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# MINIREVIEW

# CURRENT STATUS OF ARTEMISININ AND ITS DERIVATIVES AS ANTIMALARIAL DRUGS

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## Summary

Artemisinin is a promising and a potent antimalarial drug, which meets the dual challenge posed by drug-resistant parasites and rapid progression of malarial illness. This review article focuses on the progress achieved during the last years in the production of artemisinin from *Artemisia annua*. The structure, biosynthesis and analysis of artemisinin and its mode of action are described. The review also focuses on clinical studies, toxicity studies, pharmcokinetics and activity of artemisinin related compounds. The production strategies including organic synthesis, extraction from plants, in vitro cultures and alternative strategies for enhancing the yields are also discussed.

*Key Words*: artemisinin, antimalarial, biosynthesis, pharmacology, tissue culture, *Artemisia annua*

## Introduction

*Artemisia annua*, a plant belonging to Asteraceae family, is an annual herb native to China and occurs naturally as a part of steppe vegetation in northern parts of Chahar and Suiyan province ($40^0$ N, $109^0$E) in China at 1000 - 1500 m above the sea level (1). The plant now grows in many countries such as Australia, Argentina, Bulgaria, France, Hungary, Italy, Spain and United States. It is single stemmed with alternate branches reaching more than 2 meters in height. Its leaves are deeply dissected and range from 2-5 cm in length. *Artemisia* is used for the crafting of aromatic wreaths (2), as a source of essential oils used in flavouring of vermouth (3) and also as a source of artemisinin, a potent anti-malarial drug. This herb has been used for malaria therapy in China for over 1000 years. The earliest record dates back to 2000 years, in the "Fifty Two Prescriptions" unearthed from the Mawangdui Han Dynasty Tombs. Its antimalarial application was first described in *Zhouhou Beiji Fang* (" The Handbook of Prescriptions for Emergencies"), edited in the middle of fourth century by Ge Hong.

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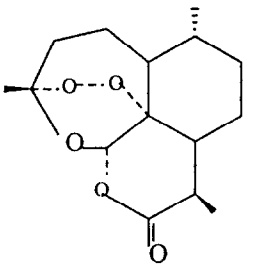

FIG. 1.

Artemisinin

Artemisinin or qinghaosu, an endoperoxide sesquiterpene lactone produced by aerial parts of *Artemisia annua* L., is effective even against multi-drug resistant strains of the malarial parasite. The isolation and characterisation of artemisinin from *Artemisia annua* is considered as one of the most novel discoveries in recent medicinal plant research. It was isolated from the plant in 1972 (4) and in 1979, its structure was determined by X-ray analysis (5). It has an empirical formula of $C_{15}H_{22}O_5$. Artemisinin has a peroxide bridge to which its antimalarial properties are attributed. It has a unique structure and lacks nitrogen containing heterocyclic ring, which is found in most anti-malarial compounds. Artemisinin is an odourless, colourless compound and forms crystals with melting point of 156 - 157 $^0$C. The molecular weight as determined by high resolution mass spectroscopy is m/e 282.1742 m+ (6).

## Biosynthesis of Artemisinin

Knowledge of the biosynthetic pathway of artemisinin, the distribution of precursors and closely related compounds is very useful and may aid in increasing the drug yield. Hence, several studies have been directed to elucidate the biosynthesis of artemisinin. The complete biosynthetic pathway for artemisinin and some of its precursors has not yet been established. Some biotransformation steps have been elucidated in vitro and in vivo Wang Yu et al., (1988) proposed an outline of the pathway of artemisinin biosynthesis (7). Artemisinic acid plays an important role in the biosynthetic pathway of artemisinin (8). Mevalonic acid serves as a primary precursor for the biosynthesis of artemisinic acid, which in turn is the precursor for artemisinin and co-occurring arteannuin B. Haung Jung et al., (1988) and Akhila et al., (1987, 1990) studied the complete pathway of artemisinin biosynthesis (9, 10, 11). The following pathway was postulated: farnesylpyrophosphate, germacrane skeleton, dihydrocoshynolide, cadinanolide, arteannuin B, artemisinin (Fig. 2). The interconversion of artemisinin to artemisitene and vice versa can also occur due to the presence of an oxido-reductase system. Akhila's studies did not indicate artemisinic acid as a precursor for artemisinin. But, after feeding $C^{14}$ labelled artemisinic acid to *Artemisia annua* twigs, radioactivity was observed in both arteannuin B and artemisinin suggesting that artemisinic acid is a common precursor for both arteannuin B and artemisinin (12). El-feraly et al., 1986; Roth and Acton 1987, 1989; Jung et al., 1990; Kim and Kim, 1992 too considered artemisinic acid to be a possible biogenetic precursor of these sesquiterpene lactones sequentially or independently (13, 14, 15, 16, 17).

Arteannuin B is considered as another precursor for artemisinin (15, 18). Brown in 1993, isolated two compounds, secocadinane and dihydroxycadinanolide in relation with artemisinin biosynthesis. The isolation of these compounds suggested an alternative biosynthetic route for artemisinin. According to this, arteannuin B gets converted into dihydroxycadinanolide which then undergoes Grobs fragmentation to yield an enol form of sesocadinane. Sesocadinane then undergoes enzymatic

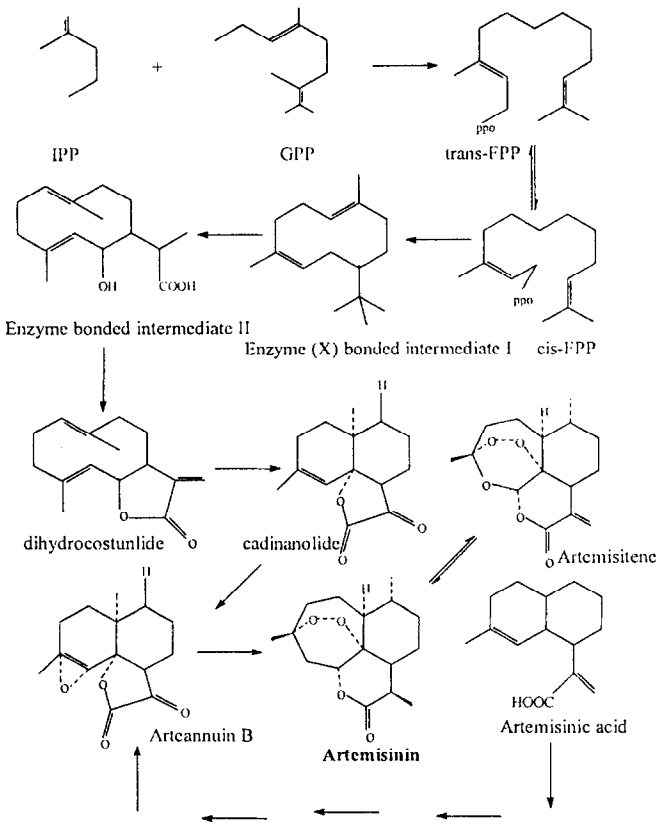


FIG. 2.

Proposed pathways for artemisinin biosynthesis.

oxygenation to yield artemisitene, which is reduced to artemisinin. This hypothesis (Fig. 3) is not supported by any biochemical evidence although it is chemically feasible (19).

Arteannuin B is considered to be an intermediate in the bioconversion of artemisinic acid to artemisinin, because it was converted to artemisinin using crude and semi-purified cell free extracts of leaf homogenates of *Artemisia annua* (20). El-Feraly et al., (1986), converted artemisinic acid to arteannuin B, by singlet oxygen generated through dye-sensitised photooxidation (13), Wang et al., (1988) converted artemisinic acid to both arteannuin B and artemisinin (7). Kim and Kim (1992), reported the transformation of dihydroartemisinic acid into artemisinin by *A. annua* tumour cell free extracts (17). However, these extracts failed to transform artemisinic acid into dihydroartemisinic acid, both of which are found in crude plant extracts. Li et al., (1994) reported synthesis of [$^{15-14}$C] labelled artemisinin in a supernatant liquid prepared from the tender leaves of ripe *Artemisia annua* with the addition of [$^{15-14}$C] dihydroartemisinic acid as starting compound (21).

Staba's group reported the conversion of IPP to both arteannuin B and artemisinin (22, 23). Sangwan et al.,(1993) achieved in vivo and in vitro transformation of artemisinic acid to arteannuin B and artemisinin using an in vitro system. Artemisinic acid was transformed into arteannuin B (1.58%) and artemisinin (3.59%). Incorporation of $Fe^{2+}$, 2-oxoglutarate and NADPH in the in vitro system improved the transformation of artemisinic acid to artemisinin. Horseradish peroxidase in the presence

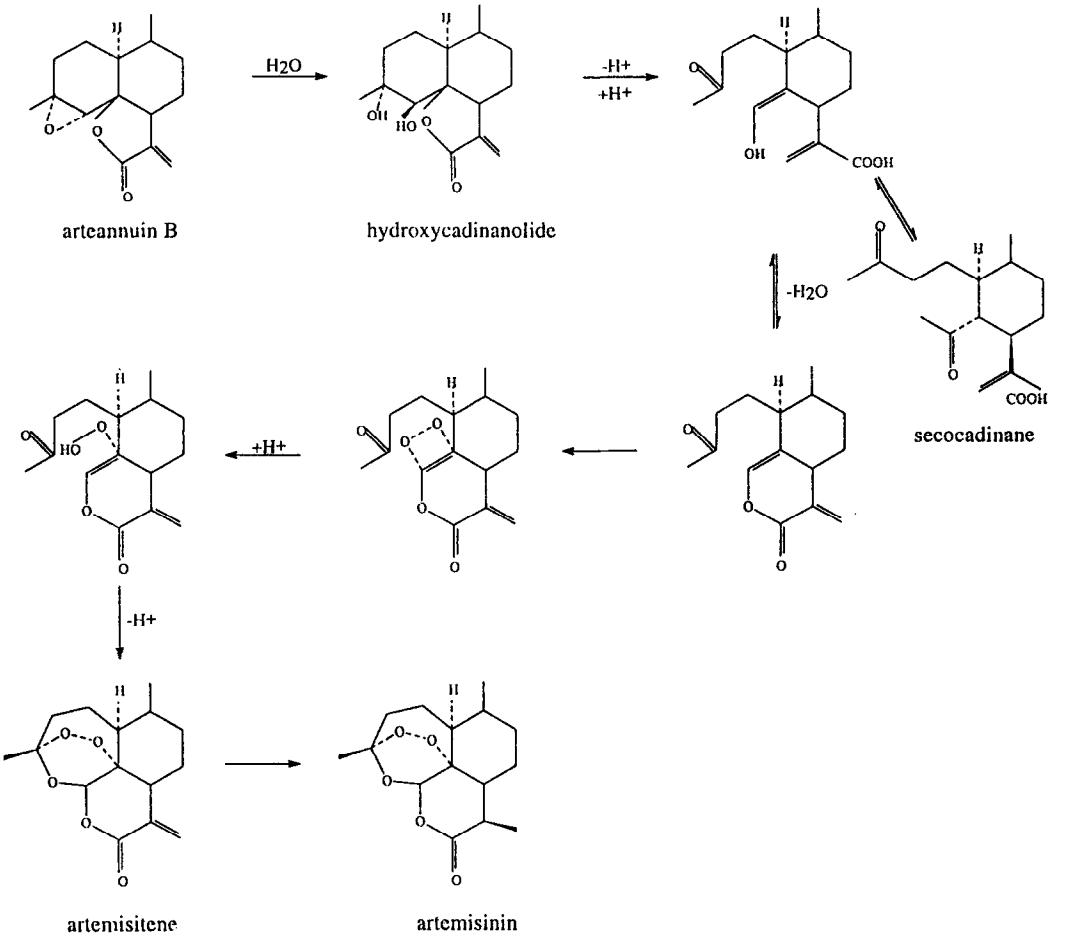


FIG. 3.

Proposed biosynthesis of artemisinin as suggested by Brown.

of $H_2O_2$ further enhanced the yields of arteannuin B and artemisinin to 4.68 and 7.19% respectively (12). Peroxidases are known to catalyse a variety of reactions in the metabolism of natural products such as oxidation of substrates with $H_2O_2$ and introduction of oxygen into a substrate (24). However, Woerdenbag et al., (1992) reported that artemisinin rapidly decomposed when incubated in medium containing peroxidase activity, indicating its instability towards these enzymes (25).

Currently the biosynthetic pathways postulated by various groups are contradictory. Intermediate products or enzymes after FPP and before artemisinic acid, arteannuin B, and artemisinin, have not been isolated in vivo. Therefore, further studies are required to understand the whole pathway.

## Analytical Procedures

Analytical methods to assay artemisinin, its biosynthetic precursors as well as metabolites, have been developed, further improved and optimized to be applied for plant material as well as for biological fluids. Analysis of artemisinin is a challenging problem as the compound is unstable, the

concentrations in the plants are low, the intact molecule has poor staining characteristics and other constituents of the plant interfere with the detection.

Various conventional and unconventional methods have been proposed and assessed to detect and quantify artemisinin. Conventional methods include Thin layer chromatography (TLC) (6, 15, 26, 27, 28, 29), gas chromatography (GC) (30, 31) and GC combined with mass spectrometry (32, 33, 34) and tandem mass spectrometry MS\MS (35), HPLC with UV detection (29, 36, 37, 38, 39) and with electrochemical detection [HPLC-EC](40, 41, 42, 43). Radio immuno assay [RIA] (44) and Enzyme linked immunosorbant assay [ELISA] (45) comprise the unconventional techniques to detect artemisinin.

Thin layer chromatography is not a reliable technique to quantify artemisinin due to the poor staining characteristics of the intact molecule and interference with other constituents of the plants. TLC is useful as an assay method only after a tedious chromatographic enrichment. Gas chromatography also has been applied for the analysis of artemisinin. However, artemisinin is a thermolabile compound and decomposes on the column. Thermal stability studies have indicated that artemisinin is stable upto $150^0$C but degrades into number of products when heated at 180 - $200^0$ C (46, 47). Sipahimalani .et al., (1991) presented a simple and rapid method for indirect detection and determination of artemisinin at nanogram levels from both plant and plant tissue cultures (31). It 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. However, no complete separation of arteannuin B, when present in plant extracts, was achieved, since it generates a peak which appears with the same retention time as one of the artemisinin degradation peaks (30, 31, 43). Woerdenbag et al., (1991) reported the separation of artemisinin from arteannuin B through gas chromatography coupled with mass spectroscopy. Chromatographic separations of artemisinin as well as its biosynthetic precursors were obtained (33). Ranasinghe et al., in 1993 reported a rapid screening method based on tandem mass spectroscopy (MS/MS) for detection of artemisinin related compounds (35). High pressure liquid chromatography with ultra violet monitoring has been used but the plethora of crude extract constituents that absorb in the low wavelength region required to detect artemisinin effectively effaces its peak. Moreover, artemisinin needs to be derivatized due to its lack of chromophores. This process can hamper the result by derivatizing the other compounds present in the crude extract. Moreover, artemisinin is sensitive to acid and base treatment (48). The most sensitive way for detecting and quantifying artemisinin in crude plant extract without any molecular breakdown or interference from other related compounds and which does not require any derivatization or sample purification is high pressure liquid chromatography with electrochemical detection [HPLC-EC]. Funk et al., (1980), first demonstrated the enhanced selectivity and sensitivity in detection and quantification of peroxides using HPLC-EC, at negative potential (49). The selective use of HPLC-EC for the assay of artemisinin was originally described by Acton et al., (1985) (40). Ferreira et al., (1994), described the method for improving the efficiency of HPLC-EC by hastening and simplifying the analysis and eliminating base line drift. This improved HPLC-EC method effectively separated and quantified dihydroartemisinin, artemisitene, artemisinin and dihydroartemisinin carboxy methyl ester (43). HPLC-EC measures artemisinin directly because the peroxide moiety undergoes electrochemical reduction. This method is highly sensitive and can detect nanogram levels of artemisinin. It is 100 times more sensitive than HPLC-UV (23). HPLC-EC is also specific for related artemisinin analogues, which have a peroxide bridge. There is no interference of other constituents of plant extract during electrochemical detection overcoming the problems associated with TLC and HPLC-UV methods. However, the reductive electrochemical detection involves very special precautions as molecular oxygen is reduced at the low cathodic potential of -0.8 V (50). However, HPLC-EC method cannot detect compounds lacking the peroxide moiety, viz. arteannuin B. The HPLC-EC method was compared to the gas chromatographic method by Ferreira et al., (1994) (43). Both methods provide good separation of artemisinin from its biosynthetic precursors. HPLC with electrochemical detection is ten times more sensitive than GC for detection of artemisinin. A reversed phase HPLC method for determination of artemisinin has been

developed. This is useful for the routine analysis of artemisinin to check its purity and can also be used for preparative scale purification of these compounds (51). Zhou et al., (1988), reported an HPLC method with polarographic detection of artemisinin and its derivatives (41). Vandenberghe et al., (1995), developed a sensitive and specific reverse-phase HPLC method using EC and UV detection for simultaneous determination and quantification of artemisinin and its bioprecursors (52). Gupta .et al., (1997) reported an HPLC-UV detection method using two different reverse phase ODS and CN columns in series for simultaneous determination of artemisinin, arteannuin B and artemisinic acid (53). Chan et al., (1997) reported a high performance liquid chromatography analysis of plasma artemisinin using a glassy carbon electrode for reductive electrochemical detection (54). Recently, two groups, Sandrenan et al., (1997) and Karbwang et al., (1997) developed an analytical method for the determination of artemether and its metabolite dihydroartemisinin based on HPLC and ECD in reductive mode (55, 56). Navaratnam et al., (1997) and Bangchang et al., (1998) described a simple and a sensitive method of simultaneous determination of artesunic acid, dihydroartemisinin and artesunate, dihydroartemisinin in biological fluids using HPLC-ECD respectively (57, 58).

The conventional methods, in most cases described above, have been specific for artemisinin but are not sensitive enough to detect levels in small samples of plant tissues, from young seedlings and from cell or tissue cultures. Radioimmuno assays [RIA] and Enzyme linked immuno sorbent assays [ELISA] are more sensitive and highly specific. A radioimmunoassay for artemisinin has been described by Song et al., (1985) and Zhao et al., (1986) (59, 44). Dihydroartemisinin-12-O-acetic acid was conjugated with bovine serum albumin and polyclonal antisera obtained by immunizing sheep with this conjugate plus Freund's Complete Adjuvant. The assay was reported to detect 2-3 ng of artemisinin and its active metabolites. Although RIA is more sensitive, the use of radioactive compounds present a series of problems of special acquisition and use requirements, uncertain stability, high cost, health hazards and disposal difficulties. Hence, enzyme immuno assays are being given more importance and are becoming increasingly popular. Ferreira and Janick (1996) developed an enzyme linked immuno sorbent assay for screening of large *Artemisia annua* populations. Artemisinin was derivatized to dihydroartemisinin carboxymethylether in three steps and linked to bovine serum albumin. The artemisinin-BSA conjugates were injected in rabbits to generate polyclonal antibodies. The assay could detect 1.5 ng/ml artemisinin and was 400 fold more sensitive than HPLC-ECD (60). ELISA is as sensitive as RIA, safer and is based on the peroxide bridge for antibody specificity to detect artemisinin and closely related compounds in crude extracts of *Artemisia annua* (45).

However, these unconventional methods are laborious since they involve generation of polyclonal and monoclonal antibodies.

## Pharmacology

Malaria has been known to man since antiquity as a disease spread by the bite of female *Anopheles* mosquito. Human malaria is caused by four major *Plasmodium* species: *falciparum, vivax, malariae,* and *ovale. Plasmodium falciparum,* the most prevalent parasite species across the globe causes cerebral malaria that is often fatal (61). The fight against malaria is ridden with several technical, logistic and economical problems. Despite considerable efforts to eradicate or control malaria, it is still the most prevalent and most devastating disease in the tropics. It threatens about 40% of world's population causing mortality, morbidity, and socio-economic loss. The continuous infestation and spread of resistance to antimalarial drugs among parasites is posing a serious threat of increase in severity of disease and death (4). Immunity to malaria builds up only after several years of recurring infection but is only partially effective. An effective vaccine is the best long term control option for malaria. But till date work on vaccine development remains at pre-clinical stage. Thus, the non-availability of an effective antimalarial vaccine and the increase in occurrence of drug resistant

parasites has necessitated the urgent development of novel antimalarial drugs to control this infection throughout the world.

Antimalarial drugs have a selective action on the different phases of the parasitic lifecycle and may be divided into casual prophylactic drugs which prevent the establishment of the parasite in the liver and blood schizontocidal drugs which attack the parasite in the red blood cell, preventing or terminating the clinical attack. Artemisinin and its derivatives are potent blood schizontocides. They are also gametocytocidal (62, 63, 64, 65). Liver stages of the parasite are not affected (66), so these drugs cannot be used as casual prophylactic agents. They are stage specific. Late stage ring parasites and trophozoites are more susceptible to artemisinin than schizonts or small rings (67, 68, 69).

**Mechanism of Action**

Artemisinin and its derivatives kill the parasites more rapidly than any other antimalarial agent due to its unique pharmacodynamic action. They are toxic to malarial parasite at nanomolar concentrations. It causes structural changes in the erythrocyte stage of parasite by affecting the membranes surrounding the food vacuole, the nucleus, the mitochondria, endoplasmic reticulum and nucleoplasm. Thus, artemisinin localizes in parasite specific membranes. Such changes lead to the formation of autophagus vacuoles and loss of cytoplasm, which kills the parasite (70, 71). The peroxide function in artemisinin and related compounds is vital for antimalarial activity and the results of in vitro studies indicate that the carboxyl function is not necessary for antimalarial activity (72). It is postulated that the killing of malarial parasite by artemisinin and its derivatives is mediated by the production of cytotoxic compounds such as free radicals and reactive aldehydes (73). This is evidenced by the following observations. Artemisinin derivatives lacking the endoperoxide bridge, a known source of oxygen free radicals, are devoid of antimalarial activity (74, 75). Moreover, the in vitro antimalarial activities of artemisinin and artesunate are enhanced by high oxygen tension and by addition of other free radical generating compounds such as doxorubicin, miconazole (76), castecin and artemitin (77). Similarly, antioxidants (free radical scavengers) such as α-tocopherol, catalase, dithiothreitol (76), ascorbate and reduced glutathione (78) block antimalarial activity. Most free radical generating drugs cause "oxidant damage" by producing oxygen free radicals (79). However, artemisinin derivatives affect malarial parasites very differently from other oxidant drugs. Instead of reacting with oxygen and producing large quantities of oxygen-containing free radicals, artemisinin itself becomes a free radical in a reaction catalyzed by iron (80).

The biochemical action of artemisinin depends on two sequential steps (81). The first step, activation, comprises an iron-mediated cleavage of the endoperoxide bridge generating an unstable organic free

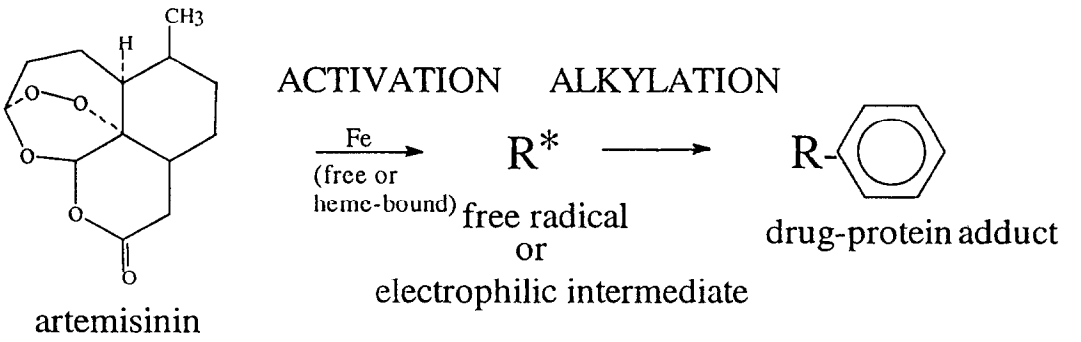


FIG. 4.

Mechanism of action of artemisinin (90).

radical and/or other electrophilic species (82). Iron activates artemisinin into a free radical. This was confirmed by electron paramagnetic resonance spectroscopy (83) and by studies of isolated erythrocyte membranes (84). However, since there are various iron pools in the infected red blood cells, it is not known for sure which ones are responsible for artemisinin activation (81). The observation that chloroquine, which binds to heme (85), antagonizes the antimalarial activity of artemisinin against *Plasmodiun falciparum* (86) suggests that the free heme pool may be important. This is evident by the fact that iron chelators antagonize the antiparasitic effect of artemisinin (83, 87). The second step, alkylation, involves the formation of covalent adducts between the drug and malarial proteins (Fig. 4). Artemisinin forms covalent complex with albumin in vitro. The covalent nature of the complexes has been confirmed by elctrospray mass spectroscopy and SDS-PAGE (88). Radiolabelled artemisinin is taken up by isolated red blood cell membranes while there is no uptake or protein alkylation when artemisinin is incubated with intact erythrocytes (89).

Thus, artemisinin derivatives are free radical generators but are different from "oxidant" free radical-generating drugs in two ways, the resulting free radicals are carbon centred and there are specific target proteins (90).

## Toxicity Studies

The use of artemisinin compounds is appealing not only because of their rapid effect and novel mechanisms but also because of their apparent safety. No serious toxicity has been observed in human patients. Artemisinin, artemether and sodium artesunate show remarkably high $LD_{50}$ values as compared to chloroquine. Artemisinin compounds may have neurotoxic potential at high doses as neurological damage was observed in experiments on dogs and cats. However, to-date there is no evidence that these compounds cause permanent neurological damage in humans (91). Genovese et al., (1998) demonstrated neurotoxicity of arteether in rats by auditory discrimination task and subsequent neurohistology (92). Histological evaluation of the brainstem of rats treated with arteether (3.125-12.5 mg/kg/day, IM, in sesame oil) for 7 consecutive days showed significant neuropathology, including chromatolysis, in the nucleus trapezoideus and nucleus superior olive. Mild neuropathology was also detected in nucleus ruber and other brainstem regions (93). Kamchonwongpaisan et al., (1997) carried out studies on rats treated with high doses of arteether and on mouse neuroblastoma cells (Neu2a) treated with 3H-dihydroartemisinin and reported that while artemisinin derivatives have neurotoxic effects in rats and alkylate proteins in neuroblastoma cells, these effects occurs only at high doses or after prolonged exposure (94). Nontprasert et al., (1998) demonstrated that at low doses (30mg/kg/day) of artesunate and artemether, no abnormalities were observed in adult Swiss albino mice. At dosage of 50 mg/kg/day, abnormalities of balance and equilibrium was observed. However, these abnormalities were reversible. However at high doses of 100 mg/kg/day the effects were irreversible. Also, intramuscular artemether was found to be more neurotoxic than intramuscular artesunate (95). However, recently Price et al., (1998) compared the therapeutic efficacy and toxicity of artesunate with that of artemether in an open randomized trials in 443 Thai patients of recrudescent multidrug resistant falciparum malaria. Both the oral artemisinin derivatives were highly effective, safe and resulted in equivalent therapeutic responses (96). Smith et al., (1997) studied the role of haem in the neurotoxicity of artemisinin derivatives by examining neurite outgrowth measured by image analysis and cellular metabolism of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) measured spectrophotometrically in neuroblastoma cell line NB2a and by examining binding of radiolabelled dihydroartemisinin to NB2a cell and rat brain proteins. In the cases of artemether, dihydroartemisinin and arteether, haemin (ferriprotoporphyrin 1$\alpha$) significantly increased the dose related inhibition of neurite outgrowth from differentiating NB2a cells and significantly increased the dose dependent inhibition of MTT metabolism. There was a two fold increase in the dose related binding of radiolabelled dihydroartemisinin to proteins from NB2a cells and 3-6 fold to rat brain. However, haemin did not enhance the neurotoxicity of desoxyarteether (97). Furthermore, Transmission electron microscopy revealed that dihydroartemisinin damaged NB2a cell

mitochondrial cristae and endoplasmic reticulum. Scanning electron microscopy revealed that dihydroartemisinin depleted the filopodia like processes projecting from the cell body and neurites (98). Thus, this toxicity may be due to an interaction of iron with the endoperoxide bridge of the derivative to produce toxic free radicals and/or other toxic metabolites. The drug induced changes in differentiating NB2a neuroblastoma cells may also have a role in the neurotoxicity of artemisinin derivatives (97, 98).

Few adverse side effects have been reported in humans. Transient heart block was noted in 1/82 patients with artesunate and 3/39 cases with artemether(99). However, no abnormalities were detected when electrocardiographic monitoring was performed for over 24 hours with 53 falciparum malaria patients treated with artemisinin derivatives (100). After oral artesunate the minor side effects reported include fever (25%), tenesumus (6%), abdominal pain (3%) and diarrhoea (1%). In animal studies the incidence of abortion was more in the first trimester (101). McGready, et al., (1998) showed that artesunate and artemether were well tolerated and no drug related adverse effect was observed during the treatment of multidrug resistant falciparum malaria of 83 pregnant women. There was no congenital abnormality in the new born children (102). However, documentation on the safety of artemisinin and its derivatives in the treatment of malaria in pregnant women is currently limited.

No major side effect has been observed even in cases complicated by cardiac, renal and hepatic disorders or by pregnancy. Artemisinin and its derivatives appear to be better alternative to the present anti-malarial drugs for the treatment of severe malaria and offer the advantage that it may cause far fewer reactions at the site of injections (91).

**Artemisinin Derivatives**

*Deoxoartemisinin* Deoxoartemisinin was successfully prepared from artemisinin in one step using $NaBH_4$ and $BF_3Et_{20}$ in THF. It showed 8 fold increased antimalarial activity in vitro against chloroquine resistant malaria as compared to artemisinin (103).

*α-Artelinic acid* α-Artelinic acid is a potent, stable and water-soluble antimalarial agent synthesised from artemisinin. Vishwakarma et al. reported blood schizontocidal antimalarial activity of α-artelinic acid against *Plasmodium knowlesi* (104).

*Artemether* Artemether, a methyl ether derivative of artemisinin was found to be more active than artemisinin (105). Artemisinin is reduced with sodium borohydride to produce dihydroartemisinin as a mixture of epimers. The mixture is treated with methanol and an acid catalyst to provide artemether (106). It is effective in cerebral malaria by intramuscular injection (107).

*Arteether* Arteether is the β-anomer of the ethyl ether of dihydroartemisinin. It was synthesized originally in China by Li et al. in 1981 (108). Brossi et al., in 1988 synthesized arteether by boronhydride reduction of artemisinin to dihydroartemisinin, etherification in boron trifluoride ethyrate, and , finally, separation of anomers (109). The drug has been formulated as a solution in sesame oil for intramuscular use, at a concentration of 160mg/ml for preclinical and clinical studies. Arteether is virtually insoluble in water, but is soluble in 50-100% ethanol in water and in a variety of oils. It has been stable in accelerated stability studies at 50°C for several months (110). It is a potent antimalarial and is very effective against drug sensitive strains of *P. falciparum* in vitro as well as in vivo in the mouse malaria model (109), and *Aotus* and *Rhesus* monkey malaria model (110). Presently, artemether has been registered in 27 countries in the world, including France, Thailand, Union of Myanmar, Pakistan, Sudan, Niger, Cote d'Ivoire, Nigeria etc..

*4-(P-Substituted Phenyl)- 4(R or S)-[10(alpha or beta)- dihydroartemisininoxy] butyric acids* 4-(P-Substituted Phenyl)- 4(R or S)-[10(alpha or beta)- dihydroartemisininoxy] butyric acids, which are dihydroartemisinin derivatives are, potential antimalarial agents with a higher efficacy and longer plasma half life than artesunic or artelinic acid. They are water soluble and have showed 2-10 fold

increase in in vitro antimalarial activity against D-6 and W-2 clones of *Plasmodium falciparum* than artemisinin or artelinic acid (111).

*Artemisinin suppositories* Artemisinin suppositories developed by the Institute of Beijing Materia Medica of the Chinese Academy of Traditional Medicine from micronized artemisinin and water soluble matrix of S-40 Polyethylene glycol monostearate with tween-80 added as suspending agents, are found to be easier to administer, cheap and very effective in treating children with severe malaria (112, 113).

*Artesunate* Artesunate is a water soluble hemisuccinate derivative of artemisinin. It is the only artemisinin analogue that can be given intravenously (114). However, since it is unstable in water, it should be contiguously reconstituted with 5% sodium bicarbonate solution prior to injection. Presently, artesunate is registered in Brazil, China, Ghana, Burma, Thailand and Vietnam (90).

*Arteflene* A synthetic derivative of artemisinin was evaluated extensively against various drug-sensitive and drug-resistant *Plasmodium falciparum* in vitro and *Plasmodium berghei* in mice. The potential therapeutic and prophylactic activities were studied comparatively with chloroquine, mefloquine, quinine as well as artemisinin and the derivatives artemether and artesunic acid. Experimentally arteflene proved to be a highly effective antimalarial drug. The suppressive and prophylactic properties were comparable to chloroquine and superior to artemisinin, artemether and artesunic acid. It was consistently rather more active against drug-resistant than against drug sensitive strains of *Plasmodium falciparum*. Drug interactions in vitro and in vivo with Chloroquine, Mefloquine and Quinine revealed a synergistic effect with arteflene (115).

*Semisynthetic artemisinin trioxanes* Recently, Poshner et al., in 1999 converted artemisinin to a series of C-10 carbon-substituted 10 deoxoartemisinin compounds. The three steps involved lactone reduction, replacement of the anomeric lactol OH by F using diethylaminosulfur trifluoride, and finally boron trifluoride promoted substitution of F by aryl, heteroaryl, and acetylide nucleophiles. All of these C-10 nonacetal, chemically robust, enantiomerically pure compounds have high antimalarial potencies in vitro against *Plasmodium falciparum* malaria parasites, and furans 5a and 5b and pyrrole 7a are showing antimalarial activity also in vivo even when administered to rodents orally (116).

A short description and their available dosage forms have been described in Table 1.

TABLE 1
Artemisinin and Derivatives

| Artemisinin Derivative | Description | Available dosage forms |
|---|---|---|
| Artemisinin | | Oral Capsules / tablets suppositories |
| Artemether | Lipid-soluble β-methyl ether of dihydroartemisinin | Oral Capsules / tablets<br>Oily solution for IM injection |
| Arteether | Lipid-soluble β-ethyl ether of dihydroartemisinin | Oily solution for IM injection (investigational) |
| Artesunate | Water-soluble hemisuccinate of dihydroartemisinin | Oral tablets<br>Powder for IV or IM injection<br>Suppositories (investigational) |
| Dihydroartemisinin | Active metabolite of artemisinin and all currently used derivatives | Oral tablets |

*Abbreviations:* IM = Intramuscular; IV = Intravenous

TABLE 2
Clinical Studies

| Ref. | Place (Year) | Sample Size Age group | Type of Malaria | Nature of Trial | Success rate | Conclusion |
|---|---|---|---|---|---|---|
| 117 | China (1979) | 2150 202 (641 children) | *P. falciparum* *P. vivax* | Randomized | 98% after 7d 85% after 5d 52% after 3d | Recrudescence rate was higher after 3d than 7d therapy |
| 118 | Oxford (1991) | Each group 28 Adults | *P. falciparum* | Randomized Comparison | Not calculated | It is equally effective IV and IM |
| 119 | Brazil (1992) | Each group 88 Adults | *P. falciparum* | Triple blind random controlled | 77% | Effective in combination with tetracycline |
| 120 | Thailand (1991) | Each group 50 Adults | *P. falciparum* | Double blind random controlled | 72% | Duration of treatment determines efficacy |
| 121, 122 | Thailand (1991) | 1000 | *P. falciparum* | Randomized comparative | 92.50% | With Mefloquin it accelerates clinical response |
| 123 | Vietnam (1995) | Not specified | *P. falciparum* | Randomized comparative | 92.5% artesunate 80% quinine | Significantly reduces mortality |
| 124 | India (1995) | 50 | *P. falciparum* | Not specified | 100% after 48 hr alpha-beta-arteether | Well tolerated, Safe, effective and rapidly acting antimalarial. |
| 125 | Gambia (1996) | 576 Children | *P. falciparum* | Open randomized | 79.5% artemether 78.5% quinine | As effective as quinine in treatment of cerebral malaria |
| 126 | Vietnam (1997) | 231 | *P. falciparum* | Double blind Randomized | 84% artemether 65% mefloquine | With mefloquine it increased the efficacy and reduced rate of recrudence |
| 127 | Vietnam (1997) | 175 Adults | *P. falciparum* | Open Randomized Comparative | 95% | No significant difference in efficacy between the 4 treatments |
| 128 | India (1997) | 50 | *P. falciparum* | Open Randomized | 98% | Rapid recovery with no side effects |
| 129 | Thailand (1998) | 443 | *P. falciparum* | Open Randomized | 94% | Safe, highly effective |

## Clinical Studies

Artemisinin and its derivatives have been very effective in treating severe falciparum malaria. These compounds have broader stage specificity of action than other antimalarials. However, the drug is effective only when it is given in a form appropriate for the given clinical situation. A complete data of drug tolerance and its adverse reactions and its success rate should be known. An improved clinical case definition would help to limit over-treatment of malaria and under-diagnosis of other conditions. Hence, clinical studies data plays an important role in determining the effectiveness of the drug and to retard the progression of the disease to its severest form. Various clinical studies conducted in past few years have been summarized in Table 2.

## Pharmacokinetics

Pharmacokinetic studies are needed to plan the drug regimens and make proper drug evaluation. However, there is a paucity of pharmacokinetic information on artemisinin. Artemisinin and its derivatives are primarily metabolized in the liver. In general, these are rapidly absorbed from the gastro intestinal tract with peak plasma levels occurring at 1 hour. These are then quickly hydrolyzed in vivo to dihydroartemisinin and eliminated rapidly from plasma with a half-life ranging from 45

TABLE 3
Pharmacokinetics of Artemisinin and Its Derivatives

| Ref. | Year | Drug & Dose (mg/kg) | Route | Method of assay | $T_{1/2\alpha}$ (h) | $C_{max}$ (ng/ml) | $t_{max}$ (h) | Cl (ml/kg /min) | $t_{1/2\beta}$ (h) |
|---|---|---|---|---|---|---|---|---|---|
| 133 | 1988 | Artemether | | RIA | | | | | |
| | | 3.2 | IM | | - | 1200 | 4.1 | 8.3 | 7.1 |
| | | 6.0 | IM | | - | 900 | 9.0 | 11.7 | 10.9 |
| | | 10.0 | IM | | - | 800 | 7.3 | 11.7 | 13.2 |
| 134 | 1988 | Artemether | | HPLC | | | | | |
| | | 6 | IM | (ECD) | 2.6 | 145 | 5.2 | 38.5 | 7.7 |
| | | 10 | IM | | 2.0 | 224 | 6.3 | 32.3 | 11.1 |
| 135 | 1989 | Artemisinin Suppository | | HPLC (ECD) | | | | | |
| | | 10 | PR | | 110 | 6.7 | - | - | 4.4 |
| 134 | 1988 | Artemisinin | | HPLC | | | | | |
| | | 10 | Oral | (UV) | 170 | 2.5 | - | - | 4.1 |
| 134 | 1988 | Artemisinin Suppository | | HPLC (ECD) | | | | | |
| | | 10 | PR | | 179 | 9.6 | - | - | 4.1 |
| 132 | 1990 | Artemisinin | | HPLC | | | | | |
| | | 5.3 | Oral | (UV) | 0.5 | 260 | 1.0 | 108 | 1.9 |
| 136 | 1986 | Artesunate | | HPLC | | | | | |
| | | 3.3-4.4 | IV | (ECD) | - | 3088 | - | 35.3 | 0.5 |
| 133 | 1988 | Artesunate | | RIA | | | | | |
| | | 2.0 | IV | | | 33000 | - | 15 | 1.4 |
| | | 3.8 | IV | | | 16000 | - | 5 | 0.8 |
| 137 | 1994 | Arteether | | HPLC | | | | | |
| | | 3.6 | IM | (ECD) | 0.6 | - | - | 0.014 | 23.1 |
| 138 | 1997 | Artemisinin | | HPLC | | | | | |
| | | 500 | Oral | (ECD) | 1.55 | - | - | - | 4 |
| 139 | 1998 | Artemisinin | | HPLC | | | | | |
| | | 500 | Oral | (ECD) | 1.16 | 364 | - | - | 2.72 |
| 140 | 1997 | Artemisinin | | | 2.61 | 360 | 1.6 | - | 4.34 |
| | | 250 | Oral | | | | | | |

minutes to 11 hours (90, 101, 130, 131, 132). However, despite considerable research in the past decade, there is very little published information on the pharmacokinetic studies of artemisinin compounds. The available data in pharmcokinetics of artemisinin and its derivatives have been summarized in Table 3.

## Production of Artemisinin

### Synthetic Approach

In view of the limited availability of artemisinin and the demand for more, the synthetic preparation of artemisinin is an attractive proposition. But due to its complex structure, the complete chemical synthesis is very difficult. Several groups of workers have reported the synthesis of artemisinin. Schmid and Hofheinz in 1983 were the first to report the total synthesis of artemisinin. They converted (-)-isopulegol to artemisinin in 13 synthetic steps with an overall yield of 5%, while Xu et al., (1983) achieved the same transformation in 19 steps with an overall yield of 0.24%. In both cases the sequence of synthesis is similar in concept. The key steps, peroxidation and ring closure, are induced by singlet oxygen and acid in each case (141, 142). A new "reconstitutional" synthesis of artemisinin has been reported by Wu et al., (1985) (143). Avery et al., (1987) gave a pathway of synthesis, which involved ozonification of vinylsilane. The key step involves the opening of the ring of a transient silyoxydioxethane to form a labile α-hydroperoxyaldehyde, which undergoes selective cyclization to give artemisinin with a yield of 37% (Fig. 5) (144).

Ravindranathan et al., (1990), described a stereo-selective synthesis of artemisinin from (+) –isolemonene (145). Avery et al., (1992), reported a 10 - step synthetic route with (R)-(+)- pulegone as the starting compound. Another efficient synthesis was reported by Lin et al., (1993) starting from (-)β-Pinene using an intramolecular Diels-Alder approach, the later synthesis was fundamentally different from the approach described by earlier workers. Each procedure for the chemical synthesis of artemisinin requires a final photo oxidation step (46, 146). Synthesis of artemisinin by various methods has been described by Ravindranathan (1994) (147). The de novo synthesis of artemisinin is complex, uneconomical and gives low yields, suggesting that isolation from plants would be a better alternative.

The other alternative of overcoming the disadvantages of complex organic synthetic reactions is the preparation of artemisinin from its closely related biosynthetic precursors. Artemisinin can be easily synthesized from artemisinic acid with an overall yield of 40% (148). The photo oxidation of artemisinic acid has been studied by several groups (13, 149, 150). Artemisinic acid is the most abundant sesquiterpene in *Artemisia annua* which occurs 8-10 folds higher than artemisinin (14, 16) followed by arteannuin B (151). Vonwiller et al., (1993), reported an efficient method for isolation of artemisinic acid from *Artemisia annua* (152). Artemisinic acid can then be converted to artemisinin greatly increasing the yield of artemisinin (142). Jung et al., (1991), proposed synthesis of deoxo artemisinin from artemisinic acid via photo oxidative cyclization (153). From deoxoartemisinin, partial synthesis of artemisinin in two steps has been described (154). Roth and Acton (1991) have also proposed a conversion of artemisinic acid to artemisinin with a 30% yield (155). It has also been proved that the introduction of peroxide bridge oxygen occurs during the triplet oxygen (air) oxidation step (154). In another study, artemisinic acid and arteannuin B could be converted to artemisinin and deoxoartemisinin in four and five reaction steps, respectively (156).

### Biotechnological Approach

In view of plants being the only valid source, a biotechnological approach has been considered as a viable alternative to produce artemisinin. This includes production by tissue cultures, cell free systems and intact plants. The first ever trial of using tissue culture technique for artemisinin production was

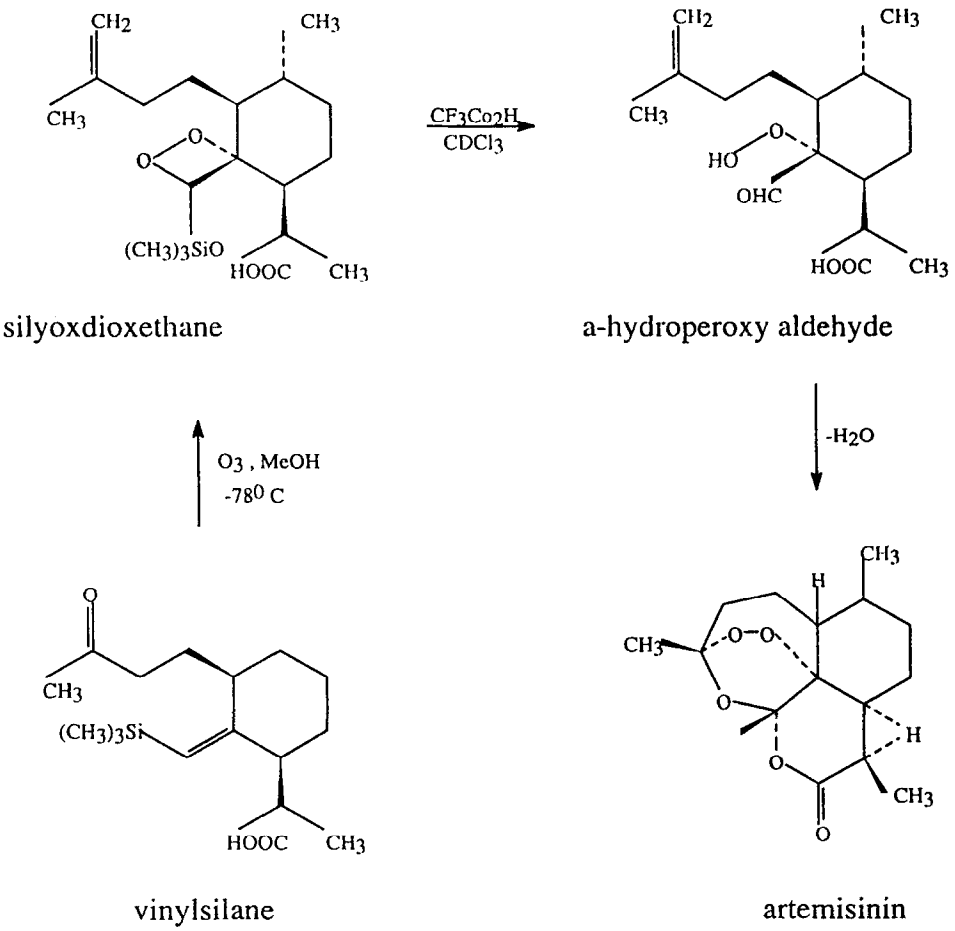


FIG. 5.

Synthesis of artemisinin from vinylsilane.

reported by He et al., (1983) (157). Various other workers have also reported successful in vitro propagation of *Artemisia annua* via shoot cultures (23, 30, 158, 159, 160, 161, 162, 163) Shoots can be easily cultured using standard protocols. Benzyladenine (BA) and coconut water are effective in inducing shoot formation (160). The shoot cultures showed better growth and produced more artemisinin on 2% Sucrose (161, 164). Vishweshwar Rao and Lakshmi Narasu (1998) reported that substitution of sucrose with other carbohydrates or a combination of sucrose and other sugars resulted in differences in growth and artemisinin production (165). Best growth could be observed in the medium supplemented with sucrose or a combination of sucrose and maltose. Least growth was observed when lactose was used instead of sucrose. Use of maltose alone as the carbohydrate source resulted in lowered yields of biomass (166). Callus cultures have been reported in media supplemented with combinations of auxin and cytokinins (17, 23, 163, 165). However, usually non friable callus is obtained. Although, the highest yields of friable callus was obtained with 4.4 μm BA and 4.5 μm 2,4-D but only 10% of the clones generate callus (158). Cell suspension cultures can be obtained with difficulty from callus cultures (161). There have been contradictory reports indicating both the presence and absence of trace amounts of artemisinin in callus and cell cultures. No artemisinin or related sesqueterpenes could be found in the callus cultures initiated from the leaves of selected high artemisinin producing clones (159). No artemisinin or arteannuin B was detected in suspension grown cells. The spent medium was devoid of these compounds. When root initiation occurred from shoot

cultures, active synthesis of arteannuin B and artemisinin was observed (30) suggesting that, a certain degree of differentiation is required for the production of artemisinin (23, 30, 162). The reason for obtaining trace amounts of artemisinin in cell cultures has been attributed to high peroxidase activity in undifferentiated cultures (25). The drug is not secreted into the culture medium but there is an isolated report of low levels of artemisinin being produced in culture medium (158). Paniego and Giulietti (1994) reported the establishment of the dedifferentiated and differentiated cultures of *Artemisia annua*. Yields of the order of 1.13 and 0.78 mg/g DW were obtained from the primary callus. They failed to detect artemisinin in cell suspension cultures and only trace amounts were found in the multiple shoot cultures (167). Woerdenbag et al., (1993), reported a high percentage of artemisinin content (0.16% of dry weight) from shoot cultures using 1/2 MS media supplemented with 0.05 mg/l NAA, 0.2 mg/l BAP and 1% sucrose (161). A concentration of 0.3 mg/lit BAP and 0.1 mg/lit NAA was found to yield highest number of multiple shoots on MS medium (166). Most workers (17, 23, 28, 30, 163) did not detect artemisinin in roots in vitro although Nair et al., (1986), and Jha et al., (1988), reported trace amounts (158, 168). Roots do not contain artemisinin but evidently enhance its production in cultured shoots (23). Removal of roots from shoots cultured in hormone free liquid medium reduced shoot artemisinin by 53% (169). Shoot proliferation in hormone free medium consistently produced roots and contained artemisinin levels as high as 0.28% in some clones (170).

Paniego *et al.* (1993), established shooty teratomas of *Artemisia annua* by infecting the stem tissue with the wild type *Agrobacterium tumefaciens* nopaline strain T 37 (171). Hairy root cultures of *Artemisia annua* have been produced by transformation with *Agrobacterium rhizogenes* MTCC 532 and LBA 9365 (172). Jaziri et al., (1995) reported low levels (0.001% dry wt) of artemisinin in hairy root cultures of *Artemisia annua* (173). Agropine and mannopine have been used as markers to confirm the transgenic nature of hairy roots induction (172, 173, 174). Transformed root cultures in dark did not produce artemisinin. However, artemisinin could be detected in green hairy root cultures (172). Recently, Vanden Eeckhout group studied various parameters such as explant type, age of source of explant, *Agrobacterium tumefaciens* strain and type of binary vector on *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua*. Good correlation between integration and expression of foreign DNA was observed. Different assays showed expression of β-glucuronidase, neomycin phosphotransferase II, superoxide dismutase and bleomycin acetyl transferase. Transgenic plants were obtained with pTJK136 vector from leaf and stem explants of 12-18 week old plants. Stable integration and expression of transgenes was also shown in the progeny (174).

Attempts were also made to improve the artemisinin production by incorporation into the culture medium certain components like casein hydrosylate; plant growth regulators; by precursor feeding; by influencing the artemisinin biosynthesis route or by elicitation. Although it was earlier reported that addition of $GA_3$ (Gibberellic acid) did not affect the artemisinin content of *A. annua* shoot cultures (23, 161, 176), Woerdenbag et al., (1993), reported the converse to be true (161). Casein hydrolysate (source of amino acids and oligopeptides), in low concentration enhances artemisinin production but prolonged exposure negatively affects biomass production and growth of shoots. Vishweshwar Rao (1997) reported that incorporation of boron, casein hydrolysate and gibberellic acid enhanced artemisinin production by 25%, 36% and 65% respectively. However, no effect on growth was observed (166). Stimulation of terpenoid synthesis in plantlet cultures of *Artemisia annua* by addition of $GA_3$ was reported by Fulzele et al., (1995), while other workers found no significant increase in artemisinin content in $GA_3$ - treated plants. However, significant effect on biomass production in plants treated with 40 and 80 mg/l of $GA_3$ was observed (2, 177). Smith et al., 1997 reported that $GA_3$ (0.01mg/l) increased the growth rate of hairy roots of *Artemisia annua* by 25% with a slight increase in artemisinin level compared to controls (178). A combination of Benzylaminopurine (BAP) (1.0 mg/l) and Kinetin (10mg/l) increased the yield of artemisinin in vitro by 3.6 and 2.6 times respectively, due to increase in dry matter production, which overcame a concurrent decrease in the artemisinin content (160).

Addition of artemisinin precursors to the medium used for tissue cultures of *Artemisia annua* resulted in increased amounts of artemisinin. Weathers et al., (1994), reported a four fold increase of artemisinin in tissues and an eleven fold increase of artemisinin in the spent medium on addition of artemisinin precursors (175). However, by feeding of mevalonic acid lactone, the growth and artemisinin content were negatively influenced (22, 23, 161). Attempts by different groups of workers to improve the artemisinin production by influencing the artemisinin biosynthetic pathway through addition of sterol synthesis inhibitors, (antifungal drugs) or mutagenic agents and fungal elicitors yielded contradictory results. Addition of naphtiphine to the medium improved artemisinin production. While 5-azacytidine, colchicine, miconazole, terbinaphine were too toxic for the cultures and negatively influenced artemisinin production (161). However, Kudakasseril et al., (1987) reported a concentration dependent increase in the levels of artemisinin and growth of shoot cultures with miconazole (22). Incorporation of different fungal elicitors did not in any way affect either growth or artemisinin production (166).

## Other Biological Activities

Artemisinin and its derivatives have been tested for possible therapeutic effect in other systems as well because of its unusual chemical structure (179). These drugs have been found to inhibit plaque formation in *Toxoplasma gondii* (180) while effective against *Leishmania major* (181). Artemisinin, its derivatives dihydroartemisinin and arteether have been found to exhibit suppression of humoral responses at high dose levels but no effect on delayed hypersensitivity to sheep erythrocytes (179). Artemisinin derivatives are active against *Schistosoma mansoni* and *Schistosoma japanicum* in vitro and in experimental animals (90). At higher concentrations, artemisinin-related endoperoxides have been found to have cytotoxicity to Ehrlich ascites tumour cells (182), human HeLa cells and against murine bone marrow using a CFU-GM assay (183). The derivatives of artemisinic acid and artemisitene were found to be cytotoxic in vitro to human hepatoma cell line SMMC-7721, human embryonic lung cell line WI 38, human gastric cancer line SCG-7901 and murine leukaemia cell line P388 (184). Unknown constituents not related to artemisinin, extracted from aerial parts of *Artemisia annua* plants were found to have immunomodulatory activity (185). Artemisinin also has potential application as a herbicide and algicide. The inhibitory concentrations are 5$\mu$M and 0.25$\mu$M respectively (186). Artemisinin also showed significant cytotoxic activity against P-388, A-549, HT-29, MCF-7, KB tumor cell lines (187) and EN2 tumour cells (188). Artemisinin was also found to be effective against avian coccidiosis when given at low levels as a food additive. It significantly reduced lesions caused by the parasite *Eimeria tenella* (189, 190).

## Conclusions

Artemisinin and its derivatives have become the most important drugs for malaria control, removing a major barrier to the economy of developing tropical countries. Despite having followed an unorthodox path of drug development, these drugs are being widely used. *Artemisia annua* is the only practical source of artemisinin since a complete organic synthesis is commercially not viable. Progress towards approval and commercialization of artemisinin and its antimalarial derivatives has been slow. The rapid increase of multi-drug resistant parasites have rendered previous malarial control methods unpractical, risky and no longer cost effective. Thus, this necessity has propelled the artemisinin based antimalarials into their present status. Artemisinin compounds appear to be well absorbed, rapidly hydrolysed to the biologically active metabolite and rapidly eliminated. They have a greater log kill per cycle than other classes of antimalarials. They also have the broadest stage specificity of drug action. However, new criteria are necessary to evaluate antimalarial drug efficacy. Low yield from plants may be overcome by breeding of high yielding plants. Manipulation of culture conditions, growth media and hormone levels to yield higher levels of artemisinin have not been successful. A

thorough study of the biosynthetic pathway and of the enzymes involved in the artemisinin production is the need of the hour. Harvesting the plant for artemisinin precursors (arteannuin B and artemisinic acid) along with artemisinin is feasible and an attractive option. These precursors occur in the levels 8-10 times higher than artemisinin suggesting the adoption of bioconversion route for the in vitro production of artemisinin from its precursors, which is at present being carried out in our laboratory.

Clearly, the discovery and development of artemisinin is the most exciting and successful breakthrough in the medicinal plant drug development for the control of malaria.

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