited (WHO, 2002; Gordi et al., 2002; Mueller et al., 2000) such that artemisinin-derived drugs are more successful than all currently known standard chemotherapies used in the fight against malaria.

**PRODUCTION OF ARTEMISININ**

Although artemisinin can be synthesized chemically, the analogues are unlikely to be economically competitive with that produced naturally in Artemisia due to complexity of the process (Ferreira et al., 2005; Xu et al., 1986; Schmid and Holheinz, 1983). The many reaction steps and low yields obtained by organic synthesis make it obvious that its extraction from the leafy biomass of \textit{A. annua} invariably appears to be the only viable option at the moment for producing cheap and large quantities of artemisinin. Therefore, the increased cultivation of the crop in plantations and the improvement of artemisinin extraction methods are the most effective strategies for producing artemisinin. However, one of the major shortcomings on the production of sesquiterpenic compounds via whole plants is the relatively lengthy growing cycle required to obtain appreciable yields (g/100 g dry weight), which can range only from 0.2 to 0.9% in many of the commercial varieties currently cultivated in different parts of the world. Usually the period from time of planting to artemisinin extraction from \textit{A. annua} is approximately 12 – 15 months. Not surprisingly, the yields derived from dried leafy biomass after such a lengthy period are considered low for commercial production, where a full ton of plant materials can only produce between 6 – 18 kg of purified artemisinin. This low yield thus appears to be one of the most intractable problems related to the production and use of artemisinin-derived drugs against malaria, especially in Africa where the plant is not ubiquitous.

\textit{A. annua} is a vigorous growing annual weed, which stores most of its active ingredients in glandular trichomes found in the leaves and inflorescence (Ferreira and Janick, 1995; Duke et al., 1994). The plant is considered to have originated and occurs naturally as part of the steppe vegetation in Northern China (Ferreira et al., 2005). However, it now grows effectively in other climatic conditions. In Asia, for example, it is well distributed and extends as a native into Southern Siberia, Vietnam, and Northern India. Outside of Asia, the plant has adapted ubiquitously to different growth conditions as seen in many parts of Europe, USA, Australia, and Argentina (Ferreira et al., 2005). In Africa it has been introduced into commercial-scale cultivation in Tanzania, Kenya, Uganda and Madagascar within the past six years and more recently in Nigeria (Brisibe, 2006), where evaluation of optimal agronomic practices and mass selection for late flowering and high artemisinin yielding lines are currently in progress. For these studies, seeds were obtained from six different countries – Brazil, China, Vietnam, India, Germany and USA. Some of these, espe-
cially the hybrid populations from Brazil, have originated plants that had a growth span of about 192 days before flowering and were up to 2.84 meters in height with an average leaf biomass yield of 324 g/plant and artemisinin concentrations as high as 0.9% (on a g/100 g dry weight basis) under humid lowland tropical conditions (Brisibe et al., manuscript submitted).

AGRO-TECHNOLOGIES FOR ENHANCED PRODUCTION OF ARTEMISININ

The availability and cost of ACTs are largely functions of the artemisinin yield in *A. annua* cultivars, which has significant effect on artemisinin cost itself that is currently a key cost driver for the production of the drugs. It is not surprising, therefore, that there is a current surge in the cultivation of the plant around the world, most notably in Africa, where high artemisinin-yielding lines have been earmarked for commercial cultivation in different countries. However, African regions mostly afflicted by malaria are within the tropics, where day lengths are short, thus likely to induce most cultivars which are not adapted to the tropics to flower early without the accumulation of sufficient leafy biomass (Ferreira et al., 2005). Interestingly, there are currently genotypes that have been developed by Mediplant in Switzerland (Delabays et al., 2001) and hybrid populations by Dr. Pedro M. de Magalhães (1999) at CPQBA, University of Campinas, Campinas, Brazil, which are late flowering and produce sufficient leafy biomass, that appear most suitable for cultivation in the tropics. The interpretation that these varieties, especially those from Brazil, can perform well within the tropics has support from our own recent studies in Nigeria which show that they can produce on average 0.9% artemisinin and can be selected further for adaptation to lower latitudes quite close to the Equator (Brisibe et al., manuscript submitted; Brisibe, 2006).

Though Artemisia is well suited to both small-scale and plantation agriculture, currently the most significant bottleneck for feasible commercial production of artemisinin anywhere in the world is the availability of seed stocks of lines suitable for the local conditions which can produce high leafy biomass and artemisinin yields. Once the problem associated with seed production has been conquered, other agronomic factors need to be optimized to maximize the production of leaf biomass and artemisinin. Four of such factors are discussed below.

**Planting**

Artemisia seeds are very small and usually commercial cultivation involves transplanting of vigorous nursery-grown seedlings to the field at the 3 – 5 leaf stage when they are about 10–15 cm in height. However, in localities where labour is scarce or expensive, seedlings can be raised directly in the field after the preparation of a fine seed bed. We observed in several trials that transplanting was clearly inferior in terms of agronomic performance and artemisinin yield of plants when raising seedlings in a nursery prior to field cultivation were compared with direct seeding in the field (Brisibe et al., manuscript submitted; Brisibe, 2006; Ferreira et al., 2005). However, irrespective of the method of establishment, it is often preferable to plant after the rains have started. This would mean that the soil has high moisture content since any moisture stress in the early and mid-vegetative growth stages of the plant tends to induce premature flowering or leaf atrophy (Brisibe et al., manuscript submitted).

Our preliminary studies have equally demonstrated that leaf biomass yield and artemisinin production will probably have a wider variation in plants generated from seeds than in those generated from asexual propagation methods such as cuttings or *in vitro* culture. Although this has not been evaluated on a large scale, however, a recent study using crop generated by cloning a Swiss variety, has proven that 0.7% artemisinin and an average of 450 g dry leafy biomass per plant can be obtained (Ferreira, unpublished). Against this backdrop crop establishment from cloned plants looks like an attractive option if the source plant is rich in artemisinin content, such as 1.4% that has been reported previously (Ferreira et al., 2005; Laughlin et al., 2002). Such vegetative propagation methods are equally useful for maintaining genetic fecundity. However, the cost benefits of crop establishment from seeds versus asexually propagated plantlets also need to be evaluated.

Undoubtedly the economics of artemisinin production *in planta* could be drastically improved by two contemporary procedures. First, a recent study showed that exposing plants grown in a greenhouse to salinity stress of 4 and 6 g/l NaCl significantly increased the level of artemisinin in the plants by about two-fold compared to the control (Qian et al., 2007). It has equally been demonstrated that intermittent harvesting of the early planted full time crops within one growing season can also enhance the production of artemisinin through an increase in leaf biomass yield (Kumar et al., 2004). Plants grown for more than 30 weeks and harvested four times as a ratoon crop produced an average artemisinin yield of 74.2 kg ha$^{-1}$, which is almost three times higher than the maximum yield of 28.5 kg ha$^{-1}$ when the plants were harvested only once within a growing season. Taken together, these observations are highly encouraging and should be of immense significance in efforts geared towards large scale production of artemisinin in Africa.

**Soil**

Presently, there are only a small number of recorded studies of the effect of soil type on the vegetative growth of *A. annua* and artemisinin yield. It is obvious from these that the plant prefers well drained sandy and loamy soils.
However, independent of the soil's physical characteristics, the pH level is seen as the most critical factor for plant growth and development. A pH level of 5.8 – 6.0 seems to be adequate for plant growth and leaf production, but values as high as 8.0 have been reported to be appropriate, depending on the cultivar (Laughlin et al., 2002). A recent study has shown that an *A. annua* farmer can succeed in growing the crop in a poor soil with a minimal pH of 5 though higher leaf biomass accumulation can be achieved at pH 6. Lime is not expensive and could thus be used as a cost effective means of increasing the production of leaf biomass through soil amelioration by simply raising the pH value from 5 to 6. Clayey soils will definitely require higher amounts of lime than sandy soils for the same increase in pH. In the future, technology already developed to genetically engineer plants to enable them grow in acidic soils could be beneficial to the cultivation of *A. annua*, thus leading to a decrease in the overall production costs of artemisinin.

**Nutrients**

Although there are not many specific experimental data on field responses of *A. annua* to fertilization with the macronutrients, there are a few examples such as the application of 60 kg of nitrogen, 60 kg of phosphorus, and 50 kg of potassium per hectare, pre-drilled in bands 150 mm apart and 50 mm below the seeds (or 75 mm below transplants) being reported to have produced good-to-high leaf yields in Tasmania (Laughlin, 1993). Our own investigations of the role of macronutrients demonstrated that nitrogen was the most important element with potassium being the least required for plant growth and leaf biomass accumulation (Ferreira, 2007), just as the application of 50 and 100 kg of nitrogen per hectare increased herbage, essential oil and artemisinin yield by 26.2 and 40.1%, respectively, compared with the untreated control without nitrogen (Singh, 2000). Collectively, the omission of any of the macronutrients including nitrogen, phosphorus, potassium, calcium, magnesium or sulphur from nutrient solutions limited artemisinin and artemisinic acid production (Figueira, 1996). Regarding micronutrients, the omission of copper and, especially, boron from sand cultures has decreased artemisinin content in plants (Srivastava and Sharma, 1990). Boron deficient plants did not flower and there was approximately a 50% reduction in artemisinin yield.

Paradoxically, experimentation involving different levels of complete fertilization have not produced the expected increase in artemisinin concentration (g/100 g of the sequiterpene), though it has increased the total artemisinin production (g/plant) due to an overall increase in leaf biomass yield. Collectively, one hallmark of the various observations is the fact that poultry droppings (Brisibe, unpublished) or soils marginally deficient in potassium enhances both an increase in artemisinin concentration (g/100 g) by 75% and the overall artemisinin yield of the plant (g/plant) by 21%, while saving on potassium fertilization (Ferreira, 2007). However, submitting the plants to a severe potassium deficiency might decrease their resistance to drought since one of the major roles of potassium in plants is in the regulation of the opening and closing of guard cells around stomata, which help decrease water loss by the plant during hydric stress.

**Application of plant growth regulators**

The application of plant growth regulators *in vivo* to enhance leaf production would be desirable since the bulk of the artemisinin is sequestered in glandular trichomes on the surfaces of the leaves, and are consequently appealing targets for enrichment. So far not much work has been done in this respect, except perhaps the few investigations which have demonstrated that exogenous application of triacontanol at 1.0 and 1.5 mg/l, chlormequat at 100 and 1,500 mg/l (Shukla et al., 1992) and GA$_3$ at 25 and 50 mg/l have been shown to increase artemisinin levels in this plant (Faroqqi et al., 1996). Interestingly, our own studies using paclobutrazol, a growth retardant employed mainly in horticulture in controlling the height of plants, at 5 and 10 mg/l, respectively, while seen to have drastically reduced the overall height of the treated *A. annua* plants equally led to a significantly increased leaf biomass yield, which may or may not have an effect on the artemisinin yield of the plants (Brisibe, unpublished).

**BIOTECHNOLOGY OF A. annua AND INCREASED PRODUCTION OF ARTEMISININ**

Considering that *A. annua* is the only viable source of artemisinin, presently the most potent and efficacious antimalarial after quinine, there is understandably a great degree of interest in enhancing the production of this bioactive compound. And although effective, the agrotechnological platform as the main production strategy seems unlikely to solve the problem of global artemisinin availability. It is obvious that there is need for an additional source of artemisinin which supply will be consistent, reliable and inexpensive. Consequently a multifaceted approach using several strategies, including the utilization of the advanced techniques emerging from classical molecular biology, industrial fermentation and genetic engineering research, would be of great interest. Some of these strategies include, but are not necessarily limited to the following.

**In vitro production of artemisinin**

There are a few reports which have demonstrated that artemisinin can be produced successfully in callus and cell suspension cultures of *A. annua* though the artemisi-
Artemisinin yields have been very disappointing (Jaziri et al., 1995). However, some other investigations have proven that artemisinin production can be enhanced by the presence of roots (Fulzele et al., 1991; Ferreira and Janick, 1996). For example, it has been reported that 0.287% dry weight of artemisinin can be produced in hormone-free medium when root production was maximized though no artemisinin or its immediate precursors were detected in the roots (Ferreira and Janick, 1996). These results are an indication that differentiated shoot cultures could serve as high-value products for pharmaceutical use, since they contain artemisinin levels comparable with those observed during agricultural production. However, the low biomass produced makes it definite that tissue cultures might not be a suitable strategy for commercial exploitation of artemisinin.

Aside from these, other procedures based on the transformation of A. annua plants using Agrobacterium rhizogenes have led to the formation and maintenance of hairy roots that can produce higher levels of artemisinin than the normal roots in in vitro cultures. De Jesus-Gonzalez and Weathers (2003) have reported the production of several stable tetraploid hairy root clones of A. annua. Some of these were discovered to have given significantly more artemisinin content than the diploid parent (Clone YUT 16). Wang and colleagues (2001) equally found that adjusting the light spectrum in transformed hairy root cultures of A. annua has a significant influence on biomass yield and artemisinin content. These authors demonstrated that a high biomass yield of 5.73 g dry weight/l and an artemisinin content of 31 mg/g plant material could be obtained under red light at 660 nm, which are 17 and 67%, respectively, higher than those obtained with white light. In a related study, Liu and others (2002) found that light irradiation influenced the growth and production of artemisinin in transformed hairy root cultures of A. annua too. When hairy roots were cultured under illumination of 3,000 lux for 16 h using several cool-white fluorescent lamps, a dry weight of 13.8 mg/l and an artemisinin concentration of 244.5 mg/l, respectively, were obtained. In addition, artemisinin content can also be successfully increased by about 57% through simply regulating the ratio of nitrate to ammonium and total initial nitrogen concentrations in transformed hairy root cultures (Wang et al., 2002). These results are useful and encouraging so far, but a lot more effort should be made using the whole plant as well as some specific tissues.

Artificial polyploidization of Artemisia annua in vivo and in vitro

Artificial polyploidization is generally known to give rise to larger reproductive and vegetative organs (Adaniya and Shira, 2001). It has also been shown to increase the production of important medicinal compounds and other secondary metabolites over those of their diploid counter-parts (Griesbach and Kamo, 1996). With this in mind, Wallaart and his colleagues (1999) successfully induced tetraploid whole plants (2n=4x=36) from the diploid A. annua plants using colchicine. They reported a polyploidy production efficiency of 20 and a 30% higher artemisinin yield in the tetraploid plants. And although the increased yields of these tetraploid clones did not reach commercially useful quantities (mg/g dry weight) of artemisinin, the work showed that there are certainly some advantages in selecting for high-yielding polyploids.

Metabolic engineering of the artemisinin biosynthesis pathway

A more recent attempt at increasing the artemisinin content has been the use of genetic engineering techniques to alter the metabolic pathway of artemisinin biosynthesis in transgenic A. annua. This has been achieved mainly through the introduction of key genes encoding for enzymes regulating the biosynthetic pathway leading to the formation of artemisinin in planta. In this connection the role of certain genes, especially those involving key enzymes in the biosynthesis of artemisinin such as farnesyl diphosphate synthase (FDS) and amorpha-4,11-diene synthase (AMS) readily comes to mind. It could be speculated that genes controlling these key enzymes can be manipulated such that the enzymes become over-expressed in A. annua. Alternatively, other enzymes which are involved in pathways competing for precursors of artemisinin, for example, squalene synthase (SQS) can be inhibited through genetic engineering such that the genetically modified plants produce more artemisinin. Efforts could equally be geared in the future towards introducing the gene for artemisinin production (from A. annua) into a much faster-growing plant species, for example, chicory or tobacco (Nicotiana tabacum) with a proportionately higher leaf biomass, possibly, to enhance higher artemisinin yield. Such efforts already appear to be largely rewarding as demonstrated recently where the introduction of a gene into N. tabacum resulted in the expression of an active enzyme and the accumulation of the first dedicated precursor of artemisinin (amorpha-4,11-diene) ranging from 0.2 to 1.7 ng/g fresh weight of leaf tissue (Wallaart et al., 2001). Chen et al. (2000) equally transformed a cDNA encoding cotton FDS (farnesyl diphosphate synthase) under the control of CaMV 35S promoter into A. annua via A. tumefaciens or A. rhizogenes. By over-expressing FDS, a key enzyme in the biosynthesis of artemisinin, in the transgenic plants, the content of artemisinin was increased by about 0.8 - 1% dry weight in these plants.

Surprisingly this feature does not seem to be unique to plants alone. Recent advances using recombinant microbes circumvented the poor performance of plant terpene cyclases by expressing a codon-optimized fold (Martin et al., 2003). In a remarkable series of metabolic engineering experiments, these authors equally used an
Figure 3. The process for the microbial production of artemisinin. Using synthetic biology, the metabolism of the microbe is engineered to produce artemisinic acid, a precursor to artemisinin. Starting from acetyl-CoA (an abundant product of the central metabolism of many microbes), the microbes produce, in turn, mevalonate, farnesyl pyrophosphate (FPP), amorphadiene, and finally, artemisinic acid. The artemisinic acid is released from the microbes and purified from the culture media. The artemisinic acid is chemically converted to artemisinin. Once the artemisinin is produced, it must be further chemically converted into a derivative such as artesunate or artemether, which are integrated into ACTs for the treatment of malaria (Adapted from Hale et al., 2007).

engineered mevalonate pathway gene from the yeast eukaryotic system, which was about 30 to 90 times more efficient than the normal pathway in E. coli. This combined approach highlights an increased production of amorpha-4,11-diene by approximately 1,000 fold (Martin et al., 2003), which taken further into the pathway would possibly lead to the production of artemisinic acid. In a more facile approach, a cytochrome P450 monoxygenase gene (CYP71AV1) isolated directly from glandular trichomes of A. annua (Teoh et al., 2006) and inserted in yeast cells performed a three-step oxidation of amorpha-4,11-diene that allowed its conversion into artemisinic acid in yields that appear suitable for large-scale fermentation (Ro et al., 2006). This metabolically synthesized artemisinic acid can be obtained easily through a simple purification process (Ro et al., 2006), which can be converted to artemisinin through a few inexpensive chemical steps in the laboratory, as illustrated in Figure 3. Coming on the footsteps of this development, it is of special pharmacological interest that efforts are currently underway to optimize the CYP71AV1 gene expression system in several prokaryotic strains in order to sustain high-level production of amorpha-4,11-diene that can be easily converted to the artemisinin precursor artemisinic acid, which can be subsequently oxidized to yield artemisinin (Hale et al., 2007). The artemisinin thus produced can be further converted through simple downstream chemistry into derivatives such as dihydroartemisinin, artesunate or artemether for possible integration with other antimalarial drugs for the production of low-cost, life-saving ACTs with a great impact on malaria mortality.
Table 1. Physico-chemical properties of artemisinin (Lapkin et al., 2006).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (g.mol$^{-1}$)</td>
<td>282.3</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>157</td>
</tr>
<tr>
<td>Thermal stability in non-polar solvents (°C)</td>
<td>150</td>
</tr>
<tr>
<td>Solubility in water at pH 7 (g.l$^{-1}$)</td>
<td>0.063</td>
</tr>
<tr>
<td>Solubility in water at pH 7 and 37°C (g.l$^{-1}$)</td>
<td>0.048*</td>
</tr>
<tr>
<td>Solubility in ethanol at 21°C (g.l$^{-1}$)</td>
<td>12</td>
</tr>
<tr>
<td>Solubility in ethyl acetate at 21°C (g.l$^{-1}$)</td>
<td>100</td>
</tr>
<tr>
<td>Solubility in hexane at 40°C (g.l$^{-1}$)</td>
<td>0.46</td>
</tr>
<tr>
<td>Solubility in hexane/ethyl acetate (5% vol) (g.l$^{-1}$)</td>
<td>33</td>
</tr>
<tr>
<td>Solubility in N,N-dimethylethanolammonium octonoate (g.l$^{-1}$)</td>
<td>82</td>
</tr>
<tr>
<td>Solubility in bis(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide</td>
<td>110</td>
</tr>
<tr>
<td>Octanol/water partitioning coefficient (log P)</td>
<td>2.94</td>
</tr>
</tbody>
</table>

*Value for triclinic crystals by recrystallisation from cyclohexane; recrystallisation from EtOH (50% vol) solution yielded orthorhombic crystals with the lower and slower solubility in water.

Table 2. Properties of different solvents for artemisinin extraction (see: www.hill-assoc.com).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility</th>
<th>Selectivity</th>
<th>Preservative</th>
<th>Polarity</th>
<th>Boiling point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>None</td>
<td>None</td>
<td>none</td>
<td>high</td>
<td>100°C</td>
</tr>
<tr>
<td>Hexane</td>
<td>Poor</td>
<td>High</td>
<td>unknown</td>
<td>non-polar</td>
<td>60-80°C</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Slight</td>
<td>Poor</td>
<td>good</td>
<td>polar</td>
<td>78°C</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>High</td>
<td>Medium</td>
<td>unknown</td>
<td>mild</td>
<td>67°C</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>Poor</td>
<td>High</td>
<td>good</td>
<td>non-polar</td>
<td>15°C (at 50 bar)</td>
</tr>
</tbody>
</table>

COMMERCIAL-SCALE EXTRACTION OF ARTEMISININ FROM A. annua

Artemisinin is soluble in several solvents including methanol, ethanol, hexane, petroleum ether, dichloromethane, chloroform, diethyl ether, and acetone-trile. The physico-chemical properties of artemisinin and some characteristics of a few of the major solvents currently used for its extraction are presented in Tables 1 and 2. It is obvious from the details presented in these tables that artemisinin is poorly soluble in water at low temperatures. However, this poor solubility in cold water can be circumvented by heating to 90 - 100°C if needed, for example, in the preparation of teas. Hot water can extract up to 75% of artemisinin from dried pulverized plant material though boiling the leaves directly in water destroys most of the vital antimalarial drug (Ferreira, unpublished).

For commercial-scale artemisinin production, most methods currently use a hydrocarbon (usually either an initial hexane or petroleum ether) solvent extraction step before proceeding to purifying the product by partitioning the first extract in solvents of different polarities before using chromatography to separate the different fractions. In the simplest hydrocarbon batch extraction procedure, dried pulverized leaves are soaked in the ratio of 1 liter to 1 kg in fresh portions of warm hexane or petroleum ether (at 30 - 45°C), with each extraction cycle lasting about 10 – 48 h (Figure 4). Interestingly, under flow conditions, where the solvent percolates through a packed leaf biomass bed at the same temperature, this duration can be reduced. However, irrespective of the duration of the extraction regime (whether batch or percolation), the efficiency of artemisinin extraction can be improved significantly by simply adding a small amount of ethyl acetate which serves as a co-solvent to the main non-polar hydrocarbon solvent. This increases the solubility of artemisinin in the solvent mixture by about two-fold as depicted in Figure 4.

Now aside from these common hydrocarbon solvents that are used and, especially, as a result of the risk to human health, poor environmental performance, and the dangers of processes involving large volumes of volatile combustible fluids, alternative processes that would be able to compete in terms of efficiency and cost and have little or none of the drawbacks associated with hydrocarbon solvents, have been tested recently as “green” extraction procedures and found to be very effective. These methods include the use of organic ionic liquids such as N,N-dimethylethanolammonium octonoate (DMEA oct) and bis(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide (BMOEA bst), super-
critical CO\textsubscript{2} and hydrofluorocarbon (HFC-134a). Artemisinin extractions based on ionic liquids and HFC-134a appear to offer the cheapest running cost compared to hydrocarbon solvents. However, the total start-up cost and working capital portfolio, including the price of solvent inventory, necessary for the maintenance of high-pressure equipment in some of these methods, may be about 100\% higher than for mixed solvent or ethanol extraction procedures, which are being currently used by major competitors in artemisinin producing countries. At the moment, methods which allow recycling of extraction solvents are also being tested to decrease pollution of the environment. For instance, most artemisinin producers in China recycle their solvents three or four times before discarding (Figure 4). Apart from the need to recycle solvents or to develop extraction methods which are environmentally friendly, there is equally the need to establish the commercial-scale extraction of artemisinic acid, dihydroartemisinic acid, and artemisinin, the three major sesquiterpenes identified from most commercial cultivars of the plant. Approximate quantifications for these sesquiterpenes indicated that there were about 5\% of artemisinic acid, 24\% of dihydroartemisinic acid, and 71\% of artemisinin from the high artemisinin-containing cultivar Artemis (Ferreira, unpublished). It is likely that both the artemisinic and dihydroartemisinic acids thus extracted can be equally converted into artemisinin through inexpensive chemical procedures (Roth and Acton, 1989). It is, therefore, of immense economic importance that such methods are optimized since they can increase the overall yield of artemisinin derivable from a given quantity of dry leafy biomass. This can be easily confirmed knowing that though artemisinic and dihydroartemisinic acids are extracted with refluxing in the extraction solvent, they are usually discarded in the partitioning steps where artemisinin is pooled into non-polar fractions. Implicit in this is the fact that as the polar fractions are discarded, so also is approximately 30\% of sesquiterpenic precursors of artemisinin, which could potentially increase the final

Figure 4. Exemplification of an artemisinin extraction and purification protocol from dry \textit{Artemisia annua} leaves with a moisture content of 8\% and a raw material to solvent ratio of 1:4. This method has an extraction efficiency of up to 75\% after 3 to 4 extractions.
CONVERSION OF ARTEMISININ INTO ITS DERIVATIVES

In spite of its current role as the most important anti-malarial drug, artemisinin is not used directly because of its poor oral availability, but modified into its so-called derivatives such as dihydroartemisinin (DHA), artesunate, artemether and arteether, which are readily bioavailable. Presently, transformation of artemisinin into these derivatives is done either by companies that are into its extraction from A. annua or by pharmaceutical companies that produce ACTs. Many of these companies use their own proprietary technologies and any new entrants into the market will either have to rely on competitors to undertake the transformation for them or have to do their own research to find a suitable protocol.

So far, DHA which was developed by Chinese scientists about three decades ago is the simplest of the artemisinin derivatives. Since then, a variety of protocols have been developed and described in the literature for the conversion of artemisinin to DHA even though most of these are not optimal for practical purposes. Two of these methods that propose to transform artemisinin to DHA involve either its reduction with sodium borohydride in ethanol or methanol (the better of the two alcohols for the purposes outlined here) at about 0 to 5°C or the reduction with DIBAL-H in dichloromethane at -78°C. However, some major disadvantages associated with the latter method involving the use of DIBAL-H are the smaller yield and the higher prices of both the solvent and the means of reduction. A study recently commissioned by Medicines for Malaria Venture (MMV) and undertaken by Buzzi, Presser and von Freyhold in Germany (2007) showed an improved protocol that can lead to an optimized production of DHA. This study also showed a viable protocol for the derivatisation of dihydroartemisinin into dihydroartemisinin hemisuccinate, which is commonly referred to as artesunate. Dihydroartemisinin can also be transformed into arteether.

Currently artemisinin and its derivatives are only available commercially in China, where more than 90% of the global total is produced, and in Vietnam to some extent, as these are the primary centers of A. annua cultivation and artemisinin extraction in the world. Up until recently, many drugs based on artemisinin derivatives were registered and sold in many malaria endemic, sub-Saharan African countries. In Nigeria and Ghana, for example, there were many of such products including Artesunat™ and Cotexcin™, which were sold as over the counter (OTC) drugs (Brisibe, 2006). A major drawback with the artemisinin derivatives, however, is the high recurrence rates (Mueller et al., 2004) and the fear of a possible development of resistance in malaria-endemic areas, especially if used as monotherapy (Nelson, 2006) for durations less than 7 days as has been recently sug-

gested by the work of Jambou and others (2005). Concerns regarding this have recently led some pharmaceutical companies to phase out the production of artemisinin monotherapy for oral treatment of malaria (http://www.who.int/mediacentre/news/releases/2006/pr23/en/index.html). Nevertheless, some studies have actually shown that such fears are misplaced and could be drastically reduced by having artemisinin derivatives utilized in combination with other anti-malarial drugs to produce artemisinin-based combination therapies (ACTs), which can be used wherever resistance to conventional anti-malarial drugs has been observed as recommended by the World Health Organization since 2001 (WHO, 2001). In line with this recommendation, about 56 countries in Africa, Asia, and South America have actually adopted ACTs as either their first- or second-line antimalarial treatment wherever the common quinoline and sulphadoxine-pyrimethamine based drugs are no longer effective (Olliaro and Taylor, 2004). Expectedly, some 29 countries have started deploying them up to date (WHO, 2006) and this has fuelled an increased demand for ACTs several folds within the past 4 – 6 years. Not surprisingly, this demand will continue to increase to several hundred million treatments within the next few years, which in itself has provoked an increase in the international demand for artemisinin derivatives, leading to supply shortages that are not likely to be met soon (Cyranoski, 2004). In order to satisfy the artemisinin demand in Africa, a few corporate organisations including East African Botanicals in Kenya, AfroAlpine Pharma Limited in Uganda, Molecular Bio/Sciences Limited and Artemisinin Development Company in Nigeria, respectively, have either embarked on or planning to embark upon commercial-scale cultivation of A. annua in the continent. Quite recently, East African Botanicals, for example, has increased the area planted to A. annua from 200 to more than 1,000 hectares in Kenya, Tanzania, and Uganda in line with the expected increase in artemisinin demand. In China, Holley Pharmaceuticals, working with their contract farmers, has announced its intent to increase annual production of artemisinin from 14 to 40 metric tons per year. It has been projected that the worldwide area needed to meet the current WHO-estimated demand for the about 400 million ACTs for 2009 is between 16,000 - 20,000 hectares, based on the estimate that one hectare of land devoted to A. annua cultivation produces enough artemisinin for approximately 25,000 adult courses of ACT (cited from Ferreira et al., 2006).

ACTs AS FIRST-LINE TREATMENT FOR MALARIA

As with the combination therapies against HIV infection and tuberculosis, those used for the treatment of malaria are equally based on the additive potential of two or more drugs such that their cumulative therapeutic efficacy is improved, which enhances a delay in the development of
resistance to the individual components. The advantages of ACTs relate to the unique properties and mode of action of the artemisinin component. Generally ACTs combine the rapid action, symptomatic relief and anti-gametocytic effect of artemisinin (or its derivatives) with the long-term effect of one or more blood schizontocidal drugs with independent modes of action and different biochemical target(s) in Plasmodium. Currently, the parasites have not yet developed true resistance to artemisinin derivatives when used in combination with other standard antimalarial drugs. Using these combinations may, therefore, help to prevent such resistance from developing and may retard the development of resistance to the synthetic partner drug(s).

Artemisinin-based combination therapies are currently available in a variety of formulations. Some of the most common combination chemotherapies currently in use include: artemether + lumefantrine, dihydroartemisinin + piperaquine + trimethoprime, dihydroartemisinin + piperaquine, artesunate + sulphadoxine-pyrimethamine, artesunate + chloroquine, artesunate + amodiaquine, and artesunate + mefloquine. Of these, artemether + lumefantrine and artesunate + amodiaquine are the only two ACTs that have been pre-qualified by the WHO for use in countries experiencing high levels of resistance to standard antimalarial drugs (Brisibe, 2006). Unfortunately, there is a significant paucity of ACTs due in part to the short supply of artemisinin and its high cost in the world market (Enserick, 2005). Consequently, ACTs remain far from being accessible to the poor, rural population in many countries at risk, especially in Africa, where they presently remain too expensive for the majority of people suffering from the effects of the malaria scourge (Mutabingwa, 2005).

CONCLUSION AND PROSPECTS FOR ACTs PRODUCTION IN AFRICA

Thomas Jefferson once wrote that "The greatest service which can be rendered any country is to add a useful plant to its culture." There is no doubt that the spotlight on international malaria therapy is presently focused on the availability of artemisinin and the supply of ACTs from a seemingly simple, yet versatile plant of Asian origin that is suddenly found at the forefront of global efforts aimed at the eradication of malaria. In the current setting, it is essential that the production of artemisinin and ACTs should be seen as the central focus. However, naturally there are bound to be problems, as there would be with most crops, when trying to increase production in areas where the crop is either not widely grown or unknown. Some of these problems include the fact that appropriate cultivars suitable for particular locations, especially in Africa where malaria is a major scourge, have to be either developed or identified while farmers have to be encouraged to cultivate the introduced crop. In themselves, these are no mean challenges.

In addition, a long standing issue concerns striking an appropriate balance between encouraging farmers in Africa to produce crops that they are traditionally accustomed to as opposed to A. annua, which is an introduced crop that is not a component of the agricultural production and processing systems they are used to in order to avert disasters associated with food security. Such a balance would not only avert disasters associated with increased production of the pharmaceutical crop in detriment to crops required for provision of calories for the citizenry but also would help sustain good nutritional balance, which is closely related to a healthy immune system that could wade off malaria episodes. These concerns notwithstanding, it is very interesting that agriculture is playing a pivotal role in the provision of artemisinin, which has become very crucial in the production and accessibility of ACTs to people at risk of malaria as well as providing the additional benefit of supporting profit from greater margins and higher values enjoyed by the local economies in malaria endemic countries in Africa, where A. annua could be cultivated.

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