Plant Molecular Biology **33:** 199–209, 1997.

Mini-review

State of the art of the production of the antimalarial compound artemisinin in plants

Els Van Geldre, Annemieke Vergauwe and Elfride Van den Eeckhout*
*Laboratorium voor Farmaceutische Biotechnologie, Universiteit Gent, Harelbekestraat 72, 9000 Gent, Belgium (*author for correspondence)*

Received 13 September 1996; accepted 16 September 1996

Key words: antimalarial drugs, *Artemisia annua* L., artemisinin

Abstract

For more than three centuries we have relied on the extracts of the bark of *Cinchona* species to treat malaria. Now, it seems we may be changing to the leaves of a Chinese weed, *Artemisia annua*, and its active compound artemisinin. Artemisinin-derived drugs have been proved particularly effective treatments for severe malaria, even for multi-drug-resistant malaria. However, this promising antimalarial compound remains expensive and is hardly available on a global scale. Therefore, many research groups have directed their investigations toward the enhancement of artemisinin production in *A. annua* cell cultures or whole plants in order to overproduce artemisinin or one of its precursors. This article provides a brief review of the state of art of the different aspects in *A. annua* research.

> 'If we take as our standard of importance the greatest harm to the greatest number, then there is no question that malaria is the most important of all infectious diseases.'
> Sir Macfarlane Burnet, Natural History of Infectious Disease.

Introduction

Malaria, one of the most devastating diseases in the tropical world, is on the increase with no clear solution nearby [53]. During the past 40 years, malaria control strategies have relied on insecticides against the vector and antimalarial drugs as prophylaxis or treatment. The emergence of resistance by both vectors and parasites has emphasized the need for improved methods of malaria treatment and biological control of the anopheline vector [90]. Due to the multiple ways in which *Plasmodium* replicates, much of the work in vaccine development to date has remained in a preclinical stadium. Therefore, there is an urgent need for new efficient antimalarial drugs for malaria prophylaxis. The first effective antimalarial drug was quinine, which was isolated from the barks of several South American species of *Cinchona*. For a long time this was the ideal drug for prophylaxis since it is not toxic, works fast and efficiently and has also a long half-life time. However, *Plasmodium* parasites have built resistance against this drug. The interest in plants as potential sources of new antimalarial drugs has been stimulated by the isolation and clinical use of the endoperoxide sesquiterpene artemisinin, the active principle of the Chinese medicinal herb *Artemisia annua* [61].

Artemisia annua (Compositae) is an aromatic annual herb that occurs naturally as part of a steppe vegetation in the northern parts of Chahar and Suiyuan Provinces (40° N, 109° E) in China, at 1000 to 1500 m above sea level [86]. However, the plant now grows wild in many other countries such as the former

Yugoslavia, Hungary (where it is cultivated for its aromatic oil), Bulgaria, Romania, Turkey, the former Soviet Union, Argentina, Italy, France and Spain [41, 42]. Ironically, the humid tropics, where malaria is endemic, appears unsuitable to cultivate the plant [41], because long days are required for the plant to reach full size before flowering is induced under short days. This plant blooms during the months of February and March, and its reproduction is by seed that can germinate in September [57]. *A. annua* can be classified as a short-day plant with a critical photoperiod between 12 and 16 h.

The cultivation aspects of artemisinin production are described [45, 46]. The essential oils from *A. annua* are being used for the flavouring of vermouth [8] and there is also some interest for the fragrance industry [12].

A. annua L. (sweet or annual wormwood, sweet Annie or simply artemisia) is the source of artemisinin or qinghaosu (QHS). This secondary metabolite is an endoperoxide sesquiterpene lactone efficient against multidrug resistant strains of *Plasmodium*, the malarial parasite. These antimalarial properties are linked with the peroxide function of this sesquiterpene lactone. In 1972, the main active principle, artemisinin was isolated and in 1979 its structure was defined by X-ray analysis [7]. It has an empirical formula of $C_{15}H_{22}O_5$ with a unique structure among antimalarial agents, lacking the nitrogen-containing heterocyclic ring system found in most antimalarial compounds. By the end of 1972, artemisinin and derivatives were used in ten regions of China by ca. 6000 patients [42]. Semisynthetic derivatives of artemisinin as developed by the Walter Reed Army Institute proved to be very effective drugs against multi-drug-resistant strains of *P. falciparum*. Studies in Thailand showed that patients with malaria improved faster after treatment with artesunate and artemether compared with other antimalarial drugs [50]. The artemisinin derivatives were all as well tolerated and had no significant side-effects. Although clinical experiences are numerous, only very little preclinical research has been done. However, on a global scale artemisinin is hardly available, and drug registration requirements are not yet met. Artemisinin derivatives are now being marketed by Rhône Poulenc in Africa, but are not approved in the USA nor in Europe. The product remains expensive and its isolation requires careful chromatographic separation from similar plant constituents. Therefore, the goal of many research groups is the enhancement of the production of artemisinin in *A. annua* cell cultures or whole plants. Interdisciplinary research treating the analytical aspects, the plant biochemistry and genetic engineering of plants is needed here. This article reports on the state of art of these different aspects in *A. annua* research.

Analytical aspects in *Artemisia annua* research

Analysis of artemisinin is difficult because the compound is unstable, concentrations in the plant are low, the intact molecule stains poorly, and other compounds in the crude plant extracts interfere in its detection. Thermal stability studies have shown that artemisinin is stable up to 150 °C but degrades into numbers of products when heated at 180–200 °C [49, 51]. Moreover, artemisinin is sensitive to acid and base treatment [98].

Thin-layer chromatography (TLC) has been used to estimate the artemisinin content [40, 51, 62, 67, 78, 82], but because of the poor staining characteristics of the intact molecule and interference with contaminating constituents of the plant, this method is not very reliable. HPLC with UV monitoring at 210 nm has been used, but the presence of constituents that absorb at 210 nm completely obliterates the peak of artemisinin [1, 48, 62, 75, 96]. Since artemisinin and dihydroartemisinin are thermally labile and lack UV or fluorescent chromophores [49, 79], as well as functional groups for derivatization, development of sensitive and specific analytical methods for determination of these compounds is a challenging problem. Two different approaches have been used for the determination of these compounds: precolumn acid- or base-catalysed decomposition to UV-absorbing compounds followed by HPLC of the decomposition products [96] and HPLC with reductive electrochemical detection (LC-EC) using either thin-layer gold-mercury amalgam or dropping mercury electrodes [99, 100]. However, the reductive electrochemical detection of artemisinin involves very special precautions as molecular oxygen is reduced at the low cathodic potential of -0.8 V employed [80]. ElDomiaty *et al.* reported a reversed-phase HPLC determination of artemisitene in artemisinin which is particularly useful for the routine analysis of artemisinin to check its purity and can in addition be used for preparative-scale purification of these compounds [21]. An HPLC method with polarographic detection of artemisinin and its derivatives has also been developed [101]. Recently, a GC method for the analysis of artemisinin at nanogram

levels (detection limits 100 ng) was described [29, 76]. This method is based on the linear relationship obtained between the concentration of artemisinin and the respective peak areas for either of the two thermally degraded products. A rapid screening method based on tandem mass spectrometry (MS/MS) is described for artemisinin-related compounds present in a crude hexane extract of *A. annua* [64]. Also, a detection method based on GC combined with MS [10, 91, 92] and radioimmunoassay or RIA have been described [97]. In 1993, Jaziri *et al*. [34] reported the establishment of an indirect enzyme-linked immunosorbent assay (ELISA) method based on the peroxide bridge for antibody specificity to detect artemisinin and closely related compounds in crude extracts of *A. annua*.

Little attention has been paid to the analysis of late precursors in artemisinin biosynthesis, namely the sesquiterpenoids arteannuic acid, arteannuin B and artemisitene. TLC has been used for the simultaneous detection of these compounds, but the compounds are not fully resolved [91]. However, a method for the simultaneous determination of artemisinin-related compounds may be valuable for plant biotechnological purposes. The formation of secondary metabolites in the *in vitro* cultured cells or tissues may then be monitored more appropriately. A GC/MS method for the analysis of artemisinin and its precursors arteannuic acid, arteannuin B and artemisitene in one run was developed [91]. Because of their thermal instability, the endoperoxide containing sesquiterpene lactones artemisitene and artemisinin were measured as their pyrolysis products. A simple method for simultaneous detection and quantification of intact artemisinin and its intact bioprecursors using HPLC-UV/EC was established by Vandenberghe *et al*. [83]. With this method all compounds are clearly resolved and detection limits are far below the naturally occurring concentrations in the plant.

Biochemistry of *Artemisia annua*

Although the complete biosynthetic pathway for artemisinin and some of its precursors has not been established, some biotransformation steps have been elucidated *in vitro* and *in vivo*. Akhila *et al*. proposed a complete biosynthetic pathway for artemisinin, starting from mevalonic acid and isopentenylpyrophosphate (IPP) [5, 6]. The following biosynthetic sequence is suggested: farnesylpyrophosphate (FPP), germacrane skeleton, dihydrocostunolide, cadinanolide, arteannuin B, artemisinin. *Artemisia annua* contains 8–10 times more artemisinic acid than artemisinin. Therefore, it has been suggested that artemisinic acid is a possible biogenetic precursor of artemisinin. However, Akhila's studies did not state artemisinic acid as a precursor for artemisinin, but others [22, 38, 39, 66, 70] consider artemisinic acid to be a possible biogenetic precursor for both arteannuin B and artemisinin, sequentially or independently. Wang *et al*. converted artemisinic acid ^{3}H-labelled at C-15 (exocyclic methylene) to both arteannuin B and artemisinin [87]. Sangwan *et al*. reported *in vitro* and *in vivo* transformation of artemisinic acid to arteannuin B and artemisinin [70]. Staba's group converted IPP to both arteannuin B and artemisinin [43, 52]. Arteannuin B has been considered another precursor for artemisinin [56, 68]. Cell-free leaf homogenate of *A. annua* leaves converted arteannuin B into artemisinin [56]. Artemisitene is an endoperoxide closely related to artemisinin and has been isolated and characterized for the first time from *A. annua* in 1985 [2]. The ratio of artemisitene to artemisinin increases from 1:10 early in the season to 1:1 when flowers develop. The transformation of dihydroartemisinic acid into artemisinin by *A. annua* tumour cell-free extracts but not by leaves or calluses cell-free extracts was reported by Kim and Kim [39]. Li *et al.* could synthesize [$^{15-14}$C]-labelled artemisinin in a supernatant liquid prepared from the tender leaves of ripe *A. annua* L. with the addition of [$^{15-14}$C] dihydroartemisinic acid as starting compound [47]. Products or enzymes after FPP and before artemisinic acid, arteannuin B and artemisinin have not been isolated *in vivo*. ElFeraly *et al*. report the isolation of two new compounds from *A. annua* [23]. *Epi*-deoxyarteannuin B, also reported by Klayman *et al*. [40], might be, at least in part, an artefact derived from artemisinic acid. Furthermore, it was also observed as a by-product of oxidizing artemisinic acid. Dehydroartemisinic acid had not been previously reported either in *A. annua* or in other sources.

Artemisinin has been reported to accumulate in leaves (89% of the total artemisinin in the plant), small green stems (trace amounts), buds, flowers, and seeds [1, 11, 28, 48, 52, 81, 96]. Artemisinin and its precursor artemisitene have not been detected in roots [11, 28, 81]. Low levels of arteannuic acid and arteannuin B were present in the side-roots.

A. annua leaves are covered with glandular trichomes [17, 18]. The glandular trichomes are composed of 10 cells differentiated into five cell pairs, with each cell pair apparently having different func-

tions as deduced from ultrastructural differences [18]. An *A. annua* biotype without glands contained neither artemisinin nor artemisitene, a possible biosynthetic precursor. The report of Duke *et al*. concludes that artemisinin is sequestered in glandular trichomes of *A. annua* because flowers and leaves, which are a rich source of artemisinin and valuable oils, have abundant glandular trichomes [19]. Ferreira *et al*. support this conclusion [28]. Artemisinin content of inflorescence in the bud stage was not higher than in leaves, but artemisinin in flowers at full bloom was 4- to 11-fold higher than in leaves [28].

The artemisinin yield estimated at different stages of development reveals a positive correlation between plant age and artemisinin yields. This is assumed to be due to both an increase in leaf yield and artemisinin content with the progressive increase in plant growth [75]. Some disagreement exists about when the highest artemisinin content occurs. Some researchers state that the artemisinin content is highest just before flowering [1, 26, 48, 91, 92]. Others find an artemisinin peak reached during full flowering [28, 54, 62, 75]. Although artemisinin is the starting material for the synthesis of other more soluble and stable antimalarial compounds, the most abundant sesquiterpene in *A. annua* is artemisinic acid, which occurs in an 8- to 10-fold higher concentrations [38, 66], followed by arteannuin B [42]. The change in artemisinin content and the biosynthetical related sesquiterpenes artemisinic acid, arteannuin B, and artemisitene in *A. annua* plants during a vegetation period, has been studied in Vietnam [93]. The highest artemisinin content (0.86% dry mass) was present in the leaves of 5-month-old plants. At this stage also the highest leaf yield was found. Subsequently, the artemisinin content gradually dropped. At the age of 5 months the highest artemisinic acid (0.16% dry mass) and arteannuin B contents (0.08% dry mass) were found as well. Artemisitene was present at all stages of development, ranging from 0.002 to 0.09% dry mass.

Artemisinin production is influenced by climatological conditions [14, 27, 52, 74]. Environmental stress, such as light, temperature, water and salt, significantly alters product yields [88].

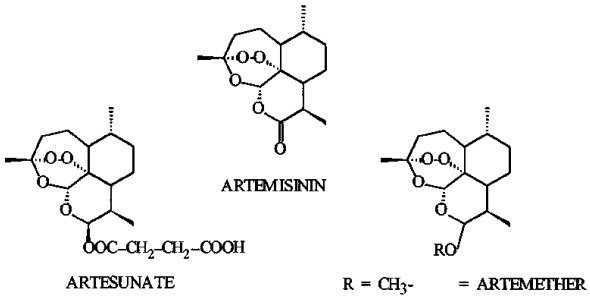

Figure 1. Artemisinin and some semi-synthetic derivatives.

Production of artemisinin

Chemical synthesis

For organic chemists it was a challenge to develop a total chemical synthesis pathway for artemisinin. Due to the complex structure (artemisinin is one of the 64 theoretical possible stereoisomers) complete chemical (*de novo*) synthesis of artemisinin is very difficult. Nevertheless, different reaction pathways, each starting from different reagents, are described [95]. The first total synthesis of artemisinin was reported in 1983 by Schmid and Hofheinz [71] who began their construction of the molecule with (−)-isopulegol. Recently, Ravindranathan *et al.* have reported a stereo-selective synthesis of artemisinin from (+)-isolemonene [65]. Another efficient synthesis was accomplished by Avery *et al*., starting from (R)-(+)-pulegone [9]. Each procedure for the chemical synthesis of artemisinin requires a final photo oxidative step. The low yield (<30%), the high cost of *de novo* synthesis and its complexity suggest that isolation from the plant will be the optimum system.

As an alternative, the preparation of artemisinin from closely related biosynthetic precursors has been tested. Artemisinin could easily be obtained from artemisinic acid, which is 8–10 times more abundant, in an overall yield of about 40% [31]. The photo oxidation of artemisinic acid has been studied by several groups [22, 37, 94] searching for a route from this relatively abundant constituent of *A. annua*. Roth and Acton proposed a conversion of artemisinic acid into artemisinin with a 30% yield [69]. Also, partial two-step synthesis of artemisinin from dihydroartemisinic acid has been described by Acton and Roth [4]. These researchers have proved unambiguously that the peroxide bridge oxygens are introduced during the triplet oxygen (air) oxidation step. Due to the complex equi-

libria to be established it is not clear how reaction conditions might be manipulated to increase the yield. Landsbury and Nowak converted artemisinic acid and arteannuin B to artemisinin in four and five reaction steps, respectively [44].

Extraction of artemisinin from plants

The large-scale extraction of artemisinin from the plant has been reported. Klayman *et al*. extracted dried leaves with petroleum ether and fractionated the extract on a silica column [40]. An alternative procedure depends upon the use of the Ito multilayer separator extractor [3]. This procedure, although very economical in comparison with the first procedure, is only suitable for small-scale extractions. ElSohly *et al*. could isolate large quantities of QHS, but the predominant artemisinic acid tends to elute with artemisinin and fractions containing artemisinin may require rechromatography to achieve the necessary purity [25]. ElSohly *et al*. described a practical and economic procedure for the isolation of artemisinin in kilogram quantities by extracting leaves with hexane followed by partitioning with a CH_3CN phase [26]. Artemisinin isolated by this way was $>$99% pure and without any artemisitene. The usual extraction method for artemisinin neglects artemisinic acid, but Vonwiller *et al*. devised an efficient method to extract both artemisinic acid and artemisinin from the same material [85]. Artemisinic acid can then be converted to artemisinin, according to Xu *et al*., which greatly increases the yield of artemisinin [94].

However, a full ton of dried leaves is needed to produce about 6 kg of artemisinin. Therefore, a total area of 40 ha must be planted to supply the raw material [33]. The low content of artemisinin in cultivated European and New World types of *A. annua* has been a limiting factor for the isolation and evaluation of artemisinin on a technical scale. Artemisinin yields of 0.06%, which are low for commercial exploitation, have been obtained from samples of *A. annua* collected in the USA. Yields of extracted artemisinin from the above-ground portions of the plant have ranged from 0.01% to 0.5% (w/w) in China [73].

In vitro *production of artemisinin*

Cell and tissue culture

Results from experiments with undifferentiated callus and cell suspension cultures of *A. annua* are disappointing with respect to the artemisinin production. At most, traces of this compound have been found [29, 32, 36, 43, 52, 55, 78, 92]. Dedifferentiated and differentiated tissue cultures of *A. annua* were carried out. The best results were obtained with 2.4-dichlorophenoxyacetic acid (4.5 μM: μ 0.02/day) and naphthalene acetic acid (NAA) (5.4 μM: μ 0.06/day). In primary callus values of 1.13 and 0.78 mg artemisinin per 9 dry weight (DW) were obtained, in cell suspension no artemisinin was found [58]. A certain degree of differentiation of the cultures is a prerequisite [29, 52]. Differentiation into shoots, or preferably shoots with roots, is necessary for significant artemisinin biosynthesis in *A. annua* shoot cultures [24, 29]. Furthermore, artemisinin contents increased when roots developed into plants with a properly developed root system [52]. Artemisinin contents in shoot cultures of *A. annua* are about 10-fold less than in the intact plant. Roots originating from leaf segments contained artemisinin if they were grown on a Murashige-Skoog (MS) or B5 medium supplemented with indolebutyric acid (IBA) or NAA at 0.05 to 2.0 mg/l [52]. The shoot cultures showed better growth and produced more artemisinin on 2% sucrose [92]. In shoot tips, Fulzele *et al*. found 0.012% (DW) [29]. Simon *et al*. reported concentrations ranging from 0.03 tot 0.05% (DW) [73]. Initial results showed also that artemisinin is present in the culture fluid from liquid suspension cultures of callus cells. This latter observation suggests the potential of this methodology for large-scale production of this drug [55].

Attempts were made to improve the artemisinin production by omittance or addition of medium components (plant growth regulators, casein hydrolysate). A combination of benzylaminopurine (BAP) (1.0 mg/l) and kinetin (10 mg/l) increased the yield of artemisinin *in vitro*, by 3.6 and 2.6 times respectively, due to an increase in dry matter production, which overcame a concurrent decrease in the artemisinin content [89]. The addition of GA_3 (gibberellic acid, a plant growth regulator that can induce blooming) and casein hydrolysate (source of amino acids and oligopeptides) to the medium improved the artemisinin production [92]. Fulzele *et al*. report the stimulation of terpenoid synthesis in plantlet cultures from *A. annua* L. by addition of GA_3 [30]. However, other scientists find only an important effect on biomass production in plants treated with 40 and 80 mg/l of GA_3 [12, 30]. The artemisinin content of the GA_3-treated plants did not reveal significant differences [30].

ELISA analysis of green hairy roots cultured in liquid medium under a 16 h light/day photoperiod

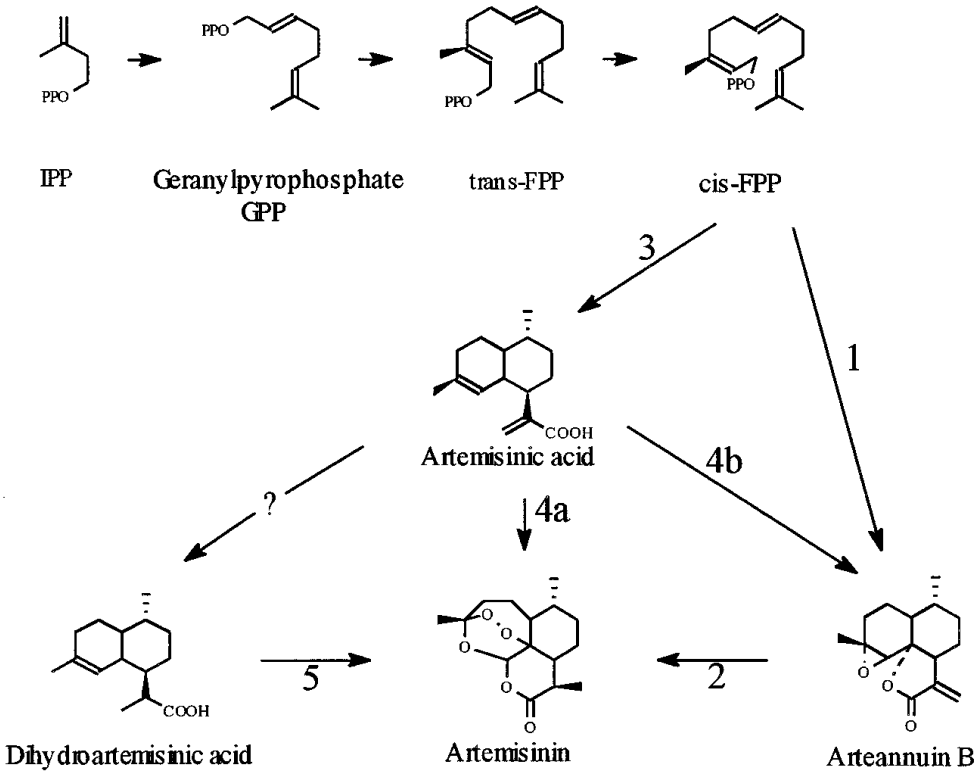

Figure 2. Proposed biosynthetic pathways for artemisinin. 1 [5]; 2 [70]; 3 [6]; 4a [22, 38]; 4b [22, 38, 68, 70]; 5 [39, 68].

showed the existence of compounds structurally related to artemisinin. Normal and hairy roots cultured in the dark, however, give no detectable levels of immuno signal [35]. This is in accordance with Martinez and Staba who did not detect artemisinin in roots [52]. However, Sumita *et al*. reported the detection of artemisinin in roots, unrooted shoots and callus [77].

The artemisinin content varies, depending on both the NAA and sucrose concentration [92]. A high fresh weight/dry mass ratio co-occurred with a high artemisinin content. Nitrate concentration and conductivity seemed suitable growth parameters. Expression of photosynthetic activity has been considered essential for the artemisinin production, as the biosynthesis of sesquiterpenoids proceeds via mevalonic acid, linked with the photosynthesis by acetyl-coenzyme A [5]. However, feeding of mevalonic acid did not induce an enhancement of artemisinin production [92].

Callus and suspension cells and medium were analysed and examined for their antimalarial activity at different stages of growth and development. Extracts of cell suspension cultures of *A. annua* exhibited antimalarial activity in *Plasmodium falciparum in vitro* [15].

Precursor feeding

Improvement of artemisinin production by precursor feeding has also been tested. Amounts of artemisinin equal to or greater than the amount produced by leaf tissue of naturally grown plants have been recovered by adding artemisinin precursors to the medium used for callus and organ cultures of *A. annua*. Addition of precursors resulted in a fourfold increase of artemisinin in the tissues and an 11-fold increase of artemisinin in the spent medium [88]. The feeding of mevalonic acid alone, however, did not induce an enhancement of the artemisinin production [92].

Investigations on whole plants

Influence of growth hormones on A. annua *cultures*

The effect of chlormequat and triacontanol on growth, plant hormones and artemisinin yield in *A. annua* has been studied [72]. Triacontanol at 1.0 and 1.5 mg/l produced a statistically significant positive effect on

artemisinin level, plant height, leaf and herbage yield. Chlormequat at 1000 and 1500 mg/l also increased artemisinin level, decreased the plant height at higher concentrations and increased the leaf and herbage yield at lower concentrations. The effect of triacontanol on the artemisinin yield might be mediated through its effect on plant growth. It appears that significant changes in plant characters result in greater availability of substrate for artemisinin biosynthesis. Chlormequat can increase the artemisinin content by 30% over untreated plants [48]. Liersch *et al*. also found some slight effects of the growth regulators on morphological criteria of glandular trichomes. The growth regulator daminozide was not able to increase the artemisinin content. Both growth regulators, chlormequat and daminozide, may have an influence on the formation of asteraceous glandular trichomes. However, it was proved that the asteraceous trichome index is not a suitable marker for the changes in correlation to artemisinin content in *A. annua* plants [48]. Fulzele *et al*. found that addition of ethephone to plantlet cultures from *A. annua* L. in bioreactor stimulated the synthesis of terpenoids in general [30].

Influence of mediators of the sesquiterpenoid biosynthesis

Attempts were made to improve the artemisinin production by influencing the artemisinin biosynthesis routing (addition of sterol synthesis inhibitors, mutagenic compounds). The addition of naphtiphine (inhibition of the enzyme squalene epoxidase) to the medium improved the artemisinin production. Other additions, 5-azacytidine (a gene regulator), colchicine (a gene regulator), miconazole (inhibition of sterol desmethylase), terbinaphine (inhibition of the enzyme squalene epoxidase), were too toxic for the cultures and did not induce an enhancement of the artemisinin production [92]. Kudakasseril *et al.* however reported a concentration dependent increase in the levels of artemisinin and growth of shoot cultures (based on dry weight) with miconazole [43]. Other sterol inhibitors such as AMO 1618 (2′-isopropyl-4′-(trimethylammonium chloride)-5-methyl-phenylpiperidine carboxylate), CCC (chlorocholine chloride), and MER 29 (4-chloro-2-(2-diethylamino,ethoxyphenyl)-2-(4-methyl phenyl)-benzene ethanol) increased both the incorporation of ^{14}C-IPP into artemisinin by cell-free extracts and the production of artemisinin in shoot cultures of *A. annua* [43]. It appears reasonable that the production of the sesquiterpenoid compound, artemisinin, might be increased. Sterol inhibitors inhibit one or more enzymes in the mevalonate pathway after the formation of FPP, which might result in a shunt forward terpenoid production rather than sterol production.

Selection of high-producing clones

Several attempts have been made for selection and breeding of high-artemisinin-yielding strains of *A. annua*. Artemisinin, which is present mainly in the leaves, varies in concentrations from 0.01% to 0.5% (based on dry weight) in China [55]. Concentrations of 0.3–0.8% have been reported for Chinese and Vietnamese plants [13, 20, 40, 52]. The average artemisinin concentration in plants of European origin range from 0.03 to 0.22% (dry leaf weight basis) artemisinin [11, 81], but a clone from China could contain up to 1.1% artemisinin [16]. In the USA, strains have been detected with mean artemisinin concentrations ranging only from 0.05% to 0.21% with individual plants producing up to 0.42% at full flowering stages [28]. Contents between plants from a population vary a lot, however, this variation is far less important. As *A. annua* is a strongly outcrossing species, hybridizations have been made between the Chinese clone and pollen issued from Italian, Yugoslavian and Spanish origins. These hybrids produced in average 0.64, 0.73 and 0.95% artemisinin, respectively, for a yield of dry leaves of about 2000 kg/ha [16]. Pras *et al*. observed that plants that are high-yielding in the laboratory continue to be so in the field [62]. A method for laboratory selection to identify high-yielding individual plants was developed for seed production and subsequent efficient cropping. The high correlation between artemisinin content for greenhouse and field plants showed that greenhouse evaluation should be an efficient procedure to estimate artemisinin content of field-grown plants. Artemisinin content for field-grown plants was either 36% higher or similar to that of greenhouse-grown plants. Greenhouse evaluation of *A. annua* under long days has been proved an efficient system to select for high artemisinin clones. The high artemisinin producing clones are characterized as tall robust plants with long internodes, open branching, dense leaves and thick stems [24]. The superior clones can be later intercrossed under short days to achieve genetic gain. While tissue cultures may be used to store and preserve clones, evaluation of artemisinin content from tissue-cultured plants is an unreliable procedure to estimate artemisinin content [60]. The wide variation

in artemisinin content of *A. annua* suggests a genetic basis for variation. A germplasm collection could be a useful source of genetic material for plant selection and breeding based upon artemisinin content. Genetic improvement of *A. annua* is difficult because the flowers are minuscule (ca. 1 mm) and systems for controlled pollination have not been devised. In addition, *A. annua* is a determinate species, which dies after seed set. Thus, plants with high artemisinin content cannot be maintained after flowering and their seeds will be the result of open pollination. Furthermore, seeds are rarely produced by self-fertilization [60], which infers self-incompatibility.

Genetic engineering of *A. annua* plants

Attempts to transform the *A. annua* plants using *A. rhizogenes* [35, 59, 63, 88] resulted in transformed hairy roots, but no transgenic plants. Shooty teratomas of *A. annua* were established by infecting stem tissue with a wild-type *A. tumefaciens* nopaline strain for studying secondary metabolisms, but no specified protocol is given [57, 59]. Until now the only successful procedure to regenerate and transform *A. annua* plants in a sufficient short time is established by Vergauwe *et al*. [84]. The transgenic plants were not different in appearance from the normally grown plants.

Future prospectives

Referring to artemisinin and its related endoperoxides, Weathers stated 'Genetic transformation and environmental conditions that favor biomass accumulations, followed by stress-induced product synthesis may increase product yields to the level where this valuable medicinal compound can be made available at an affordable price' [88]. So far, the manipulation of culture media, culture conditions and phytohormone levels have, usually, failed to permit commercial production of those phytochemicals useful in medicine and industry. This almost certainly reflects the lack of understanding of basic secondary metabolic regulation in cultured plant cells. For artemisinin a thorough study of the enzymatic pathway is very important. Enzymes involved in the artemisinin biosynthesis have to be isolated and characterized. Genes encoding these enzymes must be stimulated in the *A. annua* L. plants. Furthermore, the regulation of this pathway is still unresolved and it is not certain that all the products of the artemisinin biosynthesis are already fully known. The transformation procedure developed by Vergauwe *et al.* allows to manipulate the biosynthetic pathway of artemisinin in *A. annua* plants [84]. Transgenic plants, with sense or antisense constructs of important enzymes for the artemisinin production, will then be screened for their artemisinin production.

Acknowledgements

This research was supported by the National Fund for Scientific Research, Grant 9.0034.93. A.V. is a Research Aspirant of the National Fund for Scientific Research (Belgium).

References

1. Acton N, Klayman DL: Artemisitene, a new sesquiterpene lactone endoperoxide from *Artemisia annua*. Planta Med 5: 441–442 (1985).
2. Acton N, Klayman DL, Rollmann IJ: Reductive electrochemical HPLC assay for artemisinin (qinghaosu). Planta Med 5: 445–446 (1985).
3. Acton N, Klayman DL, Rollmann IJ, Novotny JF: Isolation of artemisinin (qinghaosu) and its separation from artemisitene using the Ito multilayer coil separator-extractor and isolation of arteannuin B. J Chromatogr 355: 448–450 (1986).
4. Acton N, Roth RJ: On the conversion of dihydroartemisinic acid into artemisinin. J Org Chem 57: 3610–3614 (1992).
5. Akhila A, Thakur RS, Popli SP: Biosynthesis of artemisinin in *Artemisia annua*. Phytochemistry 16: 1927–1930 (1987).
6. Akhila A, Kumkum R, Thakur RS: Biosynthesis of artemisinic acid in *Artemisia annua*. Phytochemistry 29: 2129–2132 (1990).
7. Anon: Qinghaosu Antimalarial Coordinating Research Group. Antimalarial studies on qinghaosu. Chinese Med J 92: 811–816 (1979).
8. Anon: Rediscovering wormwood: Qinghaosu for malaria. Lancet 339: 649–651 (1992).
9. Avery MA, Chong WKM, Jennnings-White C: Stereoselective total synthesis of (dextro)-artemisinin, the antimalarial constituent of *Artemisia annua* L. J Am Chem Soc 114: 974–979 (1992).
10. Banthorpe DV, Brown GD: Two unexpected coumarin derivatives from tissue cultures of Compositae species. Phytochemistry 28: 3003–3007 (1989).
11. Charles DJ, Simon JE, Wood KV, Heinstein P: Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. J Nat Prod 53: 157–160 (1990).
12. Charles DJ, Cebert E, Simon JE: Characterization of the essential oil of *Artemisia annua* L. J Ess Oil Res 3: 33–39 (1991).
13. Chan KL, Teo CKH, Jinadasa S, Yuen KH: Selection of high artemisinin yielding *Artemisia annua*. Planta Med 61: 285–287 (1995).

14. Chen FT, Zhang GH: Studies on several physiological factors on artemisinin synthesis in *Artemisia annua* L. Plant Physiol 5: 26–30 (1987).
15. Chiung-Sheue Chen Liu K, Yang SL, Roberts MF, Elford BC, Phillipson JD: Antimalarial activity of *Artemisia annua* flavonoids from whole plants and cell cultures. Plant Cell Rep 11: 637–640 (1992).
16. Delabays N, Benakis A, Collet G: Selection and breeding for high artemisinin (qinghaosu) yielding strains of *Artemisia annua*. Acta Hort 330: 203–207 (1993).
17. Duke SO, Vaughn KC, Croom EM, ElSohly HN: Artemisinin, a constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. Weed Sci 35: 499–505 (1987).
18. Duke SO, Paul RN: Development and fine structure of the glandular trichomes of *Artemisia annua* L. Int J Plant Sci 154: 107–118 (1993).
19. Duke MV, Paul RN, ElSohly HN, Sturtz G, Duke SO: Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int J Plant Sci 155: 365–372 (1994).
20. Dung NG, Loi DT: Selection of traditional medicines for study. J Ethnopharmacol 32: 57–70 (1991).
21. ElDomiaty MM, AlMeshal IA, ElFeraly FS: Reversed-phase HPLC determination of artemisitene in artemisinin. J Liq Chromatogr 14: 2317–2330 (1991).
22. ElFeraly FS, AlMeshal IA, AlYahya MA, Hifnawy MS: On the possible role of qinghao acid in the biosynthesis of artemisinin. Phytochemistry 25: 2777–2778 (1986).
23. ElFeraly FS, AlMeshal IA, Khalifa SI: *Epi*-deoxyarteannuin B and 6,7-dehydroartemisinic acid from *Artemisia annua*. J Nat Prod 52: 196–198 (1989).
24. Elhag HM, ElDomiaty MM, ElFeraly FS, Mossa JS, ElOlemy MM: Selection and micropropagation of high artemisinin producing clones of *Artemisia annua* L. Phytother Res 6: 20–24 (1992).
25. ElSohly HN, Croom EM, ElSohly MA: Analysis of the antimalarial sesquiterpene artemisinin in *Artemisia annua* by high-performance liquid chromatography (HPLC) with post-column derivatization and ultraviolet detection. Pharmacol Res 4: 258–260 (1987).
26. ElSohly HN, Croom Jr EM, ElFeraly FS, ElSherei MM: A large-scale extraction technique of artemisinin from *Artemisia annua*. J Nat Prod 53: 1560–1564 (1990).
27. Ferreira JFS, Simon JE, Janick J: Relationship of artemisinin content of tissue cultured, greenhouse grown, and field grown plants of *Artemisia annua*. Planta Med 61: 351–355 (1995).
28. Ferreira JFS, Simon JE, Janick J: Developmental studies of *Artemisia annua* L.: flowering and artemisinin production under greenhouse and field conditions. Planta Med 61: 167–170 (1995).
29. Fulzele DP, Sipahimalani AT, Heble MR: Tissue cultures of *Artemisia annua*: organogenesis and artemisinin production. Phytother Res 5: 149–153 (1991).
30. Fulzele DP, Heble MR, Rao PS: Production of terpenoid from *Artemisia annua* L. plantlet cultures in bioreactor. J Biotechnol 40: 139–143 (1995).
31. Haynes RK, Vonwiller SC: The development of new peroxidic antimalarials. Chem Aust 64–67 (1991).
32. He XC, Zeng MY, Li GF, Liang Z: Callus induction and regeneration of plantlets from *Artemisia annua* and changes in qinhaosu contents. Acta Bot Sinica 25: 87–90 (1983).
33. Hien TT, White NJ: Qinghaosu. Lancet 341: 603–608 (1993).
34. Jaziri M, Diallo B, Vanhaelen M, Homès J, Yoshimatsu K, Shimonura K: Immunodetection of artemisinin in *Artemisia annua* cultivated in hydroponic conditions. Phytochemistry 33: 821–826 (1993).
35. Jaziri M, Shimonura K, Yoshimatsu K, Fauconnier ML, Marlier M, Homès J: Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. J Plant Physiol 145: 175–177 (1995).
36. Jha S, Jha TB, Mahato SB: Tissue culture of *Artemisia annua* L.: a potential source of an antimalarial drug. Curr Sci India 57: 344–346 (1988).
37. Jung M, ElSohly HN, Croom EM: Practical conversion of artemisinic acid into desoxyartemisinin. J Org Chem 51: 5417–5419 (1986).
38. Jung M, ElSohly HN, McChesney JD: Artemisinic acid: a versatile chiral synthon and bioprecursor to natural products. Planta Med 56: 624 (1990).
39. Kim NC, Kim SU: Biosynthesis of artemisinin from 11,12-dihydroarteannuic acid. J Korean Agric Chem Soc Rev 35: 106–109 (1992).
40. Klayman DL, Lin AJ, Acton N, Scovill JP, Hoch JM, Milhous WK, Theodarides AD, Dobek AS: Isolation of artemisinin (Qinghaosu) from *Artemisia annua* growing in the United States: J Nat Prod 47: 715–717 (1984).
41. Klayman DL: Weeding out malaria. Nat Hist 10: 18–26 (1989).
42. Klayman DL: *Artemisia annua*: from weed to respectable antimalarial plant. In: Kinhorn AD, Balandrin MF (eds) Human Medicinal Agents from Plants, pp. 242–255. American Chemical Society Symposium Series. ACS, Washington, DC (1993).
43. Kudakasseril GJ, Lam L, Staba EJ: Effect of sterol inhibitors on the incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by a cell-free system from *Artemisia annua* tissue cultures and plants. Planta Med 53: 201–502 (1987).
44. Landbury PT, Nowak DM: An efficient partial synthesis of (+)-artemisinin and (+)-deoxoartemisinin. Tetrahedron Lett 33: 1029–32 (1992).
45. Laughlin JC: Effect of agronomic practices on plant yield and antimalarial constituents of *Artemisia annua* L. Acta Hort 311: 53–61 (1993).
46. Laughlin JC: Agricultural production of artemisinin: a review. Trans Royal Soc Trop Med Hyg 88 (Suppl. 1): 21–22 (1994).
47. Li Y, Yang ZX, Chen YX, Zhang X: Synthesis of ($^{15-14}$C) labelled artemisinin. Yaoxuebao 29: 713–716 (1994).
48. Liersh R, Soicke H, Stehr C, Tüllner HU: Formation of artemisinin in *Artemisia annua* during one vegetation period. Planta Med 52: 387–390 (1986).
49. Lin AJ, Klayman DL, Hoch JM, Silverton JV, George CF: Thermal rearrangements and decomposition products of artemisinin (qinghaosu) 1. J Org Chem 50: 4504–4508 (1985).
50. Looareesuwan S: Overview of clinical studies on artemisinin derivatives in Thailand. Trans Royal Soc Trop Med Hyg 88: S1/9 (1994).
51. Luo XD, Shen CC: The chemistry, pharmacology, and clinical applications of qinghaosu (artemisinin) and its derivatives. Med Res Rev 7: 29–52 (1987).
52. Martinez BC, Staba EJ: The production of artemisinin in *Artemisia annua* L. tissue cultures. Adv Cell Cult 6: 69–87 (1988).
53. Miller LH: The challenge of malaria. Science 257: 36–37 (1992).
54. Morales MR, Charles DJ, Simon JE: Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Hort 344: 416–420 (1993).

55. Nair MSR, Acton N, Klayman DL, Kendrick K, Basile DV, Mante S: Production of artemisinin in tissue cultures of *Artemisia annua*. J Nat Prod 49: 504–507 (1986).
56. Nair MSR, Basile DV: Bioconversion of arteannuin B to artemisinin. J Nat Prod 56: 1559–1566 (1993).
57. Paniego NB, Maligne AE, Giulietti AM: *Artemisia annua*: *in vitro* culture and the production of artemisinin. In: Bajaj YPS (ed) Medicinal and Aromatic Plants. Biotechnology in Agriculture and Forestry Vol 5, pp. 70–78. Springer-Verlag, Springer (1993).
58. Paniego NB, Giuletti AM: *Artemisia annua* L.: dedifferentiated and differentiated cultures, Plant Cell Tiss Org Cult 36: 163–168 (1994).
59. Paniego NB, Giuletti AM: Artemisinin production by *Artemisia annua* L.-transformed organ cultures. Enzyme Microb Tech 18: 526–530 (1996).
60. Peter-Blanc C: Developpement et biologie de la reproduction de l'*Artemisia annua* L. Travail de diplome, Université de Lausanne, Suisse (1992).
61. Phillipson DJ, Wright CW: Antiprotozoal agents from plant sources. Planta Med 57 (Suppl 1): S53–S59 (1991).
62. Pras N, Visser JF, Batterman S, Woerdenbag HJ, Malingré TM, Lugt CB: Laboratory selection of *Artemisia annua* L. for high artemisinin yielding types. Phytochem Anal 2: 80–83 (1991).
63. Qin MB, Li GZ, Yun Y, Ye HC, Li GF: Induction of hairy root from *Artemisia annua* with *Agrobacterium rhizogenes* and its culture *in vitro*. Acta Bot Sinica 36 (suppl.): 165–170 (1994).
64. Ranashige A, Sweatlock JD, Cooks RG: A rapid screening method for artemisinin and its congeners using ms/ms: search for new analogs in *Artemisia annua* L. J Nat Prod 56: 552–563 (1993).
65. Ravindranathan T, Anil Kumar M, Menon RB, Hiremath SV: Stereoselective synthesis of artemisinin+. Tetrahedron Lett 31: 755–758 (1990).
66. Roth RJ, Acton N: Isolation of arteannuic acid from *Artemisia annua*. Planta Med 53: 501–502 (1987).
67. Roth RJ, Acton N: The isolation of Sesquiterpenes from *Artemisia annua*. J Chem Educ 66: 349 (1989).
68. Roth RJ, Acton NA: A simple conversion of artemisinic acid into artemisinin. J Nat Prod 52: 1183–1185 (1989).
69. Roth RJ, Acton N: A facile semisynthesis of the antimalarial drug qinghaosu. J Chem Educ 68: 612 (1991).
70. Sangwan RS, Agarwal K, Luthra R, Thakur RS, Singh-Sangwan N: Biotransformation of arteannuic acid into arteannuin-B and artemisinin in *Artemisia annua*. Phytochemistry 34: 1301–1302 (1993).
71. Schmid G, Hofheinz W: Total synthesis of qinghaosu. J Am Chem Soc 105: 624 (1983).
72. Shukla A, Abad Farooqi AH, Shukla YN, Sharma S: Effect of triacontanol and chlormequat on growth, plant hormones and artemisinin yield in *Artemisia annua* L. Plant Growth Regul 11: 165–171 (1992).
73. Simon JE, Charles D, Cebert E, Grant L, Janick J, Whipkey A: *Artemisia annua* L.: a promising aromatic and medicinal. In: Janick J, Simon JE (eds) Advances in New Crops, pp. 522–526. Timber Press, West Lafayette, (1990).
74. Singh A, Kaul VK, Mahajan VP, Singh A, Misra LN, Thakur RS, Husain A: Introduction of *Artemisia annua* in India and isolation of artemisinin, a promising antimalarial drug. Indian J Pharmaceut Sci 48: 137–138 (1986).
75. Singh A, Vishwakarma RA, Husain A: Evaluation of *Artemisia annua* strains for higher artemisinin production. Planta Med 54: 475–476 (1988).
76. Sipahimalani AT, Fulzele DP, Heble MR: Rapid method for the detection and determination of artemisinin by gas chromatography. J Chromatogr 538: 452–455 (1991).
77. Sumita J, Timir BJ, Shashi BM: Tissue culture of *Artemisia annua* L.: a potential source of an antimalarial drug. Curr Sci India 57: 344–346 (1988).
78. Tawfiq NK, Anderzson LA, Roberts MF, Phillipson JD, Bray DH, Warhurst DC: Antiplasmodial activity of *Artemisia annua* plant cell cultures. Plant Cell Rep 8: 425–428 (1989).
79. Theoharides AD, Smyth MH, Ashmore RW, Halverson JM, Zhou ZM, Ridder WE, Lin AJ: Determination of dihydroqinghaosu [dihydroartemisinin] in blood by pyrolysis gas chromatography-mass spectrometry. Anal Chem 60: 115–120 (1988).
80. Theoharides AD, Peggins JO, Brewer TG, Melendez V, Boyd M: Automated sample deoxygenation and injection using a programmable autoinjector and valve-switching apparatus for HPLC with reductive electrochemical detection. LC-GC 7: 925–928 (1989).
81. Trigg PI: Qinghaosu (Artemisinin) as an antimalarial drug. Econ Med Plant Res 3: 20–55 (1990).
82. Tu YY, Ni MY, Zhong YR, Li LN, Cui SL, Zhang MQ, Wang YZ, Ji Z, Liang XT: Studies on the constituents of *Artemisia annua* part II. Planta Med 44: 143–145 (1982).
83. Vandenberghe DR, Vergauwe AN, Van Montagu M, Van den Eeckhout EG: Simultaneous determination of artemisinin and its bioprecursors in *Artemisia annua*. J Nat Prod 58: 798–803 (1995).
84. Vergauwe A, Cammaert R, Vandenberghe D, Genetello C, Van Montagu M, Van den Eeckhout E: *Agrobacterium tumefaciens*-mediated transformation of *Artemisia annua* L. and regeneration of transgenic plants. Plant Cell Rep, in press (1996).
85. Vonwiller SC, Haynes RK, King GK, Wang H: An improved method for the isolation of qinghao (artemisinic) acid from *Artemisia annua*. Planta Med 59: 562–563 (1993).
86. Wang CW: The forests of China, with a survey of grassland and desert vegetations. Harvard University Maria Moors Cabot Foundation No 5, pp. 171–187. Harvard University Cambridge, MA (1961).
87. Wang Y, Xia Z, Zhou F, Wu Y, Huang J: Xuaxue Xueabo 46: 1152 (1988); Chem Abstr 110: 111841j (1988).
88. Weathers PJ, Cheetham RD, Follansbee E, Theoharides K: Artemisinin production by transformed roots of *Artemisia annua*. Biotechnol Lett 16: 1281–1286 (1994).
89. Whipkey A, Simon JE, Charles DJ, Janick J: *In vitro* production of artemisinin from *Artemisia annua* L. Phytother Res 1: 15–25 (1992).
90. WHO, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (1994–1995). The role of artemisinin and its derivatives in the current treatment of malaria. Report of an Informal Consultation convened by WHO in Geneva, 27–29 September 1993.
91. Woerdenbag HJ, Pras N, Bos R, Visser JF, Hendriks H, Malingré ThM: Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. Phytochem Anal 2: 215–219 (1991).
92. Woerdenbag HJ, Lüers JFJ, Van Uden W, Pras N, Malingré Th, Alfermann AW: Production of the new antimalarial drug in shoot cultures of *Artemisia annua* L. Plant Cell Tiss Org Cult 32: 247–257 (1993).
93. Woerdenbag HJ, Pras N, Gia Chan N, Bang BT, Bos R, Van Uden W, Van Boi, Nguyen, Batterman S, Lugt CB: Artemisin-

in, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. Planta Med 60: 272–275 (1994).

94. Xu XX, Zhu J, Huang DZ, Zhou WS: Total synthesis of arteannuin and deoxyarteannuin. Tetrahedron 42: 819–28 (1986).
95. Zaman SS, Sharma RP: Some aspects of the chemistry and biological activity of artemisinin and related antimalarials. Heterocycles 32: 1593–1638 (1991).
96. Zhao SS, Zeng M: Spectrometric high pressure liquid chromatography (HPLC) studies on the analysis of Qinghaosu. Plant Med J Med Plant Res 3: 233–237 (1985).
97. Zhao KC, Liu CX, Liang XT, Mingguang Y, Song ZY: Development of radioimmunoassay for determination of artesunate, a derivative of the antimalarial qinghaosu. Proc CAMS PUMC 1: 213–217 (1986).
98. Zheng M, Li L, Chen S: Chemical transformations of qinghaosu, a peroxidic antimalarial. Tetrahedron 36: 2941–6 (1983).
99. Zhou ZM, Anders JC, Chung H, Theoharides AD: Analysis of artesunic acid and dihydroQHS in blood by HPLC with reductive EC detection. J Chromatogr 414: 77–90 (1987).
100. Zhou WS, Xu XX: The structures, reactions and syntheses of arteannuin (Qinghaosu) and related compounds. In: Atta UR, Rahman (eds) Studies in Natural Products Chemistry, pp. 495–527. Elsevier, New York (1988).
101. Zhou Z, Huang Y, Xie G, Sun X, Wang Y, Fu L, Jian H, Guo X, Li G: HPLC with polarographic detection of artemisinin and its derivatives and application of the method to the pharmacokinetic study of artemether. J Liq Chromatogr 11: 1117–1137 (1988).