ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURE OF *Artemisia annua* L. OF VIETNAMESE ORIGIN

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
LV	Litvay
BA	N_6 – Benzyladenine
NAA	1-naphthaleneacetic acid
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
GA_3	Gibberellins
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
rpm	Rotation per minute
ANOVA	Analysis of Variance
UPLC	Ultra-Performance Liquid Chromatography
PDA	Photodiode Array

PENUBUHAN KULTUR HALUS DAN AMPAIAN SEL *Artemisia annua* L. BERASAL DARI VIETNAM

ABSTRAK

Kultur halus daripada tiga klon *Artemisia annua* L. diinduksikan daripada eksplan daun plantlet aksenik. Medium asas MS dan LV ditambah dengan 0.5 mg/L BA, 0.5 mg/L NAA dan 0.5 g/L kasein hidrolisat telah digunakan sebagai medium induksi. Tiga klon *A. annua*, iaitu klon TC1, TC2 dan Highland menunjukkan perbezaan dalam pertumbuhan dan rupa halus. Medium asas MS didapati adalah medium induksi kultur halus yang lebih baik berbanding dengan medium asas LV. Secara umum pertumbuhan kultur halus adalah lebih baik dari segi hasil biojisim dan kerapuhan apabila diinduksikan dalam medium asas MS berbanding dengan medium asas LV. Keupayaan induksi kalus ketiga-tiga klon ini dalam medium MS yang ditambahkan dengan pikloram (0-2.0 mg/L) adalah berbeza. Klon Highland *A. annua* dipilih untuk digunakan dalam kajian seterusnya kerana kalus dapat diinduksikan dengan baik secara konsisten dalam medium MS yang ditambahkan dengan 0.5 mg/L BA, 0.5 mg/L NAA dan 0.5 g/L kasein hidrolisat, dan MS yang ditambahkan dengan 0.5 mg/L pikloram. Walau bagaimanapun, apabila kultur halus klon Highland *A. annua* disubkultur, medium MS yang ditambahkan dengan 2.0 mg/L pikloram didapati lebih sesuai sebagai medium proliferasi. Seterusnya kultur ampaian sel klon Highland *A. annua* ditubuhkan dalam medium cecair MS yang ditambahkan dengan 2.0 mg/L pikloram. Corak pertumbuhan sel *A. annua* menunjukkan bahawa pertumbuhan maksimum sel dicapai 18 hari selepas dikultur. Pertumbuhan sel dapat ditingkatkan dalam medium MS dengan kekuatan berganda sama ada dalam pencahayaan atau kegelapan. Fotokala 16 jam cahaya dapat merangsang pertumbuhan sel yang lebih tinggi berbanding fotokala lain. Elisitasi

dengan arginin (4, 6, 10 mg/L) juga boleh meningkatkan pertumbuhan sel. Manakala elisitasi dengan kasein hidrolisat (0-1.0 g/L) dan ekstrak yis (0-2.0 g/L) tidak menunjukkan perbezaan dari segi pertumbuhan sel pada hari-hari elisitasi yang berlainan. Elisitasi dengan 2.0 g/L ekstrak yis satu hari selepas inokulasi sel tidak diteruskan kerana berlaku kematian sel. Kesan elisitasi gabungan kasein hidrolisat (0-1.0 g/L) dan ekstrak yis (0-1.0 g/L) juga dikaji dalam keadaan pencahayaan dan kegelapan. Respon sel terhadap elisitasi gabungan adalah berbeza dalam pencahayaan dan kegelapan. Ini menunjukkan terdapat interaksi antara pencahayaan dan kesan elisitasi gabungan. Artemisinin tidak dapat dikesan dalam kultur halus and ampaian sel *A. annua* tetapi sesquiterpene dan sebatian lain yang tidak dikenali mungkin wujud. Sebatian-sebatian ini mungkin adalah sebatian perantara dalam biosintesis artemisinin.

ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURE OF *Artemisia annua* L. OF VIETNAMESE ORIGIN

ABSTRACT

Callus cultures from three clones of *Artemisia annua* L. were induced from the leaf explants of axenic plantlets. MS and LV basal media supplemented with 0.5 mg/L BA, 0.5 mg/L NAA and 0.5 g/L casein hydrolysate were used as induction medium. The three clones of *A. annua*, namely TC1, TC2 and Highland clones, showed difference in callus growth and appearance. MS basal medium was found to be the better callus induction medium as compared to LV basal medium. Generally callus growth was better in terms of biomass yield and friability when induced on MS basal medium than it was on LV medium. Callus induction capacity of the three clones using MS medium supplemented with picloram (0-2.0 mg/L) was different. *A. annua* Highland clone was selected for further study as the callus could be induced consistently well in MS medium supplemented with 0.5 mg/L BA, 0.5 mg/L NAA and 0.5 g/L casein hydrolysate, and MS supplemented with 0.5 mg/L picloram. However, when callus of *A. annua* Highland clone was subcultured, MS medium supplemented with 2.0 mg/L picloram was found to be suitable as proliferation medium. Subsequently cell suspension culture of *A. annua* Highland clone was established in liquid MS medium supplemented with 2.0 mg/L picloram. Growth pattern of *A. annua* indicated that maximum cell growth was achieved 18 days after culture. Cell growth could be enhanced in double strength MS medium either under illumination or darkness. Photoperiod of 16-hour light was able to stimulate higher cell growth as compared to that of other photoperiods. Elicitation with arginine (4, 6, 10 mg/L) also improved cell growth. While elicitation with casein hydrolysate (0-1.0

g/L) and yeast extract (0-2.0 g/L) did not show any distinct cell growth on different feeding days. Elicitation with 2.0 g/L yeast extract one day after cell inoculation was discontinued as cell death occurred. Effect of combined elicitation of casein hydrolysate (0-1.0 g/L) and yeast extract (0-1.0 g/L) was also studied under light illumination and darkness. Cells response to combined elicitation was distinct under light illumination and under darkness. This indicated that there was interaction between light illumination and combined elicitation effect. Artemisinin was not detected in the callus and cell cultures of *A. annua* but sesquiterpene and other unidentified compounds were probably present. This could possibly be intermediate compounds related to artemisinin biosynthesis.

CHAPTER ONE

INTRODUCTION

Artemisia annua L. (Family: Asteraceae, formerly Compositae), is an annual herbaceous plant native to Asia, most probably China (McVaugh, 1984). It is endemic to steppe vegetation in Chahar and Suiyuan provinces (40° N and 109° E) in northern China and distributed between 1000 to 1500 m above sea level (Wang, 1961). The generic name *Artemisia* refers to Artemis (Greek name for Diana), goddess of maternity, for since antiquity, plants from this genus has been used to regulate menstrual disorders, to control pangs of childbirth and as an abortifacient. Many ancient herbalists such as Theophrastus in the third century B.C., Pliny and Dioscorides in the first century, and Gerard in 1597, had recorded the existence of *Artemisia* species as reviewed by Riddle and Estes (1992).

Aerial parts of *A. annua* were prescribed as treatment for fevers in the Chinese Handbook of Prescriptions for Emergency Treatments in A.D. 340. In 1527, *huang hua hao* (identified as *Artemisia annua*) was used by Li Shi-Zhen, a Chinese herbalist to treat children's fever (Klayman, 1993). *A. annua* is an annual plant while most other species of this genus are either biennial or perennial. At least 40 volatiles can be found as constituents of the essential oil from *Artemisia annua*, making this species an interesting source of essential oil (Simon *et al.*, 1990, Charles *et al.*, 1991) However, its great significance in medicinal and thus economic interests is mainly due to it being probably the only species of *Artemisia* genus able to synthesize artemisinin (Baraldi *et al.*, 2008).

Artemisinin, a sesquiterpene lactone endoperoxide, is considered the most important secondary metabolite and bioactive constituent of *A. annua* (Heppner and Ballou, 1998, Van Agtmael *et al.*, 1999). This compound has been discovered in

leaves, small green stems, buds, flowers, seeds (Acton *et al.*, 1985, Zhao and Zeng, 1985, Liersch *et al.*, 1986, Singh *et al.*, 1986, Martinez and Staba, 1988, Madhusudanan, 1989, Ferreira and Janick, 1995b), but could not be detected in roots of field-grown plants or pollen (Pras *et al.*, 1991, Ferreira and Janick, 1995a). The greatest amount of artemisinin was found in the inflorescence. At anthesis, artemisinin content was more than 10 times the artemisinin found in leaves (Ferreira and Janick, 1995a). However other reports indicated otherwise. Baraldi *et al.* (2008) reported significantly higher artemisinin content in leaves than in inflorescences at full bloom. Liu *et al.* (2007) found that upper leaves of *A. annua* contained more artemisinin than in flower buds. Interestingly, leaves from different height of the plant gave varying amount of artemisinin (Delabays *et al.*, 2001, Liu *et al.*, 2007). Artemisinin has also been isolated from *in vitro*-grown plants (Nair *et al.*, 1986, Whipkey *et al.*, 1992, Ferreira and Janick, 1995b).

Artemisinin has been traditionally used to treat fevers, and haemorrhoids, in aromatic wreaths and currently as potent antimalarial drug (Ferreira *et al.*, 1997). Artemisinin, along with its derivatives is a proven treatment for a number of diseases besides malaria including hepatitis B and parasites that cause schistosomiasis (Dhingra *et al.*, 2000, Borrmann *et al.*, 2001, Utzinger *et al.*, 2001). Artemisinin has been reported to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon, and small-cell lung carcinoma (Efferth *et al.*, 2001, Singh and Lai, 2001) as well as drug-resistant cancers (Efferth *et al.*, 2002, Sadava *et al.*, 2002). Furthermore, it is relatively a safe drug with no obvious adverse reactions or side effects, even for pregnant women (WHO, 2003).

Malaria, one of the most devastating diseases in the tropical world, has been recorded in as far back as the sixteenth century B.C. in Egypt. In the fifth century,

followers of Hippocrates observed a connection of the disease to marshes (Klayman, 1985). In 1989, Ronald Ross, an English physician working in India, and Giovanni Battista Grassi, an Italian physician, has independently proven that the disease was spread by the bite of the female *Anopheles* mosquito (Cox, 2002). Malaria is a serious endemic disease in many part of the world, affecting 5% of the world population. About 40% of the world population is at stake of malaria. Four major *Plasmodium* species, namely *P. falciparum, P. vivax, P. malariae* and *P. ovale* are known to cause human malaria. *P. falciparum* being the most prevailing parasite species across the globe which causes cerebral malaria that is often fatal to children (Wirth *et al.*, 1986). Epidemiological data estimated that there were 221 million *P. falciparum* attacks in Africa in 1995 (Snow *et al.*, 1999). In 2002 the estimate was 365 million clinical cases in Africa (Snow *et al.*, 2005), followed by 119 million cases estimated in South East Asia. *P. falciparum* were mainly focused in the African region (70%), but the highly populated South East Asia region contributed 25% of the world's clinical attacks in 2002. Approximation of world population at risk was 2,211 million and 515 million clinical cases.

Fighting malaria is ridden with technical, logistic and economical problems. Malaria poses a major setback to the economy of developing tropical countries and successful control of this disease is important for improved world health (Wirth *et al.*, 1986). Chloroquine has been the mainstay of antimalarial drug treatment for the past 40 years, but resistance is now widespread (Bloland *et al.*, 1993). Resistance to pyrimethamine-sulphadoxine (PSD) as a successor to chloroquine developed as well (White *et al.*, 1999). And similar scenario occurred with the use of mefloquine in southeast asia (Van Vugt *et al.*, 1998) . The World Health Organization (WHO) has endorsed artemisinin-based combination therapy (ACT) as the "policy standard" for

treating malaria infections caused by *P. falciparum* (RBM, 2004, Davis *et al.*, 2005a). Artemisinin increases efficacy and has the prospective of slowing the speed of emerging and spread of resistance (Mutabingwa, 2005). International efforts have been made through programmes such as the Global Burden of Diseases to enumerate malaria-related health consequences (Murray and Lopez, 1997, WHO, 1999), the Roll Back Malaria initiative to cut the burden of malaria within six years (WHO, 1999) and the Millennium Development Goal's target to put a stop to increasing malaria cases by 2015 (UNDP, 2003), clearly reflect the magnitude of the malaria plague and the urgency to address the prevalence of this disease. Increasing severity of this disease and death rate resulting from it is compounded by continuous infestation and spread of resistance to antimalarial drugs among *Plasmodium* parasites. Even though immunity to malaria is acquired after several years of recurring infections, it remains partially effective. An effective vaccine is the best long term control option for malaria. But till date, vaccine development remains at pre-clinical stage (Dhingra *et al.*, 2000). As effective antimalarial vaccine is not available and occurrence of drug-resistant parasites is increasing, the need to develop novel antimalarial drugs to curb the spread of this disease becomes more urgent.

At present, artemisinin forms the basis of a large family of drugs recommended by World Health Organization (WHO) to fight against malaria. Artesunate, the main derivative of artemisinin, has been suggested to be employed as prescription for severe illness of pregnant women and children in low transmission area (Dondrop and Day, 2007). However, low and inconsistent yield of artemisinin, 0.01 – 0.08% (Simon *et al.*, 1990, Abdin *et al.*, 2003,) from field cultivation depending on plant materials , genotype, and seasonal and geographic variations hamper efforts to produce sufficient artemisinin to meet the annual global demand of

20 – 22 t yr^{-1} (Charles *et al.*, 1991, van Geldre *et al.*, 1997, Bhakuni *et al.*, 2001, WHO, 2005, Vaz *et al.*, 2006).

Ravindranathan (1990) has described various methods of chemical synthesis of artemisinin. However, such *de novo* synthesis is complicated, uneconomical and provides low yield of artemisinin suggesting that plant source might be a valid alternative (Dhingra *et al.*, 2000). Artemisinin yield from field cultivation in India was reported by Singh *et al.* (1986) to be 3.7 kg/ha for Washington strains of *A. annua*. Such yield from field is far too low as compared to 15 kg of artemisinin per ha yield for commercial purpose. Assuming that in future, the maximum artemisinin yield per ha is 20 kg, enough for 15,000 to 20,000 applications (Woerdenbag *et al.*, 1990), and to estimation that 500 million people are treated against malaria yearly (Snow *et al.*, 2005), an area of 25,000 ha (250 km^2) of *A. annua* plantation could be sufficient to provide the necessary amount of artemisinin to treat these patients.

Therefore the enhanced production of artemisinin via biotechnological techniques has been considered as a viable approach. *In vitro* propagation of *Artemisia annua* using shoot cultures has been reported (Nair *et al.*, 1986, Martinez and Staba, 1988, Elhag *et al.*, 1992, Woerdenbag *et al.*, 1993). Callus culture was established in medium supplemented with auxin and cytokinins combinations (Martinez and Staba, 1988, Ferreira and Janick, 1996, Rao and Narasu, 1998). Cell suspension culture had also been established from callus culture (Woerdenbag *et al.*, 1993, Chan and Nallammai, 2001, Nallammai and Chan, 2002, Baldi and Dixit, 2007). However, presence of artemisinin in callus and cell cultures has been contradictory, (Woerdenbag *et al.*, 1991, Elhag *et al.*, 1992, Woerdenbag *et al.*, 1993, Paniego and Giulietti, 1994).

Considering the importance of artemisinin which is tedious and difficult to synthesize chemically, it is important to identify the best clone of *A. annua* with high artemisinin content. Therefore the present study was conducted to serve the following aims:

a) to identify the suitable medium for callus induction and callus growth for three clones of *Artemisia annua,* of Vietnam origin, namely TC1, TC2 and Highland
b) to select one clone of *Artemisia annua* for cell suspension study based on growth and friability of callus of the three different clones
c) to study the effect of physical and chemical factors on the growth of cells in the cell suspension culture
d) to optimise the cell culture condition for improved cell growth and artemisinin yield by combining both the physical and chemical factors

CHAPTER 2

LITERATURE REVIEW

2.1 *Artemisia annua*

2.1.1 *Artemisia* species

Artemisia annua belongs to the *Artemisia* genus which consists of about 400 species worldwide (Heywood and Humphries, 1977). *Artemisia* genus is part of the Astereacea, a big family consists of more than 1,600 genera and 23,000 species. This family is the second largest family of flowering plants in terms of number of species. It includes ornamental plants that produce beautiful asters, garden perennials of which many have medicinal values and fragrance. This family has been widely known and placed in the order Asterales (Ferreira *et al.*, 1997).

The genus *Artemisia* comes under the the Anthemideae tribe of the Asteroideae, a subfamily of the Asteraceae. *Artemisia* is a fairly large genus with 100 to 200 known species and many are commonly known as some form of sagebrush or wormwood such as silver sagebrush (*Artemisia cana* Prush), birdfoot sagebrush (*Artemisia pedatifida* Nutt.) and Alaska wormwood (*Artemisia alaskana* Rydb.)(Greger *et al.*, 1986).

Species of *Artemisia* are usually shrubs, with divided leaves and inconspicuous flower. *A. annua* is a large shrub, usually single-stemmed with alternate branches. growing more than 2 m height. Its aromatic leaves are deeply dissected, ranging from 2.5 to 5 cm in length. The chromosome number is 2n = 18 (Bennett *et al.*, 1982).

2.1.2 Floral Biology

A. annua is a determinate short-day plant. Non-juvenile plants are very responsive to photoperiodic stimulus and flowering occurs two weeks after induction. Non-juvenile plants in greenhouse studies, flowered after being subjected

to 8-, 10-, or 12-hour photoperiods for two weeks, but remained vegetative under 16-, 20-, 24-hour photoperiods. In field studies conducted in northern temperate zone, the first flower buds were produced when day length was 12:57 hour. Greenhouse data further implied that flowering occurred two weeks earlier when day length was 13:31 hour (Ferreira and Janick, 1995a).

The greenish-yellow nodding flowers (capitula) are 2 to 3 cm in diameter, with calyses composed of numerous, imbricated bracts. These capitula contain numerous bisexual florets in the centre and pistillate marginal florets with stigma extrusion. Both flowers have synpetalus tubular corolla with the top split into 5 lobes in the hermaphroditic florets and into 2 to 3 lobes in the pistillate florets. The bifid stigma, also known as pollen presenter has modified acuminate epidermal cell that assist in pollen capture. Active pollen presenter in Asteraceae is characterized by growing style pushing the pollen presenter past the anthers. Pollens from the anthers are then collected and extruded for presentation and dispersion by wind. The five stamens have bilocular anthers, attached to the bottom of the corolla, inferior to the top of the style. Each stamen has a lanceolate appendix at the top, which alternates with the lobes of the corolla (Ferreira and Janick, 1995a). Ovaries are inferior and unilocular, each generating one achene, 1 mm in length and slightly nerved. Typical in anemophylous species, the pollen is tricolpate and relatively smooth with internal collumellae-tecta complex configuration in the exine. The pollen is extremely allergenic, as in other *Artemisia* species due to the presence of allergenic proteins on the exine surface (Park et al., 1993).

Insects and wind action help to cross-pollinate this plant. This method is rather unusual in the Asteraceae (McVaugh, 1984). Based on light and scanning electron microscopy examination on floral morphology, the capitula was thought to

be adapted for self-pollination (Ferreira and Janick, 1995a) but experimental data showed that self-pollination is rare and difficult to achieve, inferring the presence of self-incompatibility in *A. annua*, a common feature among members of Asteraceae family (North, 1979).

2.1.3 Glandular Trichomes

The presence of trichomes and glands containing volatile monoterpenes and sesquiterpenes are common in the Asteraceae family (Mehrotra *et al.*, 1990). Duke and Paul (1993) found biseriate glandular trichomes in the leaves of *A. annua* at the earliest stage of development but later become obscured by filamentous T-trichomes. These two types of trichomes are arranged in two rows along either side of the leaf midrib. On the abaxial surface of leaf and stems, these trichomes are arranged randomly. Glandular trichomes are also found on florets of *A. paniculata*, *A. Mauiensis* and a range of other species. Kelsey and Shafizadeh (1980) reported biseriate glandular trichomes on both leaf surfaces of *A. nova*. This type of trichome is found on floral stalks of *A. tridentata* (Slone and Kelsey, 1985), on both leaf surfaces and ovary surface of *A. umbelliformis* (Cappelletti *et al.*, 1986) and in the adaxial surface of *A. campestris* (Ascensao and Pais, 1987).

Duke and Paul (1993) has provided very detail description on the origin of biseriate glands in leaves by using light, scanning and transmission microscope. The earliest stage of gland formation observed is the 1-cell stage, in which the single epidermal cell enlarges and protrudes above the leaf surface. This cell expands and divides anticlinally and again, periclinally into four cells. Throughout these stages, vacuoles are relatively small and only few unstacked thylakoids are found in proplastids. At 6-cell stage, chloroplasts are present with stacked thylakoids with

absence of starch grains. Lack of starch grains is the only distinguishing feature between chloroplasts of glands and mesophyll tissues. Further periclinal division of the two apical cell layers results in the final 10-cell stage. After all 10 cells are formed, the cuticular surface of the gland begins to separate from the cell wall, near the tip of the gland. The onset of cuticular detachment is believed to be due to onset of secretory activity. Subcuticular space borders the six apical cells of the gland. Cytoplasm is denser at this stage than earlier, and the two basal cells contain chloroplasts and relatively large vacuoles. The apical cell pairs have no chloroplasts and the subapical 2-cell pairs contain large, amorphous chloroplasts without starch grains.

Ten-celled biseriate glandular trichomes are abundant in the bracts, receptacles and florets of the capitulum in *A. annua*. These glands which can be found on both both pistillate and hermaphroditic florets from earliest developmental stages are easily ruptured, releasing volatile oils when the florets are pressed. The floral biseriate trichomes are formed in the same way as leaf glandular trichomes.

Due to absence of artemisinin and artemisitene in glandless biotype of *A. annua,* Duke *et al.* (1994) concluded that the subcuticular spaces of the biseriate glandular trichomes in leaves are the sites of sequestration of these compounds. Extraction of artemisinin and artemisitene can be done from 5-second leaf dip in chloroform, without visible damage to other leaf epidermal cells. Artemisinin can also be extracted by a 1-minute dip of inflorescences in petroleum ether or acetonitrile (Ferreira and Janick, 1995a)

2.2 Chemical constituents of *Artemisia annua*

Extensive search for new antimalarial drugs which began in 1967, following developing resistance of *Plasmodium falciparum* to chloroquine in 1960s, led to isolation of an active malarial fraction from *Artemisia annua* in 1971. The biologically active compound was isolated and named artemisinin in 1972 and the structure was elucidated in 1979. Screening of artemisinin in other *Artemisia* species (Rucker *et al.*, 1986, Klayman *et al.*, 1984, O'neill *et al.*, 1985, Liersch *et al.*, 1986, Balachandran *et al.*, 1987) revealed that only *Artemisia apiacea* contains artemisinin other than *A. annua* (Liersch *et al.*, 1986).

Besides artemisinin, other related sesquiterpene lactones are arteannuic acid (Tu *et al.*, 1982), 6,7-dehydroarteannuic acid (El-Feraly *et al.*, 1989), arteannuin B (Tu *et al.*, 1982, Jeremic *et al.*, 1973), arteannuin C (Misra, 1986), and epi-deoxyartemisinin B (Roth and Acton, 1987, El-Feraly *et al.*, 1989), artemisitene (Acton and Klayman, 1985) and deoxyartemisinin (Xu *et al.*, 1986). The presence of guaianolides and seco-guaianolides were also reported by Huneck *et al.* (1986).

The essential oil in this plant is mainly found in its leaves and has limited perfumery and pharmaceutical value (Lawrence, 1982). Composition of this essential oil includes sesquiterpenes and mainly monoterpenes. The primary component is artemisia ketone, whose structure deviates from classic isoprene (Allen *et al.*, 1977). Other monoterpenes which are found abundantly in the essential oil are borneol, 1,8-cineole, α-pinene, thymol, carvacrol and camphor. Among the sesquiterpenes, caryophyllene and caryophyllene oxides are the most important (Banthorpe *et al.*, 1983, Lawrence, 1982, Risinger *et al.*, 1978). There was also report on the presence of α-myrcene and β-myrcene hydroperoxides (Rucker *et al.*, 1987). Furthermore, *A. annua* contains flavonoids (Baraldi *et al.*, 2008, Bhardwaj *et al.*, 1985, Jeremic *et al.*, 1973, Misra, 1986, Shilin *et al.*, 1989), long chain hydrocarbons (Ulubelen and

Halfon, 1976), acetylens (Bohlmann and Zdero, 1972) and coumarins (Anon., 1982a, Shilin *et al.*, 1989).

2.2.1 Chemical Properties of Artemisinin

Artemisinin is a rare sesquiterpene lactone endoperoxide, which can be extracted from *A. annua*. This novel compound was isolated for the first time in 1972 (Anon., 1979, Anon., 1982b) and its structure was later established using combined spectral, chemical and X-ray analysis (Zhongshan *et al.*, 1985, Liu *et al.*, 1979, Blasko *et al.*, 1988, Leban *et al.*, 1988). Sesquiterpene lactones are known for wide varieties of biological activities such as cytotoxic and antitumour activities. These group of compound also show antibiotic properties, act as phytotoxins, mammalian toxins, insect-feeding deterrents as well as causing allergic contact dermatitis (Rodriguez et al., 1976, Picman, 1986).

Zeng *et al.* (1983) reported that artemisinin is sensitive to acid and base treatment, yet stable when heated in neutral solvents at 150 °C for 2.5 min. Klayman *et al.* (1984) found out that 20% artemisinin is destroyed when it is subjected to 48-hr boiling in ethanol, whereas similar boiling treatment in isopropanol did not lead to compound decomposition. Lin *et al.* (1985) revealed that no artemisinin can be detected after heating at 190 °C for 10 min, even though the molecule is not affected at 200 °C heating temperature, above its 156-157 °C-melting point. Cleavage of peroxide bridge results in thermal decomposition, forming free radical intermediates, which either rearrange or decompose. In another study on thermal decomposition of artemisinin, Luo *et al.* (1985) refluxed artemisinin in xylene for 22 hours, causing breakage of 30% of the peroxide group. It is also light stable (Anon., 1982b)

Its empirical formula is $C_{15}H_{22}O_5$. It is colourless and odourless. It forms crystals with melting point of 156 - 157°C. Its molecular weight is m/e 282.1742 m+ as determined by high resolution mass spectroscopy (Luo and Shen, 1987).

2.2.2 Artemisinin Content in the Plant

Artemisinin is sequestered in the glandular trichome on the biseriate leaves and inflorescenses (Duke *et al.*, 1994, Ferreira *et al.*, 1997). Glandular trichomes can be found in early stage of flower formation, but artemisinin content was maximum at full blooming stage (anthesis).

The content of artemisin was higher in leaves than in inflorescences when flowers approach full bloom (Baraldi *et al.*, 2008). The presence of polymethoxyflavonoids in the phytocomplex enhances the bioavailability of artemisinin (Elford *et al.*, 1987). Castisin, a flavonoids discovered in *A. annua*, was found to inhibit some cytophysiological activities of *Plasmodium falciparum* (Yang *et al.*, 1995)

Highest level of artemisinin from cultivated plants is 0.01 to 1.4% dry weight, (van Agtmael *et al.*, 1999). Artemisinin yield is usually low and varies according to different plant parts, growing conditions, and seasonal and geographic variations (Abdin *et al.*, 2003). Variation in artemisinin concentration can be attributed to different stages in vegetative growth and diverse plant parts between strain origin (Laughlin *et al.*, 2002). There were inconsistent reports on the developmental stage at which artemisinin content is the highest: just before flowering (ElSohly et al., 1990, Acton and Klayman, 1985, Woerdenbag et al., 1991) or at the point of full flowering (Pras *et al.*, 1991, Ferreira and Janick, 1995, Morales *et al.*, 1993). Later in the season, due to loss of leaves, lower part of the plant contains only little artemisinin

(Acton and Klayman, 1985, Liersch *et al.*, 1986, Singh *et al.*, 1986). ElSohly *et al.* (1987) and Roth and Acton (1987) reported an overall drop in artemisinin content when leaves disappeared as flowers developed.

Baraldi *et al.* (2008) reported artemisinin content of 0.04 to 0.05% of plant dried weight from Italian variety. Artemisinin content from plants of other European origin was reported to be 0.03 to 0.22% (Trigg, 1990, Charles *et al.*, 1991,). Concentrations in plants of China origin vary in the range of 0.01 – 0.5 % based on dry weight (Klayman, 1985). A variety, probably Asia origin, grown in the USA contained 0.06% artemisinin in dried leaves and flowers but plant stems were devoid of this compound (Klayman *et al.*, 1984).

Artemisinin and its precursors were not found in the roots (Abdin *et al.*, 2003). Ferreira *et al.* (1995) reported artemisinin content in flowers was 4 – 5 times than in leaves. Artemisinin content also differ with plant development, indicating a possible correlation between plant age and artemisinin yield.

2.2.3 Biosynthesis of Artemisinin

The complete biosynthetic pathway of artemisinin and some of its precursors has yet to be fully understood. Comprehensive biosynthetic knowledge for artemisinin production is critical in increasing the compound yield by biotechnological means (Dhingra *et al.*, 2000).

Artemisinin belongs to the sesquiterpenes group, whose precursor is farnesyl diphosphate, FDP (C15). FDP is synthesized from mevalonic acid diphosphate via isopentenyl diphospahate (IPP) and geranyl diphosphate (Akhila *et al.*, 1987). There are two independent pathways leading to IPP formation: mevalonate pathway (MVA) stemming from acetyl CoA, and the mevalonate independent pathway (MEP)

originating from pyruvate. IPP and its isomer, dimethylallyl diphosphate (DMAPP) then lead to other terpenoids. Prokaryotes and eukaryotes use different pathway for IPP production but plants use both pathwyas (Croteau *et al.,* 2000). MVA pathway in plants occurs in the cytosol, while MEP pathway is localised in plastids. According to Adam and Zapp (1998) and Laule *et al.* (2003), there is evidence for possible exchange of IPP between these two pathways. In MEP pathway, IPP is derived from condensation of pyruvate with D-glyceraldehyde-3-phosphate. Deoxy-D-xylulose-5-phosphate synthase (DXS) catalyses the condensation of pyruvate and D-glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose-5-phosphate. In the following step, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) converts 1-deoxy-D-xylulose-5-phosphate to 2-C-methyl-D-erythritol-4-phosphate (Mahmoud and Croteau, 2001, Souret *et al.*, 2002, Souret *et al.*, 2003). In the MVA pathway, mevalonic acid is produced from the reduction of 3-hydroxy-3-methylglutaryl (HMG)-Co-A by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Mahmoud and Croteau, 2001, Souret *et al.*, 2002, Souret *et al.*, 2003).

Post-FDP production pathway is not yet completely elucidated. However it is clear that the first critical step in the artemisinin biosynthesis is the cyclization of FDP to amorpha-4,11-diene by amorphadiene synthase (ADS) (Bouwmeester *et al.*, 1999). Cytochrome 450 enzyme was believed to be responsible for the conversion of amorpha-4,11-diene to artemisinic alcohol. Further oxidation by either cytochrome 450 enzyme or dehydrogenase produces artemisinic acid. The double bond between C11 and C13 of artemisinic acid is thought to be reduced to dihydroartemisinic acid. Dihydroartemisinic acid is then further modified to artemisinin non-enzymatically (Abdin *et al.*, 2003, Wallaart *et al.*, 1999)

2.2.4 Antimalarial Activities of Artemisinin and its Derivatives

Artemisinin has proven to be effective in clinical trials in 1980s and semi-synthetic derivatives has been developed to improve the pharmalogical and antimalarial properties (Hien and White, 1993, Klayman, 1985). The unique 1,2,4-trioxane structure of artemisinin is entirely incompatible with the traditional anti-malarial structure-activity theory (Wu, 2002). It was suggested that the mode of action of these types of agents is altogether different from those of the traditional alkaloidal antimalarial agents (Anon, 1982a). The actions of artemisinin derivatives is different from that of the other antimalarial drugs, although both the artemisinin drugs and the 4-aminoquilines interact with haem (Meshnick *et al.*, 1989, Meshnick *et al.*, 1993). Most of the antimalarials show bioactivity at the late trophozoite and schizont stage, but artemisinin derivatives act at early trophozoite and ring stages. Ring stage refers to presence immature trophozoites in the erythrocytes following the invasion of schizont released from ruptured liver cells. Therefore artemisinin only affects blood-stage parasites, not liver-stage parasites or stages within mosquito, making their action stage specific (Kamchonwongpaisan and Meshnick, 1996). They are also gametocytocidal (Dutta *et al.*, 1990, Mehra and Bhasin, 1993).

Studies in monkeys have proven that artemisinin is effective in impairing life cycle and thus transmission of *Plasmodium cynomolgi B* (simian malaria) within 18 to 24 hours even in single intramuscular doses (Dutta *et al.*, 1989). Pharmacological studies and clinical observations in every type of malaria infection showed that artemisinin had direct parasiticidal action on *Plasmodium* in the erythrocytic stage but is ineffective in the exoerythrocytic, liver stage (Ferreira *et al.*, 1997). Artemisinin derivatives are highly effective against sexual and asexual forms of human-infectious *Plasmodium* species due to their most rapid initial reduction in

parasitaemia as compared to other antimalarial drugs (Hien and White, 1993, de Vries and Dien, 1996, Chen *et al.*, 1998). The endoperoxide moiety in artemisinin structure was thought to cause destructive free-radical generation within the parasite and alters the roles of key parasite proteins (Meshnick, 1998, Eckstrein-Ludwig *et al.*, 2003). This is in agreement with the observation that derivatives of artemisinin are devoid of antimalarial activity in the absence of peroxide moiety (Anon, 1982b). Several studies had shown support for oxygen-mediated toxicity. Drugs, such as miconazole and doxorubicin, were assumed to exert their effect by increasing oxygen stress, thus enhancing artesunate activity. Contrary, artemisinin activity was reduced by catalase, dithiothreitol and α-tocopherol (Krungkai and Yuthavong, 1987). Reactive oxygen intermediates were produced by artemisinin and artesunate at increased pH (Acton and Klayman, 1987, Meshnick *et al.*, 1989). Ascorbic acid and reduced glutathione, acting as antioxidants, were found to inhibit antimalarial activity of artemisinin *in vitro* (Meshnick *et al.*, 1989). *In vitro* studies had indicated that the carbonyl function was not necessary for antimalarial activity (Jung *et al.*, 1990).

The action of artemisinin and its derivatives was not antagonised by p-aminobbenzoic acid, indicating that they were different from that of both chloroquine and antifolate drugs (Sinha *et al.*, 1987). Chinese researchers had proposed that the membrane system of the parasite was the main site of artemisinin action (Anon, 1982a). Ultrastructural changes of the erythrocytic stage parasites appeared within 1 to 4 hours after exposure to artemisinin *in vivo* (Ye *et al.*, 1983). Ellis *et al.* (1985) found morphological changes in the ribosomes and the endoplasmic reticulum, along with accumulation of tritium-labelled dihydroartemisinin in the membranes. Jiang *et al.* (1985) observed mitochondrial swelling within *Plasmodium innui* parasite in

infected monkeys, but not within the host cells after exposure to artemisinin. Such changes lead to the formation of autophagic vacuoles and loss of cytoplasm, which kills the parasites (Anon., 1979, Maeno *et al.*, 1993). Browning and Brisby (1989) stated that antimalarial action of artemisinin was not due to direct effect on the lipid structure of the parasite membrane. Gu *et al.* (1986) reported haemolysis of red blood cells when exposed to artemisinin concentration exceeding those necessary for antimalarial activity. Gu *et al.* (1986) also showed that protein synthesis in erythrocytes infected with *P. falciparum* was inhibited by artemisinin, dihydroartemisinin and artemether. Inhibition of nucleic acid synthesis occurred later than protein synthesis inhibition (Li *et al.*, 1983), and hence suggested that nucleic acid was not the primary target of drug action (Gu *et al.*, 1983). Artemisinin also affects polyamine metabolism. This was shown by Zhao *et al.* (1986) who studied cytochrome oxidase activity on *Plasmodium bergheri*. In another study by Gu *et al.* (1984), accumulation of tritiated dihydroartemisinin in *P. falciparum*-infected erythrocytes was significantly higher than that in uninfected erythrocytes, indicating a selective uptake of the drug by infected cells. Efficacy of sodium artesunate was exhibited by its inhibition on activity of cytochrome oxidase (Zhao *et al.*, 1986), and glutamine transport system (Elford, 1986) in erythrocytes.

No parasite-resistance cases were reported with the use of artemisinin derivatives (White, 2004). Severity of infection did not significantly affect the pharmacokinetics of artemisinin derivatives (Davis *et al.*, 2001) and dose adjustment was not necessary for patients with hepatic or renal impairment. Important drug interactions are not known (Ilett and Batty, 2004). High recrudescence rate, usually more than 25% after short courses of artemisinin between 3 and 5 days highlights the limitations of monotherapy.

Sodium artelinate is the most active compound in the water-soluble artemisinin derivatives series. Although it is less potent than sodium artesunate, it compares favourably with this drug both *in vitro* against *P. falciparum* and *in vivo* against *P. berghei*. Sodium artelinate is also more stable than artesunate. Sodium artesunate hydrolyses in alkaline solution in a few hours, while sodium artelinate undergoes little decomposition after three months (Lin *et al.*, 1987). Arteether, artemether, sodium artesunate, artelinic acid and sodium artelinate are more stable and effective against malaria than artemisinin and dihydroartemisinin. However the latter compounds were less cytotoxic when tested *in vitro* (Woerdenbag *et al.*, 1993b). In whole animal systems, dihydroartemisinin is the most potent and most toxic compound. Although artemisinin is the least effective, it is also the easiest compound to manufacture.

2.2.5 Artemisinin-based Combination Therapies

The rationale for combining drugs with independent modes of action to prevent emergence of resistance was first developed in antituberculosis chemotherapy. This approach has since been adopted for cancer chemotherapy and for the treatment of AIDS and early HIV-1 infection (White *et al.*, 1999). While other antimalarials can reduce malarial parasite biomass by 100-fold to 1,000-fold, artemisinin and its derivatives (artesunate, artemether and dihydroartemisinin) can reduce parasite biomass by roughly 10,000-fold per two-day asexual life cycle (White, 1997). Combinations of artemisinin, or one of its derivatives, with more slowly eliminated drugs such as mefloquine or lumefantrine have proven to be highly effective, even against multidrug-resistant *Plasmodium falciparum* (Price et al., 1997, van Vugt et al., 1998). The halt of progressive mefloquine resistance on north-

western border of Thailand (White, 1997), which harbours the most resistant *P. falciparum* in the world with combination chemotherapy was attributed to two factors. First, high cure rates with combination chemotherapy eliminates most of the infection, leaving a residuum of only 10^5 parasites or 0.000001 % of the initial asexual parasites exposed to mefloquine alone. As a result of rapid reduction of parasite population in a patient, the selective pressure for the emergence of mutants with reduced mefloquine sensitivity is lessened substantially (White, 1998). Secondly, significant decrease in gametocyte carriage prevents the spread of resistance. Price *et al.* (1996) and Handunnnetti *et al.* (1996) found that recrudescent infections were associated with the increase of gametocyte carriage rate, providing a powerful selection pressure to the spread of resistance. Thus, combinations should slow the evolution of drug resistance in all malarious areas (White *et al.*, 1999).

Combination chemotherapy does protect the artemisinin derivatives from development of resistance, as opposed to argument that artemisinin derivatives should be withheld from the use of uncomplicated malaria in order to prevent the development of resistance. Parasites were never exposed to the antimalarial activity of artemisinin derivatives alone if the drug was used with another unrelated antimalarial drug. Watkins and Msobo (1993) suggested that the resistance to this group of drugs would develop fairly slow. Furthermore, use of artemisinin has been poorly regulated in many countries, a condition that would provide selective pressure to the emergence of resistance. If these drugs were used only in combination with other antimalarials, artemisinin resistance would develop much more slowly. This mutual protection will give rise to a longer useful lifespan for both components in combined antimalarial chemotherapy than if the two components were used sequentially (van Vugt *et al.*, 1998).

Artemisinin-based combination therapies (ACT) combine rapid schizontocidal activity of artemisinin derivates with a longer half-life partner drug (Davis *et al.*, 2001). Partner drugs in ACT should be well-tolerated with low toxicity, has broad spectrum of actions, able to clear asexual forms of *Plasmodium* rapidly and possess long half-life of more than four days. 'Ideal' antimalarial combinations should have components with different modes of action, prescribed as short-course regimens (three days at most) and inexpensive (Nosten and Brasseur, 2002).

Among the available derivatives, artesunate is the most favourable to be used in ACT treatment for uncomplicated malaria due to the presence of hemisuccinate group that confers water solubility and high oral bioavailability. Thus it can rapidly be converted to the potent active dihydroartemisinin (Davis *et al.*, 2001). Artesunate-mefloquine has been used widely in South-East Asia countries with 95% efficacy. However, neuropsychiatric effects of mefloquine are problematic (Hien *et al.*, 2004). Artemether-lumefantrine, though recommended by WHO as the first line ACT combination has not been proven to be as effective as other types of ACT due to bioavailability and efficacy problems (Taylor and White, 2004, Omari *et al.*, 2004) and high treatment failure rate of 28% in 14 days (Poravuth *et al.*, 2002). On the other hand, dihydroartemisinin-piperaquine is probably the most promising form of ACT (Davis *et al.*, 2005a). Indochinese studies have shown cure rates above 95% in 28 days (Denis *et al.*, 2002, Hien *et al.*, 2004)

Before the development of ACT, combinations of chemotherapeutic agents such as quinine-tetracycline, sulfadoxine-pyrimethamine and atovaquone-proguanil, were used to improve cure rates and protect component drugs against resistance. However the application of these antimalarial combinations were hampered by frequent side-effects, long course regimens (quinine-tetracycline), increasing high-

grade resistance (sulfadoxine-pyrimethamine) and high cost (atovaquone-proguanil) (Wichmann *et al.*, 2004).

The prospect of ACT is promising. Pyronaridine and naphthoquine, two novel partner drugs are being assessed and preliminary results are encouraging (WHO, 2001) and combination of artesunate with chlorproguanil-dapsone, two old antimalarial drugs is being developed (Alloueche *et al.*, 2004). Van Vugt *et al.* (2002) found that combination of atovaquone-proguanil and artesunate was effective with multi-drug resistant *Plasmodium falciparum*. The future challenge in development of ACT is to build up a potent, safe, easy to administer and inexpensive range of antimalarial combinations (Davis *et al.*, 2005b).

2.3 Malaria

2.3.1 History

Malaria is one of the most important infectious diseases in the world, and its history extends into antiquity. It is generally believed that malaria arose with primates evolution in Africa and evolved with humans, spreading with human migrations throughout tropics, subtropics, and temperate regions of the Old World. Later the disease stretched on to the New World with explorers, missionaries and slaves. Klayman (1989) gathered some controversial suggestions of the possibility that malaria appearance in the pre-Columbian America was related to slaves importation from Africa.

Malaria has been recorded since the sixteenth century B.C. in Egyptian writings. Symptoms reported includes shivering, fever and spleen enlargement. In the fifth century B.C., followers of Hippocrates noted the recurrence of fevers at regular intervals and found a connection of the disease to marshes. In the seventeenth

century, Italians thought that breathing bad air (*mal aira*) from the swamp was the cause of the disease. The term 'malaria' became part of English medical literature in the first half of nineteenth century. The Spanish name for malaria (*paludisme*) and the Spanish name (*paludismo*) are in fact derived from *palus* in Latin, meaning marsh. These records indicate high malaria infection rates in/around swamp areas as stagnant water in these areas provides natural breeding ground for *Anopheles* mosquito, the vector of *Plasmodium* parasites that cause malaria (Klayman, 1985).

The discovery of the malaria parasite and breakthrough in understanding its mode of transmission are among the most exciting events in the history of infectious disease. According to Cox (2002), the parasites in the blood were first observed in 1880 by Alphonse Laveran, a French army surgeon. As cited by Cox (2002), Patrick Manson identified mosquito as the vector of malarial parasites in 1894 based on his knowledge that filarial worms, another blood parasites, were also transmitted by mosquitoes and because of the known association between malaria and mosquitoes breeding ground, the marshy places. Ronald Ross, an English physician and Battista Grassi, an Italian physician, independently proved that the bite of *Anopheles* female mosquito as the cause of malarial infection, thus solving the mystery of malaria transmission. Also cited by Cox (2002), Ross in 1897, found oocytes of *P. falciparum* in an anopheline mosquito that fed on a patient with gametocyte form of malaria parasite. Giovanni Battista Grassi, Amico Bignami and Giuseppe Bastianellli illustrated the developmental stages of malarial parasites in anopheline mosquitoes. As found in Cox (2002), the life cycle of of malarial parasites was only elucidated nearly 50 years later by Henry Shortt and Cyril Garnham in 1947, who showed that a phase of division in the liver preceded the development of parasites in the blood.

Prior to 1947, the 10-day whereabouts of the parasites development , which could not be seen in the blood, was not known.

Life cycle of *Plasmodium* parasites is very complex, beginning with injected sporozoites into the blood of its host by the female anopheline mosquito. The sporozoites enter the liver and multiply, releasing thousands of daughter forms of merozoites into the blood. The merozoites invade red blood cells, where another phase of multiplication occurs. Some merozoites develop into male and female gametocytes, the sexual stages, to be taken up by female mosquitoes during feeding on humans. Fertilisation between these gametocytes occurs within the gut of the mosquitoes. Zygotes develops into oocysts, where another phase of multiplication occurs. As a result, sporoziotes are produced and upon reaching the salivary glands of the mosquito, are injected into a new host (Cox, 2002).

2.3.2 Mortality and Morbidity

In 1990, global incidence of malaria was estimated to be 213 million cases (Murray and Lopez, 1996). In 1998, an estimate of 273 million clinical cases or 28.2% increase was reported, out of which 90% of global cases was borne by Africa (WHO, 1999). Snow *et al.* (2005) using a comprehensive standard global approach estimated a staggering 515 million clinical *Plasmodium falciparum* malaria episodes in 2002. At a regional level, 70% of clinical events came from African region and South East Asia contributed 25%. They produced a cartography of malaria risk, with figures up to 50% higher than those reported by World Health Organisation (WHO), claiming that WHO statistics has been understated due to its dependence on passive national notifications to WHO regional offices and Africa's weak system of reporting

infectious diseases. If such argument is valid, then the magnitude of global malarial prevalence is indeed much greater than that estimated by WHO.

Death rate following a clinical attack of *P. falciparum* appears to be higher in Africa than in South-East Asia and western Pacific. Occurrence of life-threatening complications due to *P. falciparum* malaria in Africa (Snow, 1997) is at least ten-fold as compared to similar occurrence in India (Prusty and Das, 2001) and Vanuatu (Maitland, 1997). Reasons for such huge disparity were unclear but might include limited access to proper treatment (Alles *et al.*, 1998) and cross-*Plasmodium* species defence against severe disease outcomes (Mayfong *et al.*, 2004).

Malaria has been wiped out from the United States and Cuba but remain prevalent largely in Africa. Large areas in Asia, Central and South America do record high incidences of this disease as well (Nussenzweig and Long, 1994). Travellers, migrant workers, military personnel and people living around international airports contribute to about 1000 cases reported annually in the United States (Wirth *et al.*, 1986)

2.3.3 Control of Malaria in Malaysia

Malaria eradication Programme was started in Peninsular Malaysia in 1967 and substituted with Malaria Control Programme in 1980 which was further extended to Sabah and Sarawak in 1986. In Peninsular Malaysia, malaria control was focused on three states where the borders of Kelantan, Pahang and Perak merged. Population in this area is largely aboriginal Orang Asli. Primary health-care approach to malaria control was introduced in 1995. In Sabah, Five-Year Action Plan for malaria control launched in 1996, yielded great success. In 1995, Sabah accounted for 84.2% of malaria burden in the country. By 2003, the state share of burden was reduced to