CHAPTER II

LITERATURE REVIEW

1. Human Malaria

Malaria remains an important cause of mortality in tropical regions. At the end of 2004, 107 countries and territories had areas at risk of malaria transmission. Some 3.2 billion people lived in areas at risk of malaria transmission. An estimated 350–500 million clinical malaria episodes occur annually; most of these are caused by infection with *P. falciparum* and *P. vivax*. *Falciparum* malaria causes more than 1 million deaths each year. It also contributes indirectly to many additional deaths, mainly in young children, through synergy with other infections and illnesses (WHO, 2005). The cause of the disease is the infection of protozoa, *Plasmodium* parasites, which require two hosts to complete their life cycle, one is human and the other is female *Anopheles* mosquitoe. A bite of the infected female *Anopheles* mosquitoes transmits the infection. The parasites inhabit not only human but also apes, monkeys, redents, birds, and lizards. Presently, only four species of *Plasmodium* are known to infect human: *P. falciparum, P. vivax, P. malariae* and *P. ovale.*

P. falciparum is commonly found in the tropical and subtropical areas. The parasite is responsible for the most deadly and acute human malaria, which is sometimes called as "Jungle Fever" or "Malignant Tertian Malaria". *P. vivax* is responsible for the majority of malaria cases and spreads out not only in the known warm and tropical regions but also in the temperate areas. It is generally known as "Benin Tertian " or " Vivax malaria ".

For *P. malariae,* this causes chronic malaria with relapse after prolonged period of time. It is known as " Quartan " or " Malariae ". For *P. ovale*, this causes " Tertian " or " Ovale Tertian Malaria " which is similar to but milder than Benin Tertian.

Plasmodium parasite has a complicated life cycle dwelling in two different hosts, one is a vertebrate and the other is a mosquito of the genus *Anopheles* (Figure 1). Human malaria disease occurs where *Anopheles* vectors breed in nature and where human carriers of the sexual form are available to these mosquitoes. Figure 1 shows the life cycle of malaria palasite of *P. falciparum* in human. Infection of the parasite is introduced through the bite of female *Anopheles* mosquitoes sporozoites enter the host liver. The parasite develops to schizont and 5-7 days latter, the parasites in liver cells rupture and release thousands of merozoites which will invade erythocytes. Some meroziotes intrude the liver cells again and repeat the whole cycle in the host liver. This region of the liver results in a relapse, which occurs in *P. vivax, P. malariae,* and *P. ovale* but not exist in *P. facfiparum.* (Geldre *et al.*, 1997). The parasite will develop and replicate within the erythrocytes, where they proliferate asexually or differentiate into gametocytes, the sexual form of parasite. During the cause of development, the red blood cells rupture due to fever attack.

Mature gametocytes do not develop further in the human host until being ingested by mosquitoes. When the mosquitoes are fed on infectious human and took the gametocytes with the blood meal, the gametocytes are passed into mid-gut of the mosquitoes. The fertilization of gametes then quickly occurs and forms a zygote, which eventually develops into sporozoites. After that, sporozoites migrate to the salivary glands and ready to transmit to a new human host (Agtmeal *et al.,* 1999).

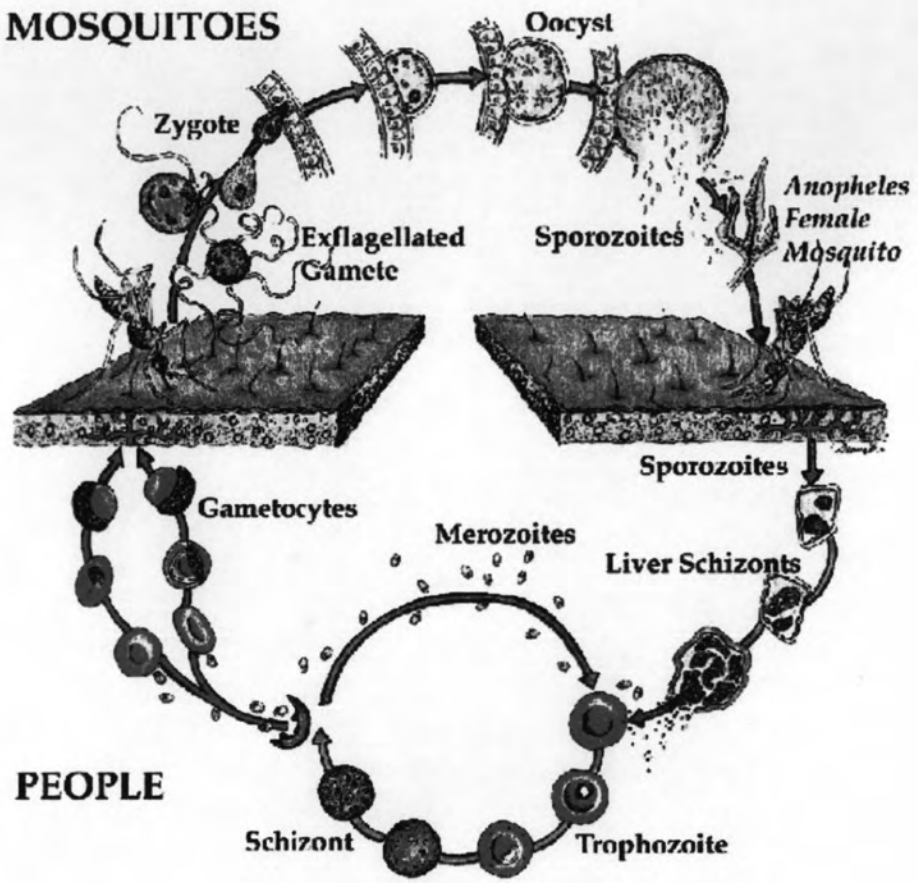


Figure 1. The life cycle of malaria.

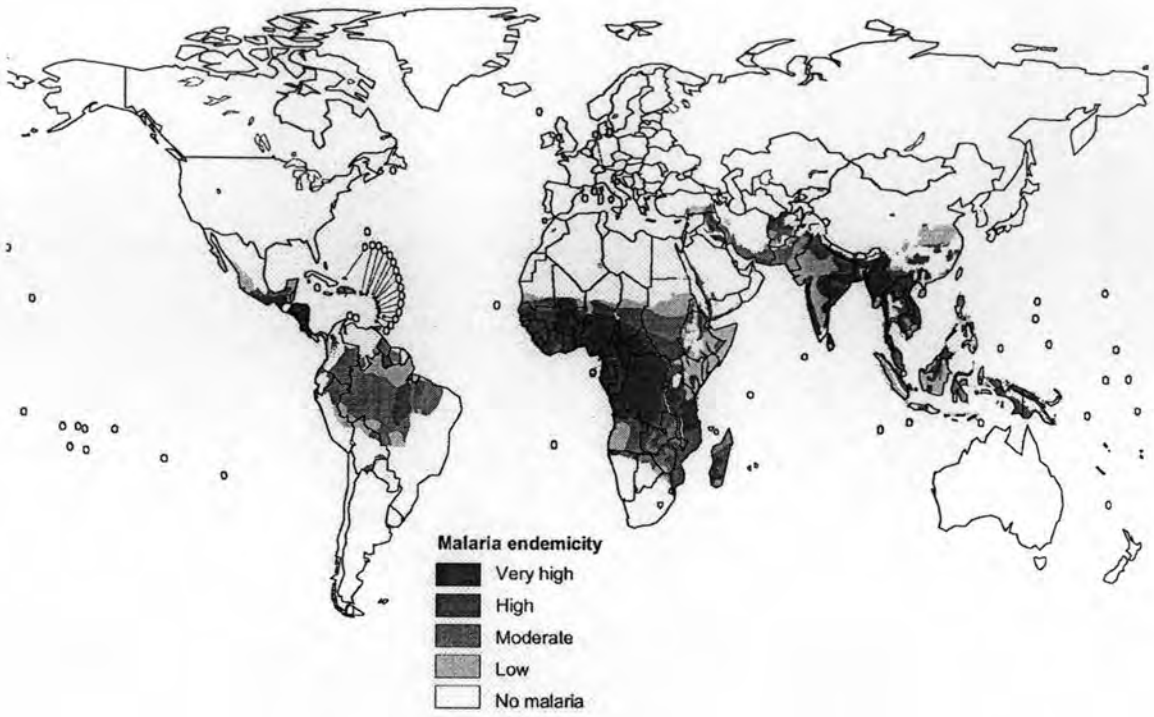


Figure 2 Global distribution of malaria transmission risk, 2003 (WHO, 2005)

Malaria has a world wide distribution (Figure 2). The areas of malaria distribution are being found in tropical areas, throughout sub-Saharan Africa and to a lesser extent in South Africa, Southeast Asia, the pacific islands, India and Central and South American. At the end of 2003, the distribution of malaria was found in 107 countries and territories (Figure 2). In Thailand, malaria is forest-related and most prevalent along the international borders, especially between Thailand and Myanmar and Cambodia.

Among the parasites, *P. falciparum* is a multidrug resistant strain to chloroquine (CQ), sulphadoxine-pyrimethamine (SP) and mefloquine (MO) monotherapies. All of these drugs have been ineffective and quinine is slowly losing its potency. In 2003, major cases of malaria were found in Tak area with 10,278 patients or 28 % of all malarial patients in Thailand (Table 1).

Table 1 The malaria cases in selected subnational areas in Thailand in the year 2003 (WHO, 2005)

Province	Case	%
Tak	10,278	28
Yala	3,051	8
Kanchanaburi	2,659	7
Chanthaburi	2,628	7
Mae Hong Son	1,929	5
Chiangmai	1,732	5
Prachuap Kiri Khan	1,437	4
Ubon Ratchathani	1,186	3
Nakhon Sri Thammarat	1,166	3
Chumporn	1,080	3

2. Anti-Malarial Drugs

Quinine is an ancient drug derived from the bark of cinchona tree. The compound is known to be the first antimalarial drug used for more than 350 years. The compound became the major treatment for malaria because quinine is quickly lethal to plasmodia. However, quinine stimulates insulin production and may cause hypoglycaemia, a particular risk in pregnancy (Ashley *et al.*, 2006). Due to its serious side effects, quinine has been replaced by a new synthetic drug of chloroquine. Chloroquine is a 4-aminoquinoline that acts mainly on the large ring-form and mature trophozoite stages of parasite. The compound is more effective and used to prevent and cure *P. falciparum* completely, and can also suppress the other three malarial species. This compound is less toxic than quinine but high toxic in overdoses when it may cause fatal cardiac arhythmias when given intravenously. Subsequently, the resistance to chloroquine of the parasites occurred by the mechanism associated with reduced drug concentrations within the parasites due to reduced ingress or increased influx (Figure 3) (Phillips 2001). Due to an increased treatment failure, chloroquine is now replaced by pyrimethamine/sulfodoxine. The antifolates proquanil and pyrimethamine are usually used in combination with sulphonamides. Mefloquine is a

quinoline methanol compound with a long terminal elimination half-life. The compound has been used widely in Southest Asia and South America (Phillips 2001). However, side-effect are frequent and include dizziness, nausea, vomiting, diarrhea and abdominal pain.

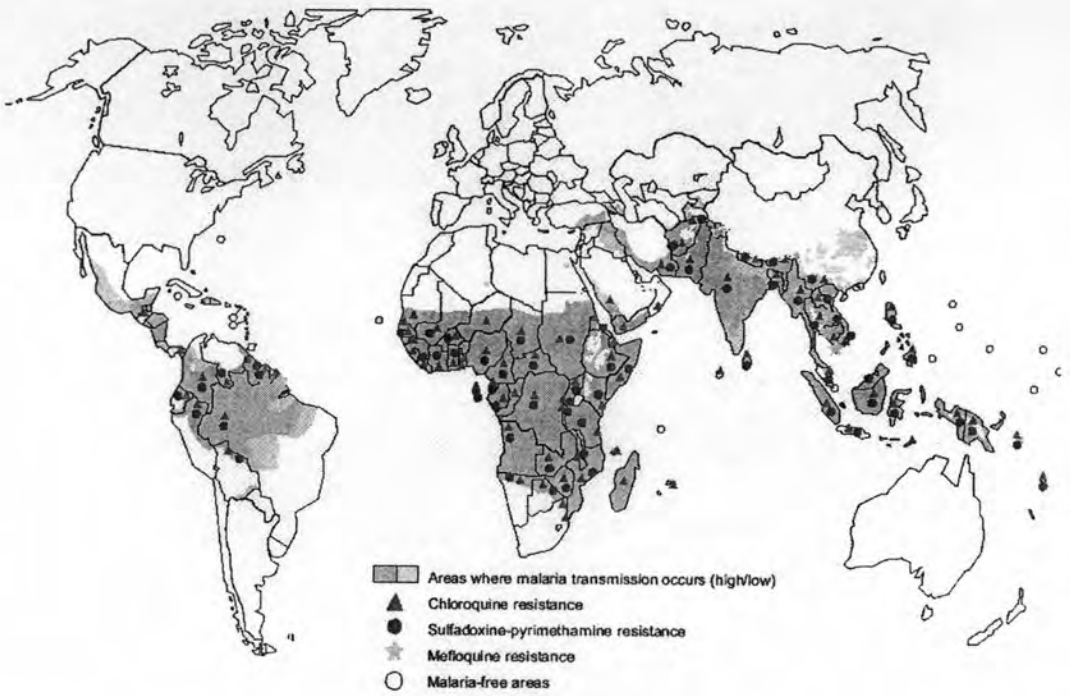


Figure 3 Drug resistance to *P. falciparum* from studies in sentinel site, up to 2004 (WHO, 2005)

The resistance to the compound has been observed in treatment of infections caused by *P. falciparum* malaria in Sountheast Asia as shows in Figure 3. Primaquine, an 8-aminoquinoline, is active against exoerytrocytic as well as latent exoerythrocytic stage of all malarial parasites and therefore used to treatment of relapsing malaria (Zakeri et al., 2002). It is contraindicated in pregnancy or in individual with glucose-6-phosphate dehydrogenase (G6PD) deficiency, as it may cause massive haemolysis (Clyde, 1981). Artemisinin, susquiterpene lactone, has

been used to treat the malarial parasites. It has low acute toxicity after oral or subcutaneous administration (Klayman, 1985).

For high effective malarial treatment, combination therapy with antimalarial drugs has been used to treat malaria (WHO, 2001). The concept of combination therapy (CT) is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite.

Artemisinin-based combination therapies (ACTs) continue to be the mainstay of treatment of uncomplicated *falciparum* malaria. For the next 8–10 years, no alternative medicines to the artemisinin derivatives able to offer similar high levels of therapeutic efficacy are expected to enter the market (WHO, 2001). In January 2006, WHO appealed to manufacturers to stop marketing oral artemisinin monotherapies and instead to promote quality ACTs in line with WHO.

3 Artemisinin

3.1 Structure and chemical properties

Artemisinin is a sesquiterpene lactone containing a peroxide bridge occuring in the leaves of *A. annua*. Its chemical name is [3*R*-(3α,5aβ,6β,8β,8aα,9α,12β, 12a*R**)]-Octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepi-10(3H)-one. It has many common names, including artemisine, arteannuin, huanghuaosu, QSH, ginghaosu or qing hau sau. It has a formular of $C_{15}H_{22}O_5$ with a molecular weight of 282.35. The structure of artemisinin is shown in Figure 4 (**1**).

Artemisinin has no UV or fluorescent chromophore in its molecule. It is soluble in apotic solution and slightly in oil.

Table 2 When, and to which first-line drug, malaria-endemic countries change policy following the development of drug resistance (WHO, 2002).

Drug	Countries that changed treatment policy[a]						
CQ to ACT				Cambodia		Myanmar Zambia Zanzibar	Burundi
SP to ACT					S. Africa		
CQ to CT					Rwanda		
CQ to CQ+SP			Ethiopia[c]	Uganda[b] Zimbabwe		Eritrea[b,c]	
CQ to SP	Malawi S. Africa	Kenya Botswana	UR Tanzania[b]		Burundi[b] DR Congo[b]		
	<1993	1998	1999	2000	2001	2002	2003
	Year of policy change						

[a] Thailand and Viet Nam changed to ACTs before 1993, having gone sequentially from CQ, through SP and mefloquine.

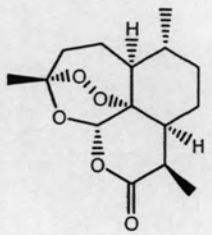
[b] Interim treatment policies while preparing to adopt ACTs.

[c] Adoption of CQ + SP was the result of prevalence of both *P. falciparum* and *P. vivax* malaria.

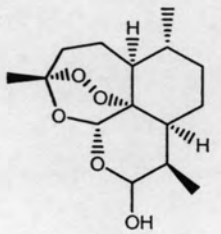
3.2 Derivatives of artemisinin

There have been many attempts to modify the chemical structure of artemisinin to obtain new artemisinin derivatives with enhanced antimalarial activity. Generally, the resulting semisynthetic derivatives still retain the endoperoxide moiety as it is essential for the activity. So far, the most promising artemisinin derivatives have been dihydroartemisinin, artemether, arteether, artesunate and artelinic acid (Klayman 1993) (Figure 4). Artemisinin treated with sodium borohydride led to dihydroartemisinin, which is twice as active as the parent compound (Luo *et al.*, 1984). Consequently, dihydroartemisinin can be converted into its ethers, carboxylic esters, carbonates and sulfonates (Torok and Ziffer, 1995). The magnitude of potency has been shown to be in the following order: carbonates > ester > ether > artemisinin.

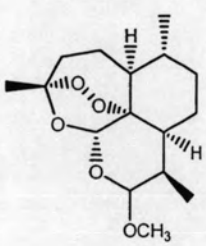
Ether derivatives i.e. artemether and arteether which are oil-soluble have been synthesized by treating dihydroartemisinin with methanol in the presence of boron trifluoride etherate (Brossi *et al.*, 1988). The oil-soluble ethers can also be produced



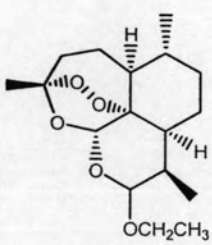
Artemisinin (**1**)



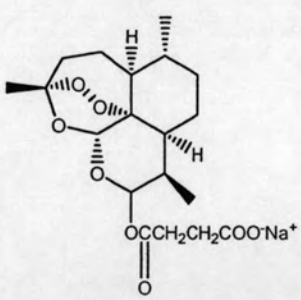
Dihydroartemisinin (**2**)



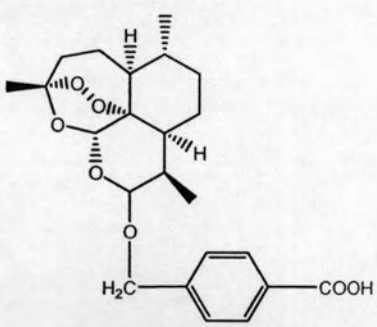
Artemether (**3**)



Arteether (**4**)



Sodium artesunate (**5**)



Artelinic acid (**6**)

Figure 4. The chemical structures of artemisinin and its derivatives.

for intremuscular injection (Phillips 2001). They are 2-3 times more potent than artemisinin. Another ester derivative called "dihydroartemisinin hemisuccinate" is prepared by neutralizing the precursor to the sodium salt of artesunic acid (sodium artesunate) which is readily water-soluble. It can, therefore, be administered by

intravenous injection, which is distributed more readily through blood circulation resulting in a rapid onset of action. This artesunate has been reported to be even more potent than dihydroartemisinin (Torok and Ziffer, 1995). However, some synthetic products such as iso-artemisitene and epi-artemisinin are less active than the parent compound *in vitro* (Brossi 1988).

3.3 Extraction of artemisinin from *A. annua*

Klayman (1985) has been reported a large-scale extraction of artemisinin from *A. annua*. Petroleum extraction has been performed with died leaves and fractionated the extract on a silica column. Other methods depend upon the use of multi-layer separator (Acton 1986). This procedure, although very economical in comparison with the first procedure, is only suitable for small-scale extractions. In 1987, El-Sohly *et al.*, could isolate large quantities of artemisinin but predominant artemisinic acid tends to be eluded with artemisinin and fractions containing artemisinin may require rechromatography to achieve the necessary purity (El-Sohly *et al.*, 1987). For evaluation of *A. annua* strains for high artemisinin production, Singh *et al.*, (1988) has used n-hexane at room temperature dried leaves of *A. annua* and concentrated by using column chromatography over silica gel. Two years latter, Charles *et al.*, (1990) has reported that the isolation method was developed for a large-scale production of artemisinin. The method involves hexane extraction of the unground dried leaves followed by partitioning the extract with between 20 % aqueous CH_3CN and purification the CH_3CN fraction by chromatography on silica gel filtration column. The purity of artemisinin obtained by this way is approximately 99 % and without any artemisitene detected. However, a full ton of dry leaves is need to produce about 6 kg of artemisinin. Therefore, a total area of 40 hectare must be used for plantation to

supply the raw material (Hien and White 1993). The low content of artemisinin in *A. annua* plants cultured European has been a limiting factor for isolation and evaluation of artemisinin on a technical scale. Artemisinin yield of 0.06 %, which is too low for commercial exploitation, has been obtained from samples of *A. annua* collected in the U.S.A.. Yields of extracted artemisinin from the above ground portions of the plant have ranged from 0.01 % to 0.5 % (w/w) in China (Singh *et al.*, 1988). Recently, the extraction of artemisinin has been reported by using supercritical fluid extraction (SFE) and analyzed by supercritical fluid chromatography (SFC). These conditions avoided degradation of the analyst and gave clean extracts ready to be analyzed by SFC. Results were compared with two conventional liquid solvent extraction processes. The SFE-SFC method was successfully applied to six samples of *A. annua* containing various concentrations of artemisinin and artemisinic acid (Kohler *et al.*, 1997). Compared with Soxhlet method, supercritical CO_2 extraction and normal stirring extraction, microwave-assisted extraction (MAE) of artemisinin from *A. annua* L saves a lot of time, gives high extraction rate. The time used in MAE is only 12 min with 92.1% extraction rate, while Soxhlet method and normal stirring extraction need several hours with only about 60% extraction rate. Supercritical CO_2 (SC-CO_2) extraction gives the lightest color of extractive but lowest extraction rate (Hao *et al.*, 2002). In 2003, a study has been conducted to enhance the dissolution rate of artemisinin in order to improve the intestinal absorption characteristics by mean of supercritical fluid technology (Nijlen *et al.*, 2003). In addition, recently, Quispe-Condori *et al.*, (2005) has reported that the highest artemisinin global yield (0.7 % in dry basis) was obtained at the 50°C and 300 bar for SC-CO_2 extraction. Very recently, Tzeng *et al.*, (2007) has reported that adding of 16.25 % ethyl alcohol

to the SC-CO_2 extraction at 40 °C and 3500 psi yields the largest amount of artemisinin with 96 % recovery.

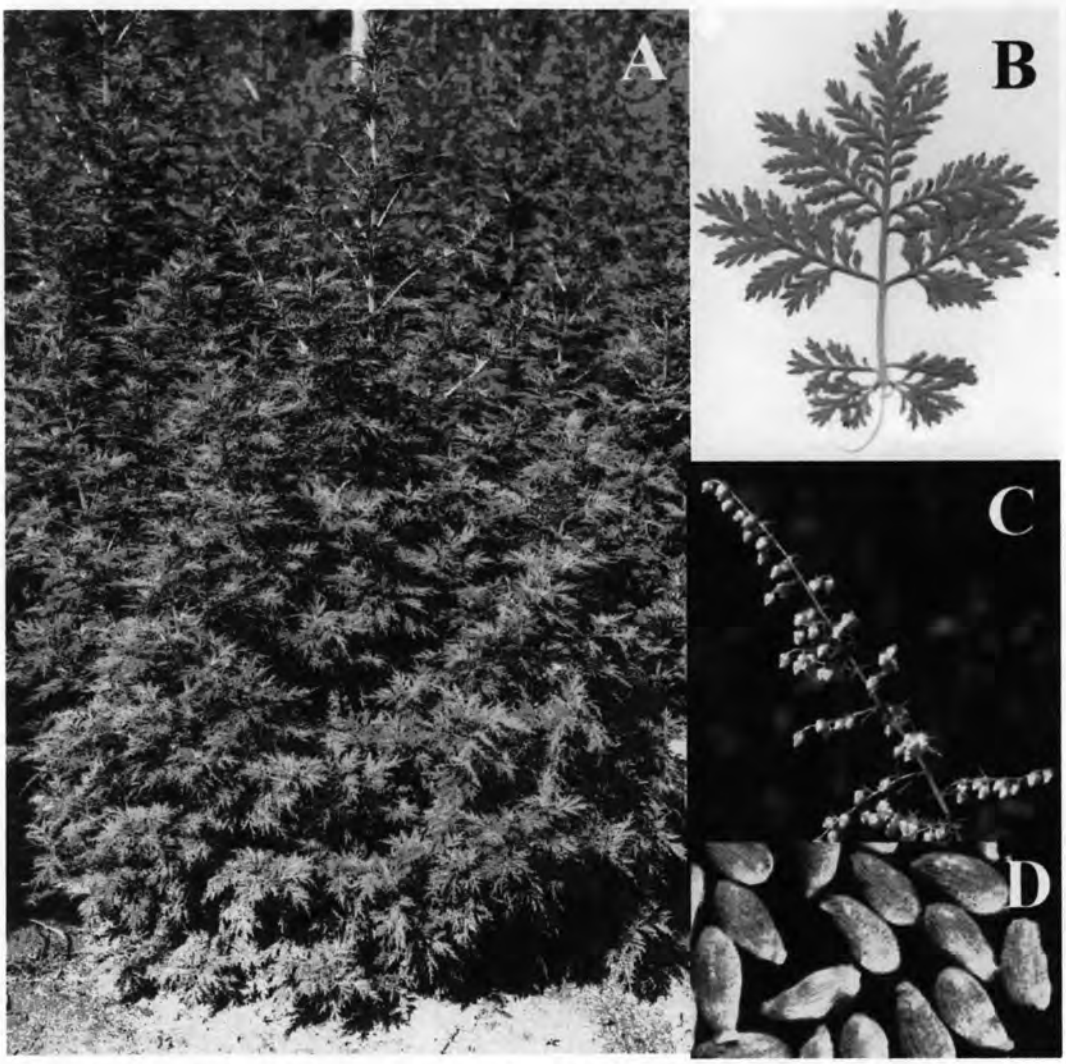


Figure 5. The plant of *Artemisia annua* L. (A) with the part of Leaves (B), Inflorescences (C) and Seeds (D) (http://www.mediplant.ch/anglais /ficheartemis_eng.html)

4 *Artemisia annua*

4.1 Botanical aspects

Artemisia annua Linn. is an annual plant and a member of the Asteraceae family. It is commonly known as sweet annie, annual wormwood and sweet wormwood (Brown 2001). Scince 1967, it has been intensively investigated in the

search for novel antimalarial drug in China (Klayman 1985). The plant is usually single-stemmed reaching about 30-200 cm in high with alternate branches and alternate, deeply dissected, aromatic leaves ranging from 2.5 to 5.0 cm in length. Ting yellow nodding flowers (capital) only 2 or 3 mm across are display in loses panicles containing little nectar and pistillate marginal (ray) florets. The involucre is imbricate with several rows of bracts. The central flowers are perfect and can be either fertile or sterile (Ferreira and Jinck 1996) (Figure 5).

Although *A. annua* is a traditional herb for antimalarial drug in China, where it is widely distributed as a weed mostly in temperature regions, the plant can be grown in many areas of the world for research purposes such as in South America, the U.S.A., Canada, Europe (e.g. Yugoslavia, Hungary, Bulgaria, Romania, Italy, France, Spain, Turkey and Russia) and Asia (e.g. Vietnam, India, Singapore and Thailand).

4.2 Artemisinin content in *A. annua*

The accumulation of artemisinin in *A. annua* has been reported to be in the leaves and in the inflorescences (Charles *et al.,* 1990; Werdendag *et al.,* 1993). Ferriera *et al.*, (1995) have reported that artemisinin is present 4 to 11-folds higher in florescences at the full blooming stage compared with leaves. Stem contains trace amount or no artemisinin while roots and pollens do not (Charles *et al.,* 1990). It has been found that more than 80 % of artemisinin in the leaves with difference percentage of distribution: shoots leaves, upper leaves, middle leaves, and lower leaves as 41.7, 25, and 22.2 percent respectively (Charles *et al.,* 1990). Liersch *et al.,* (1986) has reported that the formation of artemisinin during one vegetation period cultivated in many sources of Argentina, China, U.S.A. and Belgium. They have found that artemisinin content is in the range from 0.04 to 0.1 percent dry weight.

Sigh and coworkers have investigated the artemisinin content of *A. annua* strains at different growth stages (Singh *et al.,* 1988). The results have shown that the highest artemisinin content is 0.094 percent dry leaves. Three years latter, El-Sohly *et al.* (1990) have described a large-scale extraction technique of artemisinin from *A. annua*. In addition, artemisinic acid and arteannuin B, and other two sesquiterpenes, have also been isolated. The artemisinin yields obtained from many partitioning systems have been shown the range of 0.07 to 0.12 percent. Studies of germplasm variation in terms of artemisinin content have shown that highest artemisinin content among accession ranges from 0.003 to 0.21 percent and among individual plants ranges from 0 to 0.39 percent (Charles *et al.,* 1990). Jaziri *et al.,*(1993) have reported that the highest artemisinin content up to 1.12 percent dry weight is found in the upper leaves of the plants which are cultivated under hydroponic conditions. In Vietnam, the highest artemisinin content has been found to be present in the leaves of 5 month-old plants. In addition, the same age of the plant has been found to have the highest artemisinic acid and arteannuin B contents, 0.16 and 0.08 % dry weight respectively whereas artemisitene is present all stages of development, ranging from 0.002 to 0.09 % dry weight. (Woerdenbag *et al.*, 1993). Vandenberghe *et al.,* (1995) have reported a determination of artemisinin and its bioprecursors. The results have shown that artemisinin, artemisinic acid, arteannuin and artemisitene in plant leaves after 3 months were 0.071 %, 0.41 % 0.11 % and 0.008 % dry weight, respectively.

A. annua plants obtained from micropropagation by tissue culture technique have been found to contain high amount of artemisinic acid (0.8 % dry). In these plants, the combined content of artemisinin and its intermediates artemisinic acid and artemisinin B has been found to be 1.35%. (Gupta *et al.*, 1996). In Japan, a production of artemisinin and related sesquiterpene during a period of plant

cultivation has been published (Kawamoto *et al.,* 1999). Studies on seasonal and positional variations have shown that the maximum content of artemisinin, artemisinic acid, arteannuin B and artemisitene are 0.28 %, 1.0 %, 0.13 % and 0.003 % dry weight (Kawamoto *et al.*, 1999).

In Thailand, artemisinin content in *A. annua* leaves has been shown to vary from 0.074 to 0.115 percent dry weight. Similarly, the plants cultivated at Doi-tung Highland Agricultural Extension Center in Chiangria have also been shown to contain artemisinin in the same range of 0.07 to 0.124 percent dry weights (Atchara, 1996).

The effect of water stress on the accumulation of artemisinin has also been reported. It has been shown that greater soil water stress (lower soil water potential) during a period of two weeks before leaves harvesting leads to reduced leaf artemisinin content. In terms of post harvest handling of *A. annua* plants, artemisinin has been found to retain to a greater extent when plants are dried under ambient conditions compared with using forced air at 30°C to 80 °C for the shortest time period (12h). Prolonged drying generally results in further losses in artemisinin (Charles *et al.*, 1993).

Selection of high artemisinin producing plants has been investigated during twelve (0.13-0.31 % dw) and thirteen (0.12-0.39 %) weeks old during preflowering period (Chan *et al.*, 1995). Statistical comparison of artemisinin content from various clones has shown that they may be grouped as high (0.41-0.42 % dw), moderate (0.25-0.26 % dw), and low (0.13 %) artemisinin yield.

So far, there have been several reports on *A. annua* plants producing high artemisinin content but the results are still controversial. Many workers have claimed that good harvesting period should be performed just before flowering (Klayman *et al.* 1985; Liersch *et al.,*1986; Singh*et al.,*1988; El-Sohly *et al.,* 1990; Woerdenbag

et al., 1991; Chan *et al.*, 1995) or at the times of flowering (Acton *et al.*,1985; Liersch *et al.*,1986; Singh *et al.*,1988; Chaeles *et al.*,1990; Pras *et al.*,1991; Jain *et al.*,1996). Some groups have reported that the highest content is obtained when the plants are harvested before flower bud formation (Ferreira *et al.*, 1995 and Gupta *et al.*, 1996) and the content changed within the plant over the growing season (Acton *et al.*,1985 and Liersch *et al.*,1986).

Due to the low concentration of artemisinin detected in the plants, tissue culture systems have been considered as alternative way for artemisinin production. The artemisinin accumulation has been detected in shoot culture but lower than plant of *A. annua*. He *et al.*, (1983) have first reported the artemisinin production in *A. annua* in tissue culture. The yield was 0.008 % in shoots from the callus of this plant from Chinese origin. Martinez and Staba (1988) has extensively monitored in the shoot culture and have found that artemisinin content was between 0.3 % and 0.03 % dw. In 1990, Simon *et al.*, (1990) have reported that artemisinin is present in ranging from 0.03-0.05 % dw.

In addition, the effect of plant growth regulators has been studies. In 1992, artemisinin content has been reported to contain 0.012 % dw in plantlet cultures grown with MS medium containing 1 ppm NAA and 0.1 ppm kinetin (Elhag *et al.*, 1992), 0.08-0.16 % dw for shoot culture established on MS medium containing 0.2 mg/l BA and 0.05 mg/l NAA (Woerdenbag *et al.*, 1993). Ferreira *et al.*, (1993) have reported that the artemisinin content in range of 0.014-0.290 % dw was detected in *in vitro* culture plantlets. Artemisinin content has been shown to increase yield significantly when gibberellic acid (GA_3) is added in plant growth conditions (Farooqi *et al.*,1996; Siyapatantakirutimana *et al.*,1996). Moreover, biomass of the plant has been observed to increase with GA_3 treatment (Siyapatantakirutimana *et al.*, 1996).

In hairy root culture of *A. annua*, the effect of GA_3 has been shown to have significant increase in biomass and in artemisinin content (Smith *et al.*, 1997).

On the other hand, the transformation of *A. annua* plant by using *Agrobacterial rhizogenas* has been reported in transformed hairy roots, but not transgenic plants (Weathers *et al.*, 1994; Jaziri *et al.*, 1995; Paniego and Giuletti, 1996; Qin *et al.*, 1994). Vergauwe *et al.*, (1996) has established an *A. tumefaciens*-mediated transformation system. The regenerated plantlets have been showed to have artemisinin content up to 0.17 % dw, a little higher than that present in leaves of *in vitro* plantlets (0.11 % dw).

In addition, the artemisinin biosynthetic enzyme has been studied in transgenic *A. annua*. There have been reports that give emphasis on the enzyme farnesyl diphosphate synthase. Chen *et al.*, (2000) have transferred a cDNA encoding farnesyl diphosphate synthase and placed under a CaMV 35s promoter into *A. annua*. Analysis of artemisinin has shown that artemisinin content increase about 3-4 times from the control shoots in range of 1.0 % dw. These studies show that although manipulation of FPS increased artemisinin, the yields were still in adequate to significantly increase artemisinin production in plants.

5. Determination of Artemisinin Content in *A. annua*

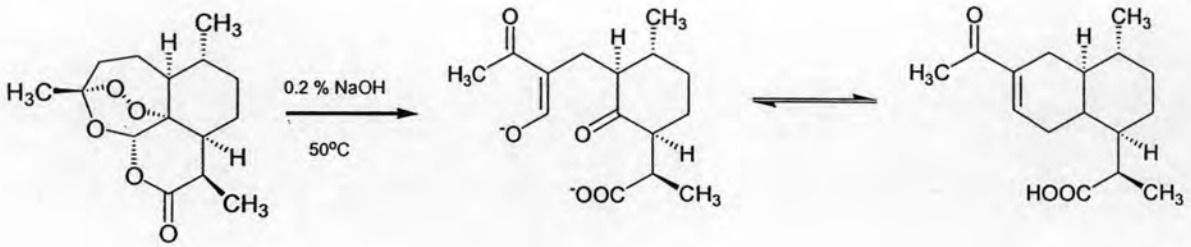
Practically, a determination of artemisinin content is difficult owing to the unstability of the compound. Study on thermal stability has shown that artemisinin is stable up to 150 °C but degrades into numbers of products when heated at 180-200 °C (Gelder *et al.*, 1997). Besides, artemisinin is sensitive to acid and base treatment (Klayman 1985). In addition, the low content of artemisinin in the plant and the

interference of other compounds in the crude plant extracts usually affect artemisinin analysis. Particularly, artemisinin has no any UV or fluorescent chromophore in the molecule and, therefore, the analysis of the compound can not be carried out by common techniques.

High performance liquid chromatography (HPLC) with UV monitoring at 210 nm has been used. However, the presence of the other constituents that also absorb strongly at 210 nm completely interferes the peak of artemisinin (Acton and Klayman 1985; Liesh *et al.*, 1986; Singh *et al.*, 1988). Therefore, a development of sensitive and specific analytical method for determination of artemisinin is a challenging problem.

Artemisinin decomposition has been used precolumn acid or base-catalyzed to form UV-absorbing compounds followed by HPLC analysis of the decomposition product. Zhao and Zeng (1986) have used NaOH for artemisinin decomposition to Q_{292} followed by neutralization with acetic acid to form to Q_{260} before subjected to HPLC analysis with UV monitoring at 260 nm (Figure 6). In addition, KOH has been used to decompose of the compound which can be monitored at 286 nm by HPLC-UV (Edlund *et al.,* 1984). Alternatively, an electrochemical HPLC detection procedure has been developed that can be used for determination of artemisinin in crude plant extracts (Figure 7) (Acton *et al.,* 1985; Charles *et al.,* 1990). An alkaline hydrolyzed derivative of artemisinin has been used to analytical target by HPLC with diode array detection (DAD) method for detect Q_{260} (Wallaart *et al.*, 1999) and capillary electrophoresis (CE) coupled with flow injection (FI) for detect Q_{292} (Chen *et al.*, 2002; Chen *et al.*, 2004). However, studies on pharmacokenitic of the artemisinin drugs and its derivatives by HPLC method with reductive electrochemical detection

of artemisinin must take special precautions as molecular oxygen can be reduced at the low cathodic potential of –0.8 V employed (Sipahimalani *et al.*, 1991).



Artemisinin Q_{292} Q_{260}

Figure 6 The reaction of pre-column artemisinin derivatization for HPLC-UV analysis of artemisinin (Zhao and Zeng 1986).

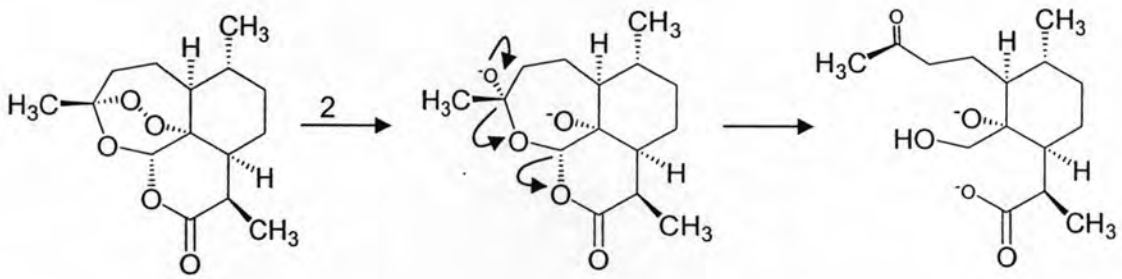


Figure 7 Electrochemical reaction occurs during artemisinin analysis HPLC-ECD technique (Acton and Klayman 1985).

On the other hand, the analysis of artemisinin employing HPLC with chemiluminesence (CL) detection in the absence of hydrogen peroxide has also been reported (Green *et al.,* 1995), as well as using the HPLC evaporative light scattering detector (ELSD) (Avery *et al.,* 1999; Peng *et al.*, 2006; Liu *et el.*, 2007).

Gas chromatography (GC) method for the analysis of the artemisinin compound at nanogram levels has been proposed (Sipahimalani *et al.,* 1991; Peng *et al.*, 2006; Tzeng et al., 2007). The technique 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.

Thin-layer chromatography (TLC) has been used to estimate artemisinin content by using 2 % vanillin as a spraying agent (Tu *et al.,* 1982) or by visualization using 60 % sulphuric acid and heating. Under these conditions, reference artemisinin appears as a yellow spot in daylight and a fluorescent blue spot when examined under UV light at 254 and 330 nm (Tawfig *et al.,* 1989). However, this protocol gives poor staining characteristics of the intact molecule and interference with contaminating constituents of the plant, this method is not, therefore, very reliable. Recently, TLC-quantitative analysis of artemisinin content in *A. Annua* crude extract has been reported. A TLC method has been developed to analyze artemisinin and its derivatives, artemether, and arteether, using a silica-gel plate and reversed-phase-C18. After development, all products are visualized after dipping in a 4-methoxybenzaldehyde dipping reagent. The color development conditions are 110 °C during at larst 8 min, for quantitative analysis. The artemisinin is measured by linear scanning at 565 nm (Gabriels and Plaizier-Varcammen, 2003; Gabriels and Plaizier-Varcammen, 2004). In addition, very recently, a simple TLC-densitometric technique has been developed for the rapid and accurate analysis of artemisinin in a large number of *A. annua* plantlets culture *in vitro* (Koobkokkruad *et al.*, 2007). This method is based on the structural conversion of artemisinin on siliga-gel plate by ammonia to form 10-azadesoxyartemisini (Figure 8), a chromophore-containing compound (λ_{max} 320 nm) that can be detected by UV-base TLC-densitometry. The technique appeared to be accurate and sensitive as compared with pre-column reaction-HPLC technique. The results show that this method can be cheap and simple technique for the accurate screening of high artemisinin-producing plants.

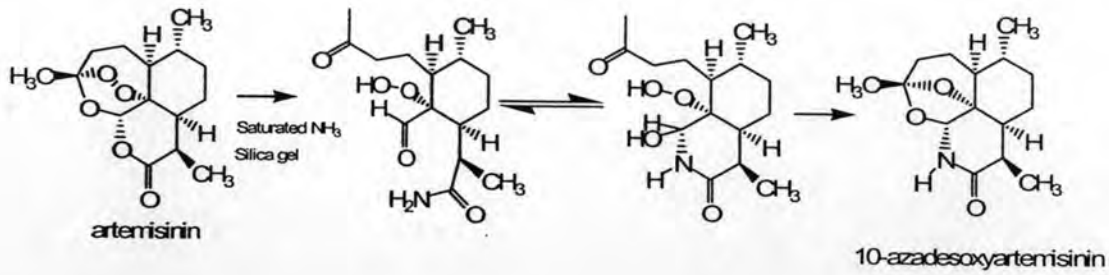


Figure 8 Proposed structure conversion of artemisinin to 10-azadeoxyartemisinin on a silica gel layer following exposure to ammonia vapor for 2 h at 100 °C (Koobkokkruad *et al.*, 2007)

For other methods, a rapid screening method based on tandem mass spectrometry (MS/MS) has been described for determination of artemisinin-related compounds present in crude hexane extracts of *A. annua* (Ranasinghe *et al.*, 1993). An enzyme-linked immunosorbent assay (ELISA) has also been developed for the detection and semi-quantitative determination of artemisinin and its structurally related compound in crude extracts of *A. annua* (Jaziri *et al.*, 1993). For this case, the peroxide linkage in the artemisinin molecule was critical in determining the antibody specificity. This ELISA technique has also been used to detect and quantify artemisinin in different organs of greenhouse-grown plants and in eight clones of *A. annua* grown in tissue culture (Ferreira and Janick 1996). GC-MS method for the analysis of artemisinin and its biosynthetic precursors has also been developed (Bouwmeester *et al.*, 1999; Wallaart *et al.*, 1999). Because of their thermal instability, the endoperoxide containing sesquiterpene lactones artemisinin and artemisitene have been measured as their pyrolysis products. Alternatively, supercritical fluid extraction (SFE) and analyzed by supercritical fluid chromatography (SFC) using a capillary column, coupled with a flame ionization detector (FID) has been reported for extraction and determine artemisinin (Kohler *et al.*, 1977). In addition, a packed-column SFC-couple with a atmospheric pressure

chemical ionization mass spectrometry has been studied for artemisinin determination (Dost and Davidson, 2003). Finally, a simple method for simulataneous detection and quantification of intact artemisinin and its bioprecursors using HPLC-UV/EC has been established by Vandenberghe *et al.,* (1995). The method allows all compounds be clearly resolved with detection limits below the naturally occuring concentrations in the plant. Recently, reversed phase-HPLC-electrospray (ESI) quadrupole time of flight (Q-TOF) tandem mass spectrometry (MS/MS) has been developed for the quantitation of artemisinin, arteannuin B, artemisitene and artemisinin acid in *A. annua* (Van Nieuwerburgh, *et al.*, 2006). The results of revered phase-HPLC ESI Q-TOFMS/MS presented the artemisinin and some of its structural analogs present in the leaves of *A. annua* are localized entirely in the subcuticular space of glands on the surface of the leaves.

6. Artemisinin Biosynthesis

Artemisinin is a natural product in the group of terpinoids d that is formed from isopentenyl diphosphate (IPP) (Akhila, 1987). For the terpenoid compounds found in nature, the common bioprecursor of two independent pathways following to IPP exist: the mevalonate pathway (MVA) originating from acetyl CoA, and the mevalonate independent pathway (MEP) stemming from pyruvate. IPP and its isomer, DMAPP, then lead to all other terpenoids (Fig. 1; Croteau et al., 2000). Enzymes sequentially link some number of IPP molecules with their dimethylallyl diphosphate (DMAPP) isomer to produce larger terpenes with 5XC atoms (where X = the number of IPP or DMAPP). Eukaryotes other than plants use the MVA isoprenoid pathway, while prokaryotes with some exceptions (Boucher and Doolittle, 2000) use the MEP pathway to separately produce IPP and DMAPP (Lichtenthaler, 1999). However,

plants use both the MVA and the MEP pathways (Croteau et al., 2000). It is now well know that in plants the mevalonate pathway is located in the cytosol, while the MEP pathway is localized to plastids (Figure 9; Croteau et al., 2000). There is evidence suggesting crosstalk between cytosol and plastids and a possible exchange of IPP between these two pathways (Figure 9; Adam and Zapp, 1998; Laule et al., 2003). In 1986, Kudakasseril *et al.* have shown the incorporation of ^{14}C-isopentenyl pyrophosphate (^{14}C-IPP) into artemisinin by cell-free extracts and the production of artemisinin in shoot cultures of *A. annua*. A cell-free system has been developed from shoot culture of the plant capable of incorporating ^{14}C-IPP into the pretoleum ether soluble compounds and artemisinin (Kudakasseril *et al.*, 1986). As a result, IPP has been proposed to be a one of bioprecursors in the biosynthesis of artemisinin. As with essentially all sesquiterpenes, the biosynthesis of artemisinin follow to involves the mevalonate pathway and includes the cyclization of farnesyl diphosphate. This is supported by the incorporation of mevalonate into artemisinin (Akhila *et al.*, 1987, 1990), although it is not clear whether the DXP pathway can also contribute 5-carbon precursors, as occurs in other sesquiterpene biosynthetic systems (Adam and Zapp, 1998).

In addition, El-Feraly *et al.*, (1986) have reported the possible role of artemisinic acid (ginghau acid) in the biosynthesis of artemisinin. In this study, artemisinic acid has been found to be a major sesquiterpene consituent of *A. annua* that can be converted to arteannuin B by singlet oxygen ($^1O_2$) generated by sensitivity photo-oxidation. The formation of this compound was monitored by high-pressure liquid chromato-graphic analysis, and the identity of the isolated material was established by direct comparison. Since $^1O_2$ is known to play a role in biogenetic reactions, it appears that artemisinic acid can serve as a biogenetic precursor for

artemisinin. However, El-Feraly's studies could not explain the presence of the precursors of artemisinic acid and some primary metabolites that led to the formation of artemisinin.

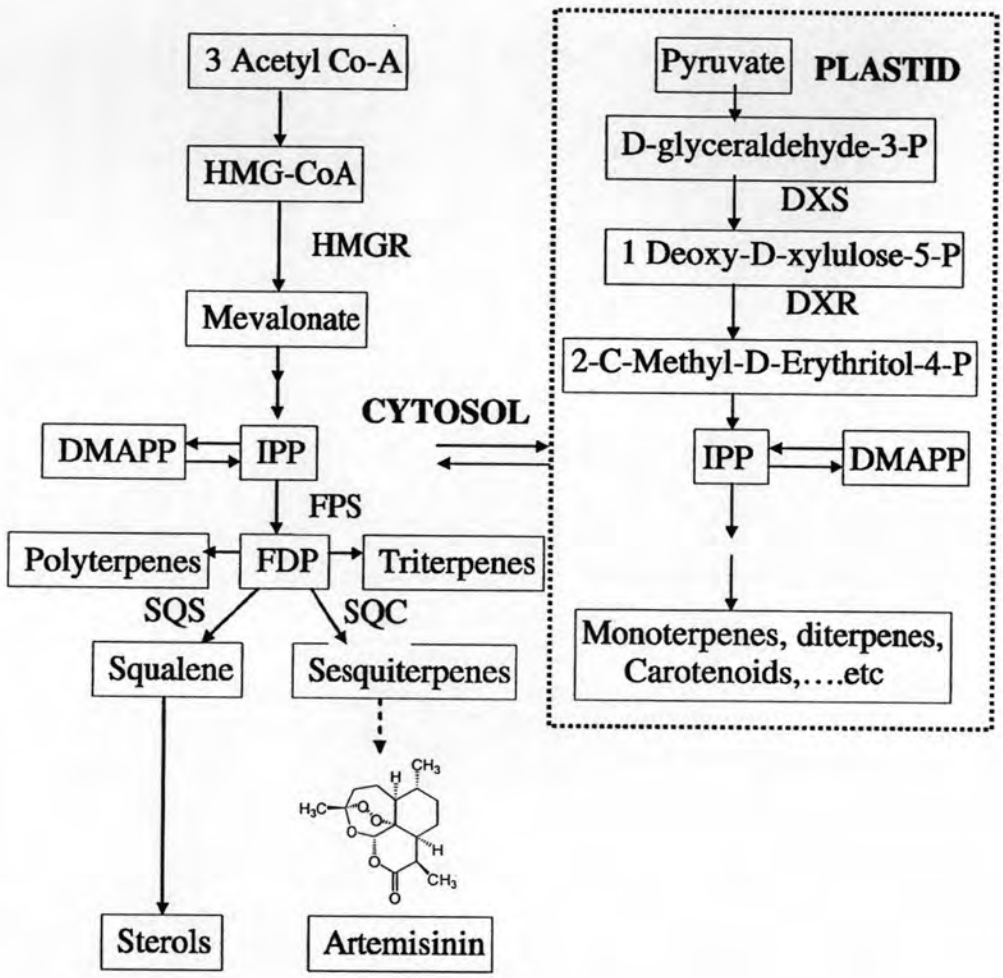


Figure 9 Simplified terpenoid biosynthetic scheme leading to artemisinin. The two arms of terpenoid biosynthesis and some of the regulatory enzymes that catalyze different reactions: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPS, farnesyl diphosphate synthase; SQC, sesquiterpene cyclase; SQS, squalene synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate (Weathers *et al.*,2006).

The enzyme product has been shown to be present in low content in the plant *A. annua*. Because the low abundance of the amorpha-4,11-diene, it has been suggested that the cyclization of FPP is a rate-limiting step in artemisinin biosynthesis (Bouwmeester *et al.*, 1999). The cDNA encoding of amorpha-4,11-diene synthase has been isolated from *A. annua* genome (Mercke *et al.*,2000). This clone contains a 1641-bp open reading frame coding for 546 amino acids (63.9 kDa). When expressed in *Escherichai coli*, the recombinant enzyme has been shown to catalyze the formation both olefinic (97.5 %) and oxygenated (2.5 %) sesquiterpenes from farnesyl pyrophasphate. GC-MS analysis has identified as 91.2 % amorpha-4,11-diene. Moreover, the enzyme amorpha-4,11-diene synthase has been cloned by PCR amplification of genomic DNA with a pair of primers, which designed from the conserved region of sesquiterpene synthase of several plants (Chang *et al.*, 2000). The PCR technique has produced 2106 bp of a full length the cDNA sequence including 1641 bp of open reading frame for 546 amino acid. The soluble fraction of *E. coli* harboring of the gene has been shown to catalyze the cyclization of fanesyl pyrophosphate to produced a sesquiterpene, which is identified through GC-MS analysis as amorpha-4,11-diene (Chang *et al.*, 2000). Recently, Wallaart *et al.*, (2001) have reported the isolation of a cDNA clone encoding amorpha-4,11-diene synthase. The amino acid sequence exhibites high identity (50 %) with a putative sesquiterpene cyclase of *A. annua*. When expressed in *E. coli*, the recombinant enzyme catalyses the formation of amorpha-4,11-diene from farnesyl pyrophasphate.

The formation of the sesquiterpene carbon skeleton, amorpha-4,11-diene is catalyzed by amorpha- 4,11-diene synthase (Bouwmeester *et al.*, 1999) for which corresponding cDNAs have been cloned (Chang *et al.*, 2000; Mercke *et al.*, 2000; Kim *et al.*, 2000; Wallaart *et al.*, 2001). The non-descript arrangement of the

amorphadiene product (see Figure 11) be lies the unique structural features that ultimately allow for the formation of the 1,2,4-trioxane moiety (Sy and Brown, 2002). The numerous amorphane type sesquiterpenes in *A. annua* in which C-12 is oxidized to a carboxyl group and the C11-C13 bond is either single or double suggest early C-12 oxidation. Indeed, Bertea *et al.*, (2005) have shown the conversion of [^{3}H]-FDP to amorpha-4,11-diene in intact *A. annua* glandular tricromes and amorpha-4,11-diene was converted by a putative cytocrome P450 dependent hydroxylase to artemisinic alcohol. The resuls showed that *A. annua* leaf microsomes converted amorphadiene to artemisinic alcohol in the presence of NADPH. The next step in this pathway are the oxidation of artemisinic alcohol at C12 by a dehydrogenase yielding artemisinic aldehye and the subsequent reduction of the C11-C13 carbon-carbon double bond in artemisinic aldehye to be dihydroartemisinic aldehye (Bertea *et al.*, 2005).

The latest step in artemisinin biosynthesis, the evidence of artemisinic acid has been reviewed by Li *et al.*,(2006). This includes the suggestion that C11-C13 double bond has been reviewed by Li *et al.* (2006). This includes the suggestion that C11-C13 double bond reduction occurs at the level of an intermediate beyond artemisinic acid, such as arteannuin B or artemisitene. On the other hand, the co-occurrence of dihydroartemisinic acid with high artemisinin artemisinin levels suggests that even if double bond reduction could occur at a very late intermediate, it also occurs in less oxidized precursors. The double bond reduction at C11,13 is of general interest biochemically, given the relative rarity of enzymes catalyzing double bond reductions (Kasahara *et al.*, 2006).

A case has also been made for dihydroartemisinic acid as a late precursor of artemisinin. Labeled dihydroartemisinic acid is incorporated into artemisinin *in vivo*, a

sequence which can occur in the absence of enzymes (Brown and Sy, 2004; Haynes et al., 2006; Sy and Brown, 2002; Wallaart *et al.*, 1999).

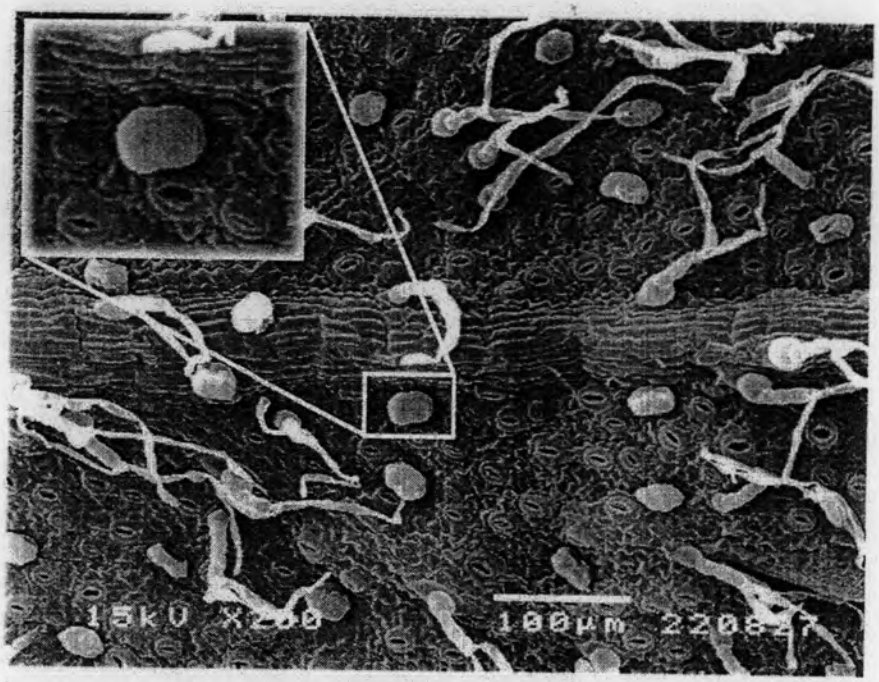


Figure 10 A scanning electron microscope view of *A. annua* leaf. Each microscopic oval is a specialized cell called trichome (insert), Bar=100 µm (unpublished, photograph by Thongchai Koob-kokkruad)

Upstream of dihydroartemisinic acid, the order of oxidations and reduction of artemisinic alcohol in route to dihydroartemisinic acid is still not settled. Bertea *et al.* (2005) have provided biochemical evidence for the fate of artemisinic alcohol in *A. annua* using GST cell-free extracts. Single step conversions labeled TS (for trichome supernatant) in Figure 10 have been reported (Beater *et al.*, 2005). While the data provided is confounded by the presence of endogenous intermediates, the results have been interpreted to indicate that the main pathway to dihydroartemisinic acid is via artemisinic aldehyde and dihydroartemisinic aldehyde, although the direct double bond reduction of artemisinic aldehyde has been not observed.

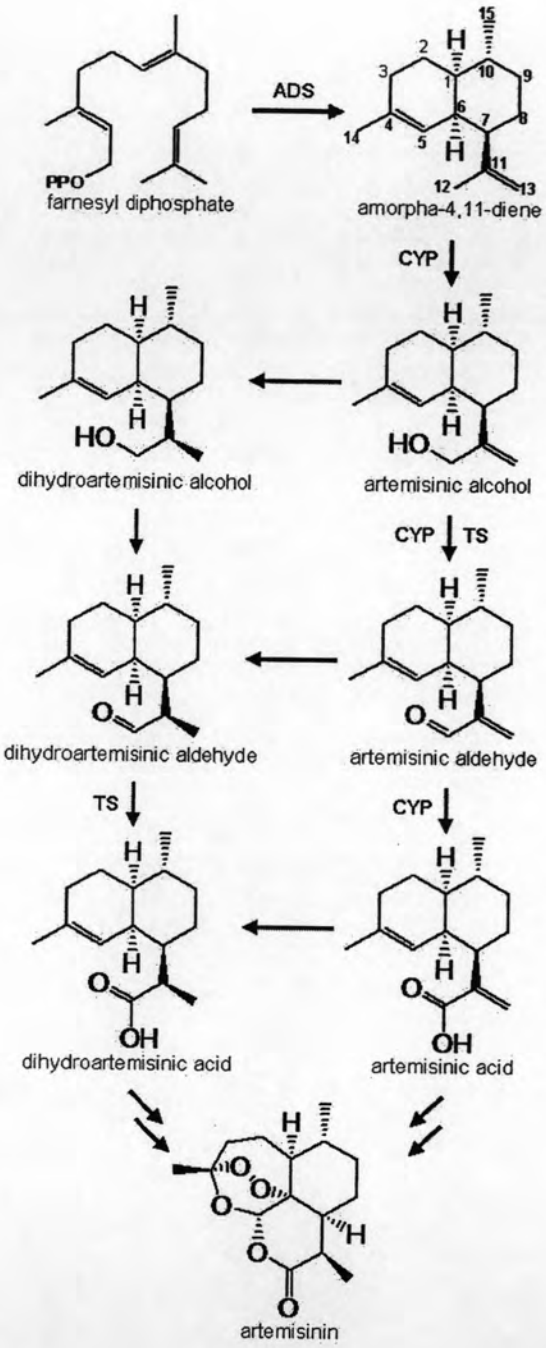


Figure 11 The proposed biosynthetic pathway of artemisinin from farnesyl diphosphate to artemisinin via amorpha-4,11-diene, artemisinin alcohol, arteminic aldehyde, dihydroartemisinic aldehyde, and dihydroartemisinic acid. Other possible intermediates are dihydroartemisinic alcoholand artemisinic acid (Covello *et al.*, 2007)."CYP", reactions catalyzed by CYP71AV1; "TS", Trichome Supernatant-catalyzed single step reactions supported by evididence from cell-free GST Extracts (Bertea *et al.*, 2005)

7. *Agrobacterium*-Mediated Genetic Transformation of Plants

Agrobacterium tumefaciens, specific of gram-negative soil bacteria in the family Rhizabiacea, are known for their pathogenesis in many plants. The infected plant then produces tumors or galls. The key of tumor production is a large (200 kb) circular DNA called the turmor-inducting (Ti) plasmid contains uncontrols growth genes, crown gall disease(Gaudin *et al.*, 1994), opine-catabolism genes. Opines are actually synthesized in the host plant under expression of T-DNA. The bacterium then uses the opines, amino acid derivatives used almost exclusively by the bacteria as a nitrogen source. In term of the genetic transformation process, the vir region, located on the *Agrobacterium* Ti plasmid, encodes most of the bacterial virulence (Vir) proteins used by the bacterium to produce its T-DNA and to deliver it into the plant cell. In wild-type *Agrobacterium* strains, the T-DNA region (defined by two 25 base pair direct repeats termed left and right T-DNA borders) is located in cis to the vir region on a single Ti plasmid. In disarmed *Agrobacterium* strains, where the native T-DNA region has been removed from the Ti plasmid, a recombinant T-DNA region usually resides on a small, autonomous binary plasmid and functions in trans to the vir region (Draper *et al.*, 1988). The transformation process begins with the bacterium–plant attachment (Figure 12; step 1), followed by induction of the expression of the vir region by specific host signals (Figure 12; steps 2 and 3). A single-stranded (ss) T-DNA molecule (T-strand) (Figure 12; step 4) is then produced by the combined action of the bacterial VirD1 and VirD2 proteins (Filichkin and Gelvin, 1993). In bacterial cells, the T-DNA exists as a ssDNA–protein complex (immature T-complex) with one VirD2 molecule covalently attached to the 50 end of the T-strand (Ward and Barnes, 1988). This complex, along with several other Vir proteins (Vergunst *et al.*, 2000), is exported into the host cell (Figure 12; step 5) by a

VirB/D4 type IV secretion system (Christie, 2004), a step that requires interaction of the bacterial T pilus with at least one host-specific protein (Hwang and Gelvin, 2004). Once inside the host-cell cytoplasm, the T-DNA is thought to exist as a mature T-complex (T-complex), in which the entire length of the T-strand molecule is coated with numerous VirE2 molecules. These molecules confer to the T-DNA the structure (Abu-Arish *et al.*, 2004) and protection (Citovsky, 1989) needed for its travel (Figure 12; step 6) to the host-cell nucleus. It is mainly during the last steps of the transformation process — namely, transport through the cytoplasm (Figure 12; step 6), nuclear import (Figure 12; step 7), intranuclear transport (Figure 12; step 8), T-DNA uncoating (Figure 12; step 9) and integration (Figure 12; step 10) that the *Agrobacterium* utilizes various cellular mechanisms to accomplish the genetic transformation of its host.

The application of *A. tumefaciens* for plant genetic engineering of plants, Ti plasmids are too large size and cannot be readily made smaller, because they contain few unique restriction sites. Consequently, a smaller, intermediate vector initially receives the insert of interest and the various other genes and segments necessary for recombination, replication, and antibiotic resistance. When engineered with the desired gene elements, this vector can be inserted into the Ti plasmid, forming a co-integrate plasmid that can be introduced into a plant cell by transformation. To produce transgenic plants, an intermediate vector of manageable size is used to clone the segment of interest. In the method, the intermediate vector is then recombined with an attenuated ("disarmed") Ti plasmid to generate a cointegrate structure bearing the insert of interest and a selectable plant kanamycin-resistance marker between the T-DNA borders, which is all the T-DNA necessary to promote insertion (L, left-hand region; R, right-hand region). Also spliced into the vector are a selectable bacterial

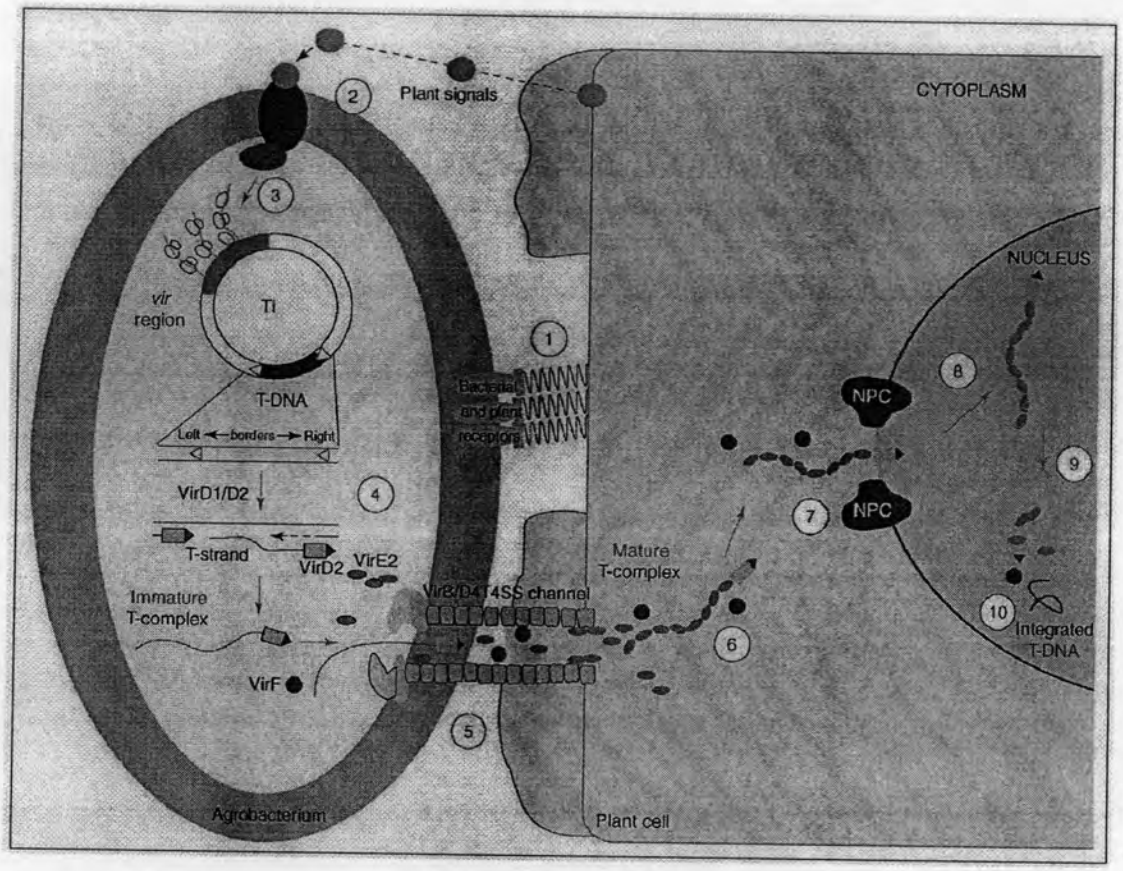


Figure 12 A model for the *Agrobacterium*-mediated genetic transformation (Tzfira and Citovsky, 2006), the transformation process comprises 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (2). Following activation of the vir gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10). A detailed model of the host cellular mechanisms and the role of plant-specific factors (from Tzfira *et al.*, (2004) with permission).

gene (spc^R) for spectinomycin resistance; a bacterial kanamycin-resistance gene (kan^R), engineered for expression in plants; and two segments of T-DNA. One segment carries the nopaline-synthesis gene *(nos)* plus the right-hand T-DNA border

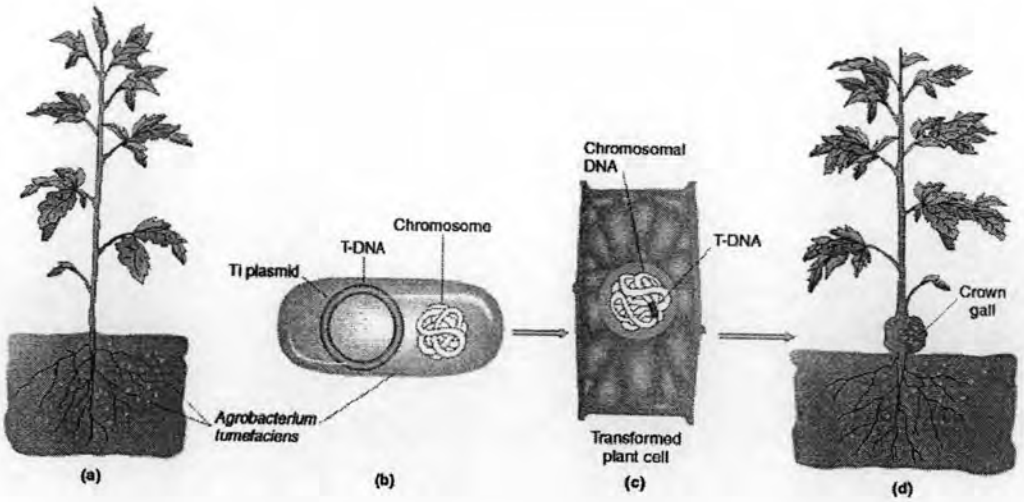


Figure 13 In the process of causing crown gall disease, the bacterium *A. tumefaciens* inserts a part of its Ti plasmid a region called T-DNA into a chromosome of the host plant.

sequence. The second T-DNA segment comes from near the left-hand border and provides a section for recombination with a homologous part of the left-hand region, which was retained in the disarmed Ti plasmid. After the intermediate vectors have been introduced into *Agrobacterium* cells containing the disarmed Ti plasmids (by conjugation with *E. coli*), plasmid recombinants (cointegrates) can be selected by plating on spectinomycin. The selected bacterial colonies will contain only the Ti plasmid, because the intermediate vector is not replicated in *Agrobacterium.* Then the antibiotic selected bacteria containing the plasmid are used to infect cut segments of plant tissue. When bacteria infected into plant cell, a part of plasmid between the leaf and right T-DNA border sequence can be inserted into the plant chromosome. For screen the transgenic plant, the plant tissues are then growing on medium containing kanamycin. The only plant cells that have the kan^R gene from T-DNA transfer will grow on the antibiotic medium. Then the plant cell can be induced to form shoots and roots, at which time they are transferred to soil where they developed into transgenic plants.

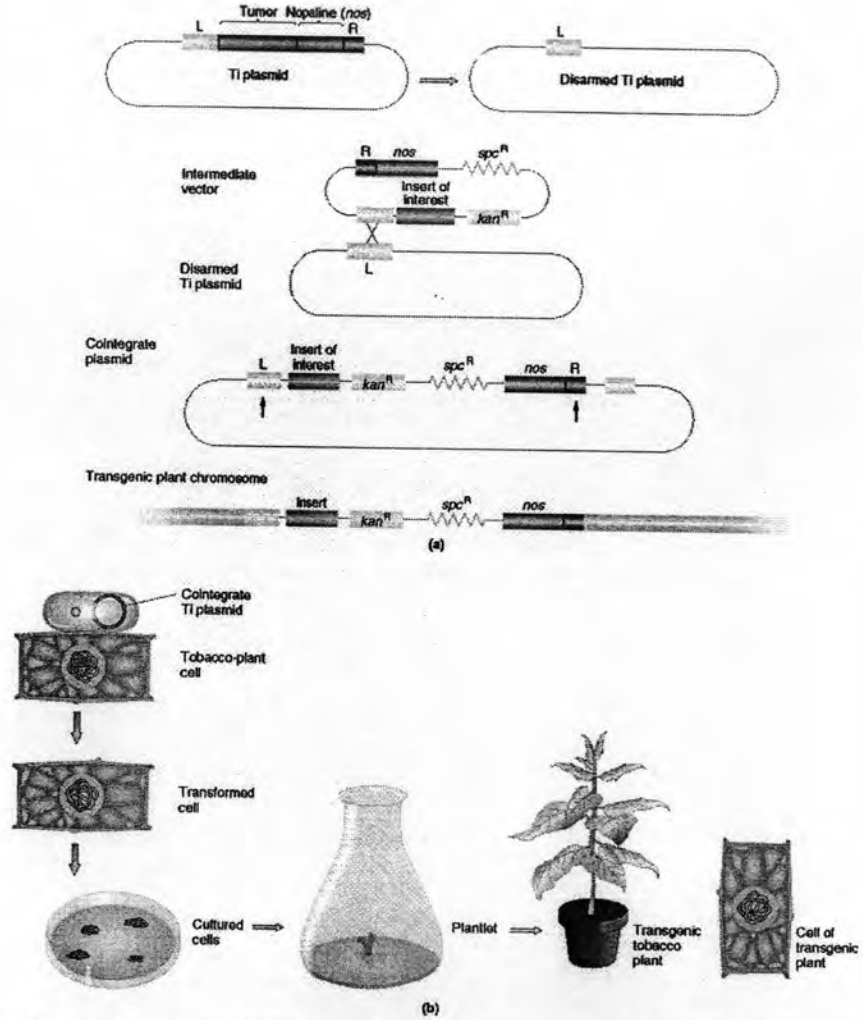


Figure 14 A construction of Ti plasmid for *A. tumefaciens*-mediated transformation (a) To produce transgenic plants, an intermediate vector of manageable size is used to clone the segment of interest. In the method shown here, the intermediate vector is then recombined with an attenuated ("disarmed") Ti plasmid to generate a cointegrate structure bearing the insert of interest and a selectable plant kanamycin-resistance marker between the T-DNA borders, which is all the T-DNA necessary to promote insertion. (L, left-hand region; R, right-hand region.) (b) The generation of a transgenic plant through the growth of a cell

2.7 Phytochemical Studies of *A. annua*

Studies on the phytochemical constituents in *A. annua* have started since 1977 (Allen *et al.*, 1977). A number of compounds have been isolated since then and are summerized in Table 3.

Table 3 Chemical constituents in *A. annua*

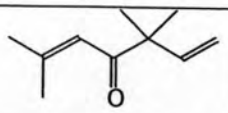
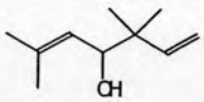
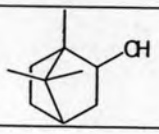
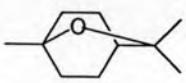
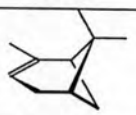
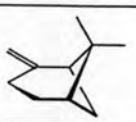
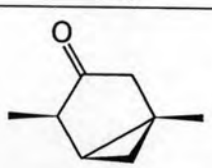
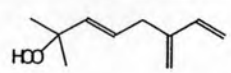
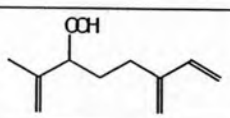
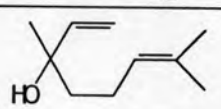
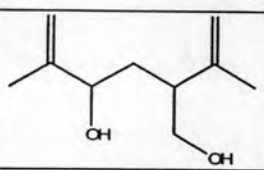
Chemical group	Chemical substance	Chemical structure	References
Monoterpene	Artemisia ketone		Allen *et al.*, 1977
	Artemisia alcohol		
	Borneol		
	1,8-cineole		
	α-pinene		
	β-pinene		
	Isothujone		
	α-myrcene hydroperoxide		Rucker *et al.*, 1987
	β-myrcene hydroperoxide		
	Camphor		Bouwmeester *et al.*, 1999
	Linalool		
	4-Hydroxy-2-isopropenyl-5-methylene-hexan-1-ol		Brown *et al.*, (2003)

Table 3 Chemical constituents in *A. annua* (continued)

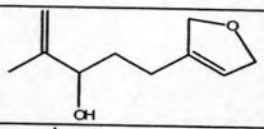
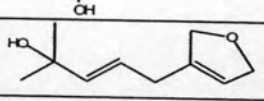
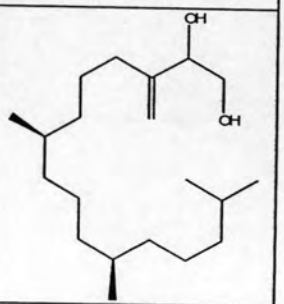
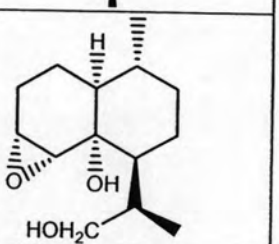
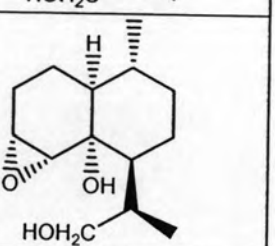
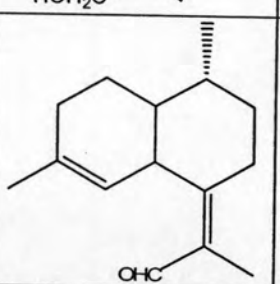
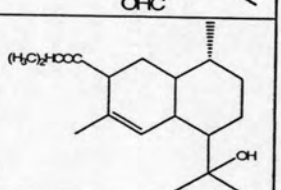
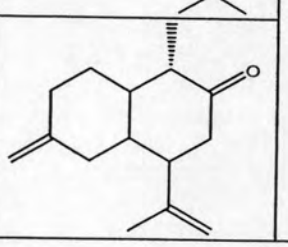
Chemical group	Chemical Substance	Chemical structure	References
Monoterpene	1,10-Oxy-α-myrcene hydroxide		Brown *et al.*, (2003)
	1,10-Oxy-β-myrcene hydroxide		
Diterpene	Phytene-1,2-diol		
Sesquiterpene	4α,5α-Epoxy-6a-hydroxy amorphan-12-ol		
	4α,5α-Epoxy-6a-hydroxy amorphan-12-ol		
	Cadin-4(7),11-dien-12-al		Ahmad and Misra, (1994)
	3-Isobutyrl cadin-4-en-l1-ol		
	Cudin-4(15),11-dien-9-one		

Table 3 Chemical constituents in *A. annua* (continued)

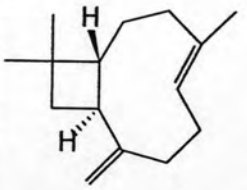
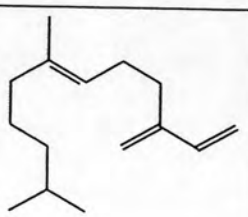
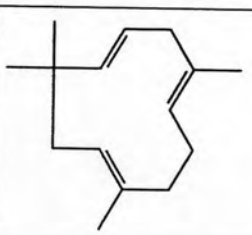
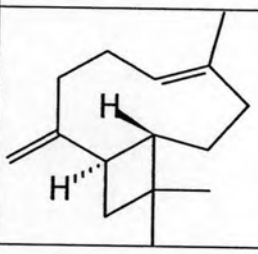
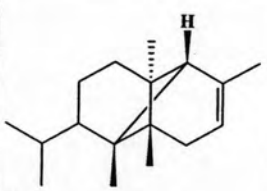
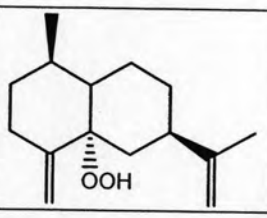
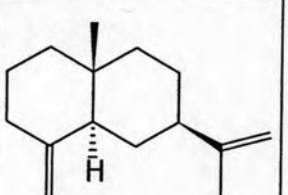
Chemical group	Chemical Substance	Chemical structure	References
Sesquiterpene	β-Caryophyllene		Simon *et al.*, 1990; Bouwmeester *et al.*, 1999
	Trans-β-farnesene		Bouwmeester *et al.*, 1999
	α-Humulene		
	β-Caryophyllene		
	α-Copaene		
	5α-Hydropreoxy-eudesma-4(15),11-diene		
	Selina-4,11-diene		

Table 3 Chemical constituents in *A. annua* (continued)

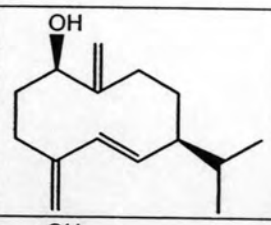
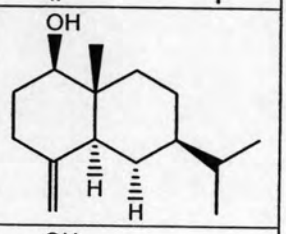
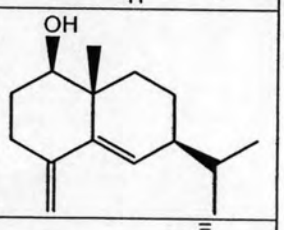
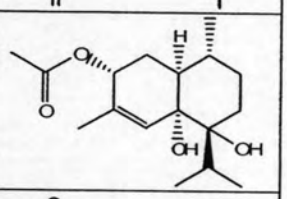
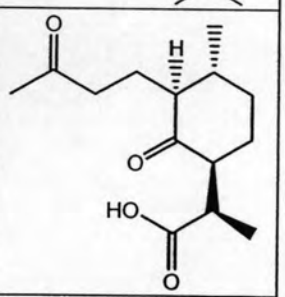
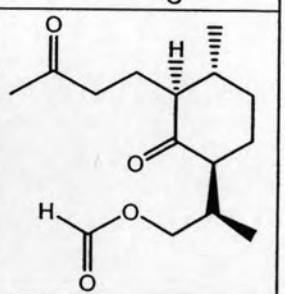
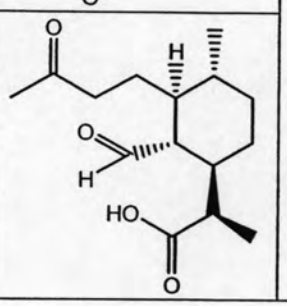
Chemical group	Chemical Substance	Chemical structure	References
Sesquiterpene	1β-Hydroxy-4(15),5E-10(14)-germacretriene		Brown *et al.*, 2003
	1β-Hydroxy-4(15),7-eudesmadiene		
	1β-Hydroxy-4(15),5-eudesmadiene		
	3α-7α-Dihydro amorpha-4-ene-3-acetate		
	1-Oxo-2β-[3-butanone]-3α-methyl-6β-[2-propanoic acid]-cyclohexane		
	1-Oxo-2β-[3-butanone]-3α-methyl-6β-[2-propanolformyl ester]-cyclohexane		
	1α-Aldehyde-2β-[3-butanone]-3α-methyl-6β-[2-propanoic acid]-cyclohexane		

Table 3 Chemical constituents in *A. annua* (continued)

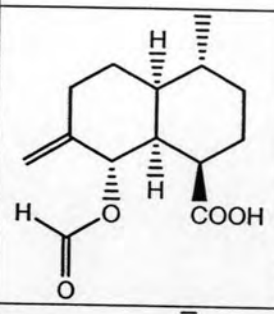
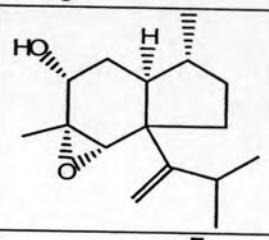
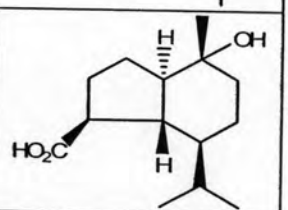
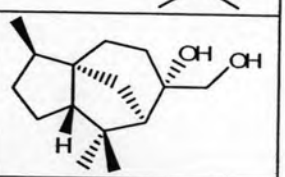
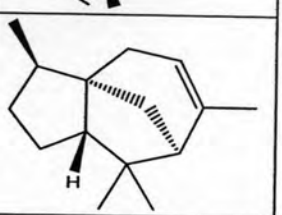
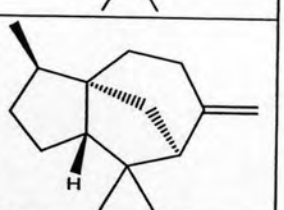
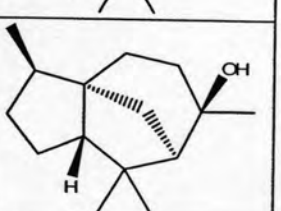
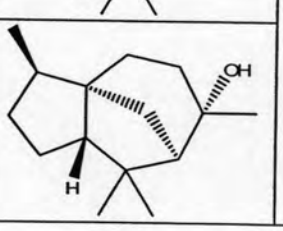
Chemical group	Chemical Substance	Chemical structure	References
Sesquiterpene	Norannuic acid formyl ester		Brown *et al.*, 2003
	3α-Hydroxy-4α,5α-epoxy-7-oxo-(8[7→6]-abeo-amorphane		
	15-Nor-10-hydroxy-oplopan-4-oic-acid		
	3α,15-Dihydroxy cedrane		
	(-)-α-Cedrene		
	(-)-β-Cedrene		
	(+)-Cedrene		
	(-)-epi-Cedrene		

Table 3 Chemical constituents in *A. annua* (continued)

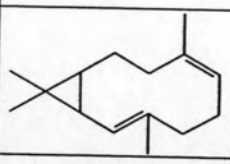
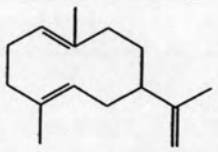
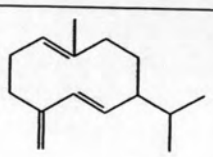
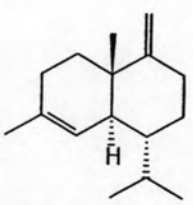
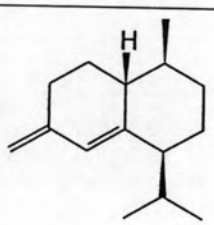
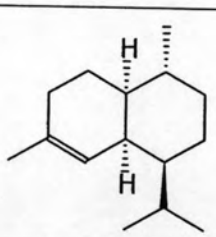
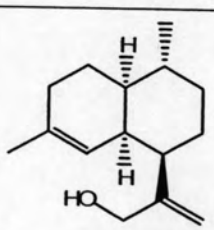
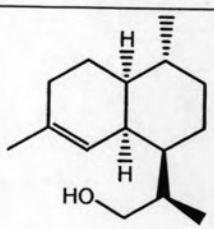
Chemical group	Chemical Substance	Chemical structure	References
Sesquiterpene	Biocyclogerma-crene		Bouwmeester *et al.*, 1999
	Germacrene A		
	Germacrene D		
	γ-cadinene		
	Muurola-4,11-diene		
	Amorpha-4,11-diene		
	Artemisinic alcohol		Bertea *et al.*, 2005
	Dihydroartemisinic alcohol		

Table 3 Chemical constituents in *A. annua* (continued)

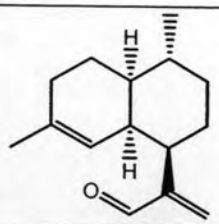
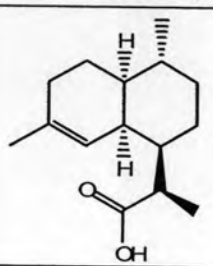
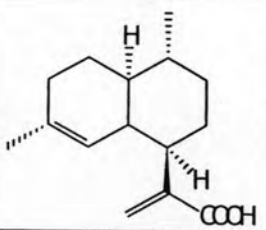
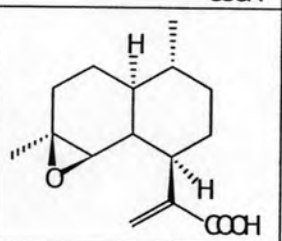
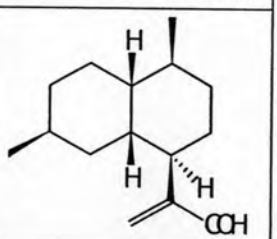
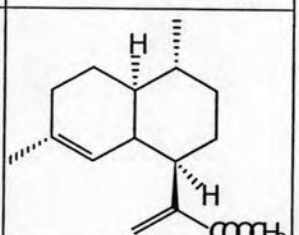
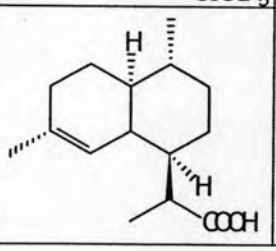
Chemical group	Chemical Substance	Chemical structure	References
Sesquiterpene	Artemisinic aldehyde		Bertea *et al.*, 2005
	Dihydroartemisinic acid		
	Artemisinic acid		Tu *et al.*, 1982; Roth and Acton, 1987
	Epoxyartemisinic acid		Tu *et al.*, 1982
	Artemisinol		
	Artemisic acid methylester		
	Dihydroartemisinic acid		Roth and Acton, 1989 ; Wallaart *et al.*, 1999b; Bertea *et al.*, 2005

Table 3 Chemical constituents in *A. annua* (continued)

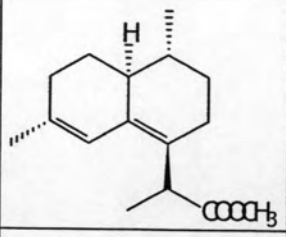
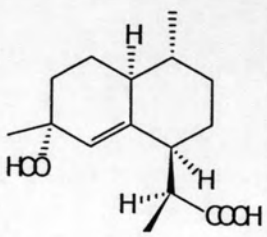
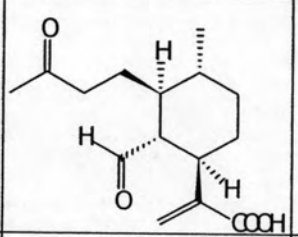
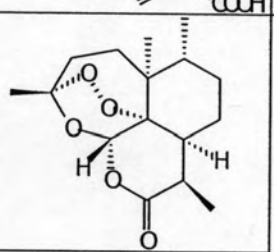
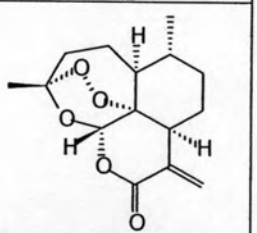
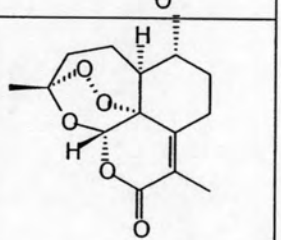
Chemical group	Chemical substance	Chemical structure	Reference
Sesquiterpene	6,7-dehydroartemisinic acid		El-Feraly *et al.*, 1989
	Dihydroartemisinic acid hydroperoxide		Wallaart *et al.*, 1999a
	Secocadinane		Browm 1994
	Artemisinin		Tu *et al.*, 1982; Klayman 1985 Acton and Klayman 1986
	Artemisitene		
	Isoartemisitene		

Table 3 Chemical constituents in *A. annua* (continued)

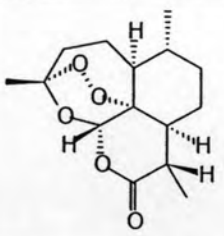
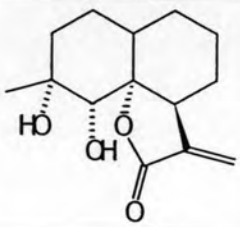
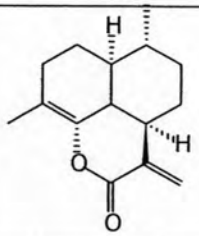
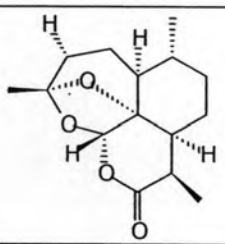
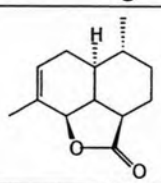
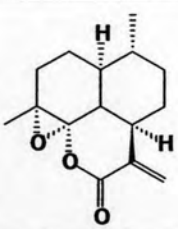
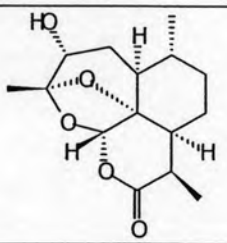
Chemical group	Chemical substance	Chemical structure	Reference
Sesquiterpene	9-epi-artemisinin		Tu *et al.*, 1982; Klayman 1985 Acton and Klayman 1986
	Dihydroxycadia-nolide		Browm 1994
	Epi-deoxyartean-nuin B		Roth and Acton 1987 El-Feraly *et al.*, 1989
	Deoxyartemisinin		Tu *et al.*, 1982
	Arteannuin A		
	Arteannuin B		
	Hydrodeoxyartemi-sinin		

Table 3 Chemical constituents in *A. annua* (continued)

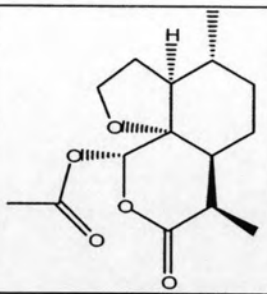
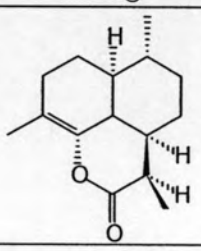
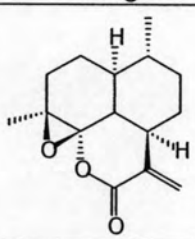
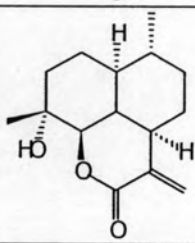
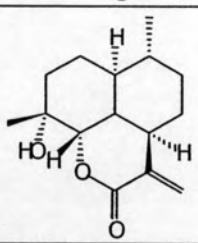
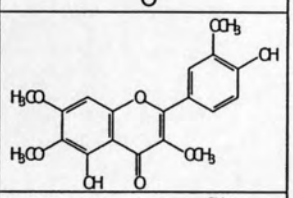
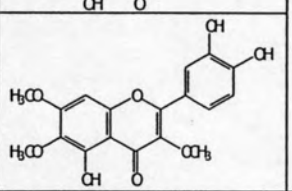
Chemical group	Chemical Substance	Chemical structure	Reference
Sesquiterpene	Artemisinin G		Brown *et al.*, (2003)
	Dihydro-epi-deoxyarteannuin B		Wallaart *et al.*, 1999
	Arteannuin C		Tu *et al.*, 1982
	Arteannuin E		
	Arteannuin F		
Flavonoid	5,4'-dihydroxy-3,6,7,3'-tetramethoxy-flavone		
	Chrysosphenol		

Table 3 Chemical constituents in *A. annua* (continued)

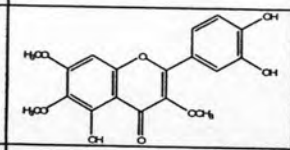
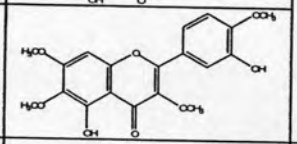
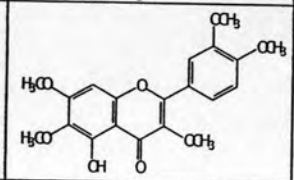
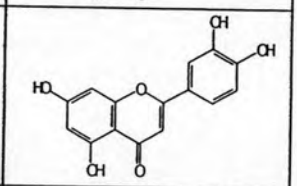
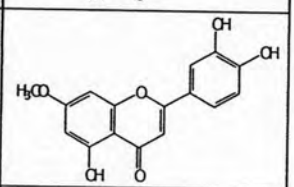
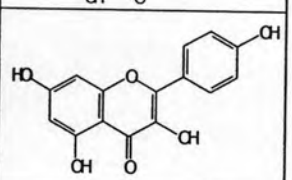
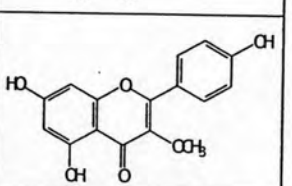
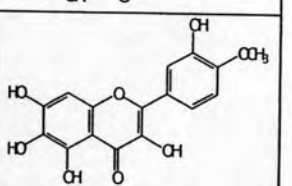
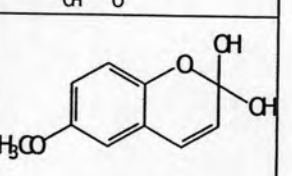
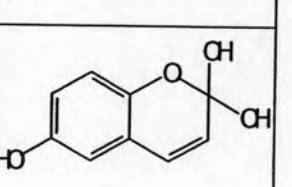
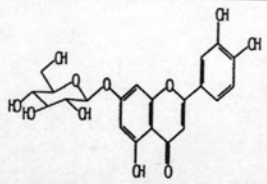
Chemical group	Chemical substance	Chemical structure	Reference
Flavonoid	Chrysosplenol D		Brown *et al.*, 2003
	Casticin		
	Artemetin		Yang *et al.*, 1995
	Luteolin		
	Luteolin-7-methylether		
	Quercetin		
	Quercetin-3-methylether		
	Quercetagetin-4'-methylether		
Chromene	2,2-dihydroxy-6-methoxychromene		
	2,2,6'-trihydroxy-chromene		

Table 3 Chemical constituents in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
Flavonoid glucoside	Luteolin-7-o-glucoside		Yang *et al.*, 1995
	Isoquercetin