ศักยภาพในการผลิต Artemisinin ในต้นกลายพันธุ์ชิงเฮา (*Artemisia annua*)

นายธงชัย กูบโคกกรวด

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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POTENTIAL OF ARTEMISININ PRODUCTION

IN *ARTEMISIA ANNUA* MUTANTS

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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Artemisinin เป็นสารต้านมาลาเรียกลุ่ม sesquiterpene lactone ที่พบในต้น *Artemisia annua* L. (ชิงเฮา) ซึ่งถูกนำมาศึกษาเพื่อเพิ่มปริมาณการผลิต artemisinin ต้นกลายพันธุ์ของชิงเฮาจำนวนมากถูกสร้างขึ้นโดยการฉายรังสีแกมมา และขยายพันธุ์ด้วยเทคนิคเพาะเลี้ยงเนื้อเยื่อพืช เทคนิค TLC-densitometric ถูกพัฒนาขึ้นเพื่อการวิเคราะห์ปริมาณ artemisinin ได้อย่างสะดวกและรวดเร็วในต้นกลายพันธุ์ของชิงเฮาจำนวนมากว่า 200 ตัวอย่าง เทคนิคนี้ได้เปลี่ยนโครงสร้างของ artemisinin บนแผ่นซิลิกาเจลโดยใช้แอมโมเนีย (NH_3) ให้เป็นสารที่ดูดกลืนแสงซึ่งสามารถวัดความยาวคลื่นด้วยเทคนิค TLC-densitometry การตรวจหาโครงสร้างสารดังกล่าวพบว่าเป็น 10-azadesoxyartemisinin ซึ่งดูดกลืนแสงสูงสุดที่ 320 นาโนเมตร การศึกษานี้ได้นำตัวอย่างสกัดหยาบของต้นกลายพันธุ์มาทำการแยก artemisinin บนแผ่นซิลิกาเจล โดยใช้ตัวทำละลายผสมของเฮกเซน เอทิลอะซิเตตและอะซิโตนในสัดส่วน 80:5:5 หลังจากนั้นนำแผ่น TLC ดังกล่าวไปบ่มกับแอมโมเนียที่อุณหภูมิ 100 องศาเซลเซียส นาน 2 ชั่วโมง แล้วนำมาวัดการดูดกลืนแสงด้วยเครื่อง TLC-densitometer ที่ความยาวคลื่น 320 นาโนเมตร เทคนิคนี้ให้ความถูกต้องและความไวสูงในการวิเคราะห์โดยเปรียบเทียบกับเทคนิค HPLC ตัวอย่างต้นกลายพันธุ์ของชิงเฮาจำนวน 205 ตัวอย่างถูกนำมาวิเคราะห์และพบว่ามีปริมาณ artemisinin ในแต่ละต้นที่แตกต่างกันมาก ตั้งแต่ปริมาณร้อยละ 0.02 ถึง 3.46 โดยน้ำหนักแห้งของสารสกัดหยาบ นอกจากนี้การศึกษากิจกรรมของเอนไซม์ amorpha-4,11-diene synthase (ADS) เอนไซม์ตัวแรกในวิถีชีวสังเคราะห์ของ artemisinin ยังแสดงให้เห็นถึงความสัมพันธ์ของกิจกรรมของเอนไซม์ดังกล่าวและปริมาณ artemisinin ในต้นกลายพันธุ์ การศึกษานี้เป็นการยืนยันว่า amorpha-4,11-diene synthase มีความสำคัญในการผลิต artemisinin ในต้นชิงเฮา ในแง่การศึกษาฤทธิ์ต้านเชื้อมาลาเรีย ต้นกลายพันธุ์ที่มีปริมาณ artemisinin แตกต่างกันนั้นแสดงให้เห็นถึงความสัมพันธ์กับฤทธิ์ต้านเชื้อมาลาเรีย อย่างไรก็ตามต้นกลายพันธุ์บางส่วนมีฤทธิ์ต้านเชื้อมาลาเรียสูง แต่มีปริมาณ artemisinin ต่ำ ซึ่งตรวจพบว่ามีสารบางชนิดปรากฏขึ้นในต้นกลายพันธุ์ และนั้นอาจเป็นสารที่มีการตอบสนองต่อการต้านเชื้อมาลาเรียดังกล่าว

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THONGCHAI KOOBKOKKRUAD : POTENTIAL OF ARTEMISININ PRODUCTION IN *ARTEMISIA ANNUA* MUTANTS. THESIS ADVISOR : ASSOCIATE PROFESSOR WANCHAI DE-EKNAMKUL, Ph.D., THESIS CO-ADVISOR : CHALERMPOL KIRDMANEE, Ph.D., 79 pp. ISBN 974-17-2942-1

Artemisinin, a well-known antimalarial sesquiterpene lactone present in the plant *Artemisia annua* L. (Qinghao), was investigated for its yield improvement in the plant. Various mutants of *A. annua* were generated by gamma ray irradiation and propagated by using plant tissue culture techniques. A simple TLC-densitometric technique was developed for rapid analysis of artemisinin content in more than 200 samples of the obtained mutants. This new analytical method is based on the structural conversion of artemisinin on a silica gel plate by a NH_3 to form a chromophore-containing compound that can be detected by UV-based TLC-densitometric analysis. Structure elucidation of the NH_3-treated artemisinin product indicated that compound was 10-azadesoxyartemisinin which has its λ_{max} at 320 nm. Various crude extract samples of *A. annua* mutants were subjected to artemisinin separation on a silica gel plate using the solvent system of hexane: ethylacetate:acetone, 80:5:5. The TLC plate was then exposed with ammonia vapor at 100°C for 2 hours before being scanned by TLC-densitometer using the wavelength of 320 nm. The technique appeared to be accurate and sensitive as compared with a complicated HPLC-UV technique. Among 205 mutant samples tested, the artemisinin content appeared to be highly variable, ranging from 0.02 to 3.46 % w/w of their dry weight of the crude extracts. Study on the enzyme activity of amorpha-4,11-diene synthase (ADS) showed a correlation between this enzyme activity and the artemisinin content for a number of mutants. This confirms that amorpha-4,11-diene synthase is important in the production of artemisinin in *A. annua*. The selected mutants with varied artemisinin content also showed essentially a correlation with their antimalarial activity. However, a few mutants with high antimalarial activity but low artemisinin content were also observed. An extra spot of compound was detected in these mutants and it might be another compound responsible for the antimalarial activity.

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ABBREVIATIONS

TLC	=	thin-layer Chromatography
ADS	=	amorpha-4,11-diene synthase
HPLC	=	high performance liquid chromatography
FPP	=	farnesyl pyrophosphate
IPP	=	isopentenyl pyrophosphate
DTT	=	dithiothrietol
Mops	=	3-(N-morpholino)-propanesulfonic acid
BSA	=	bovine serum albumin
NADPH	=	β-nicotinamine dinucleotide phosphate (reduced form), tetrasodium salt
$Na_2HPO_4.12H_2O$	=	dihydrogen orthophosphate
$NaH_2PO_4.H_2O$	=	sodium dihydrogen ortophosphate
$MgCl_2$	=	magnesium chloride
MoO_4	=	sodium molipdate
m	=	meter (s)
cm	=	centimeter (s)
mm	=	millimeter (s)
nm	=	nanometer (s)
l	=	liter (s)
ml	=	milliliter (s)
μl	=	microliter
%	=	percent (part per 100), percentage
min	=	minute (s)
mg	=	milligram (s)
MS medium	=	Murashige and Skoog (1962) medium
pH	=	The negative logarithm of the concentration of hydrogen ions
Rf	=	distance spot moved/distance solvent moved (TLC)
UV	=	ultraviolet light
Vis	=	visible light

v/v	=	volume/volume (concentration)
w/v	=	weight/volume (concentration)
w/w	=	weigh/weigh (concentration)
^{o}C	=	degree Celsius
δ	=	chemical shift
g	=	gram (s)
mg	=	milligram (s)
μg	=	microgram (s)
ng	=	nanogram (s)
NMR	=	nuclear manetic resonance
13C-NMR	=	Carbon-13 nuclear manetic resonance
1H-NMR	=	Proton- nuclear manetic resonance
mM	=	millimolar (concentration)
μmol	=	micromole
cpm	=	count per minute
Ci	=	curie
mCi	=	millicurie
μCi	=	microcurie
h	=	hour (s)
g	=	centrifugal force (relative to gravity)
λmax	=	wavelength at maximuma absorption
ELISA	=	enzyme-linked immunosorbent assay
GC	=	gas chromatography
MS	=	mass spectrometry
EC	=	electrochemical detection
et al	=	et alii
cDNA	=	complementary deoxyribonucleic acid
bp	=	base-pare

CHAPTER I

GENERAL BACKGRUOD

1. INTRODUCTION

Today, malaria is still a major health problem in the world. The disease is prevalent in almost 100 countries, accounting for 40 % of the world's population (Phillips, 2001). It affects in an estimation of three to five hundred million people, causing more than a million deaths per year or one child in every 30 seconds. Malarial disease is caused by a group of protozoa parasites belonging to the genus of *Plasmodium*. Only four species are known to infect human: *P. falciparum, P. vivax, P. malariae* and *P. ovale*. The infection of parasite is introduced through the bite of female *Anopheles* mosquitoes. Nowadays, a control of malaria has been relied on using insecticides against the vectors and antimalarial drugs for the treatment. However, resistance on the drugs developed by both the vectors and parasites has emphasized the need for improved method of malarial treatment and the biological control of the *Anopheles* mosquito (Geldre *et al.,* 1997). The problems of drug resistance are greatest in South-East Asia, where there is evidence for resistance or reduced sensitivity against all antimalarial drugs, including mefloqine, halofanttrine and even quinin. Interestingly, treatment of severe malarial now relies on the use of the latest natural antimalarials, namely artimisinin and its derivatives.

Artemisinin or qinghaosu was first isolated from *Artemisia annua* L. plant (Klayman 1985). The plant has been used in traditional Chinese medicine as a remedy for chills and fevers for more than 2000 years. However, the content of artemisinin in the leaves of this plant is present in relatively low level and is insufficient for using the compound in the treatment of the resistant strain causing the malarial disease. Therefore, there have been several attempts in many laboratories to increase the production of the artemisinin (Geldre *et al.,* 1997). Some high artemisinin production plants have been selected from the field, greenhouse, and hydroponic conditions.

However, one major obstacle on the selection of high artemisinin producing plant is the analysis of artemisinin in numerous of the *A. annua* plant samples. This is due to the absence of an UV or fluorescence chromophore in the molecule of artemisinin. So far, the techniques available for the analysis of artemisinin includes high performance liquid chromatography with UV detection (HPLC-UV) (Zhao and Zeng 1986), HPLC-elctrochemical detector (Acton *et al.,* 1985), gas chromatography (GC) (Sipahimalani *et al.,* 1993), Mass spectroscopy (MS) (Ranasinghe *et al.,* 1993) and GC-MS. In addition, immunodectection of artemisinin has been developed (Jaziri *et al.,* 1993; Ferriera and Janick 1996) as well as the thin-layer chromatography (TLC) which is easily, simply and quickly for artemisinin detection. However, both the immuno-and TLC-techniques can not be used for quantitative purpose because there is no reliable straining procedure to react specifically on artemisinin molecule. Therefore, the development of sensitive, specific and easy analytical method for determination of artemisinin is still a challenging problem.

In order to increase a possibility in selecting high-artemisinin producing plants of *A. annua*, this study proposes to develop a simple TLC-desitometric method for quantitative analysis of artemisinin in *A. annua* plants. It is expected that the developed method will be simply, accurately and sensitively for working on large number samples of *A. annua* mutants. The mutants will be obtained by gamma-ray irradiation which can generate a wide range of artemisinin content in the mutants. In addition, this work is also proposes to study a correlation between the enzyme activity catalyzing the first step of artemisinin pathway and the level of artemisinin content in the mutants. It has been reported that amorpha-4,11-diene synthase is such an enzyme of the pathway that convert farnesyl pyrophasphate to amorpha-4,11-diene (Bouwster *et al.,*1999). Finally, the crude extracts of some selected mutants will also be tested with *Plasmodium* parasites to confirm the antimalarial activity of the varied artemisinin-containing mutants.

2. LITERATURE REVIEW

2.1 Biology of Malaria

2.1.1 Human Malaria

Malaria remains an important cause of mortality in tropical regions. 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.

2.1.2 The Life Cycle of Malaria

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,

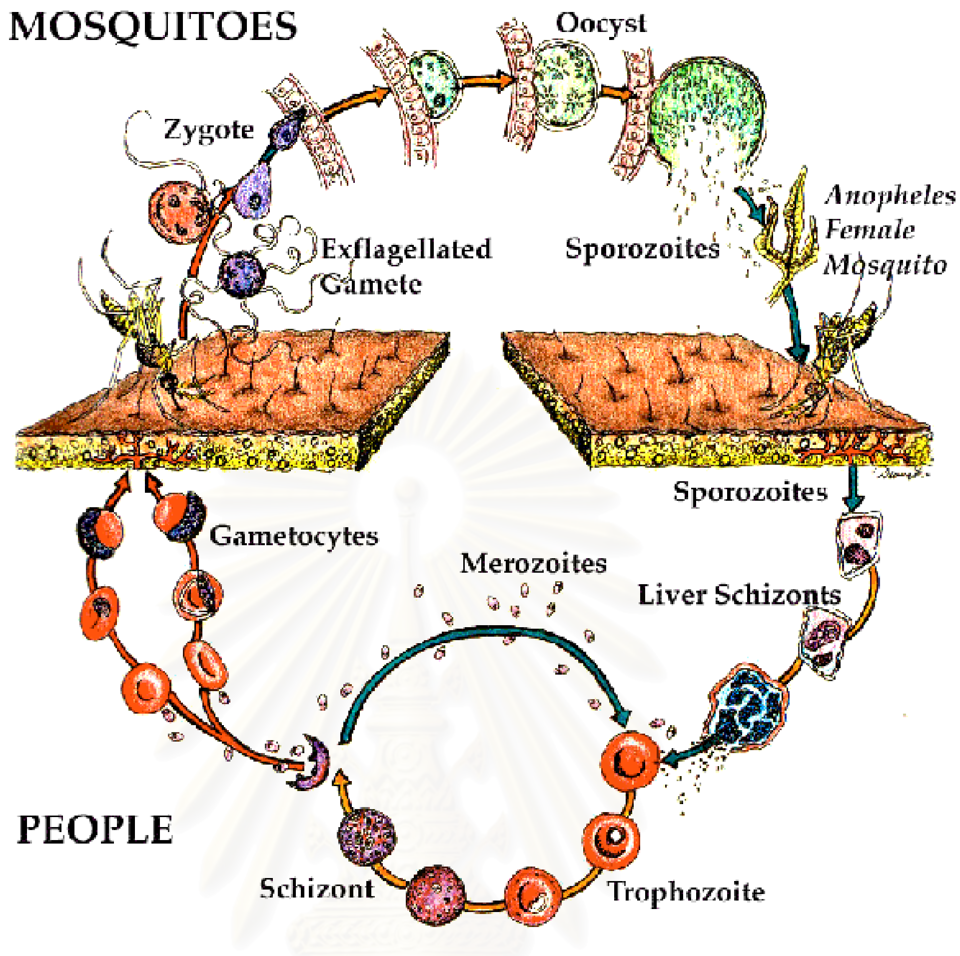


Figure 1. The life cycle of malaria.

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).

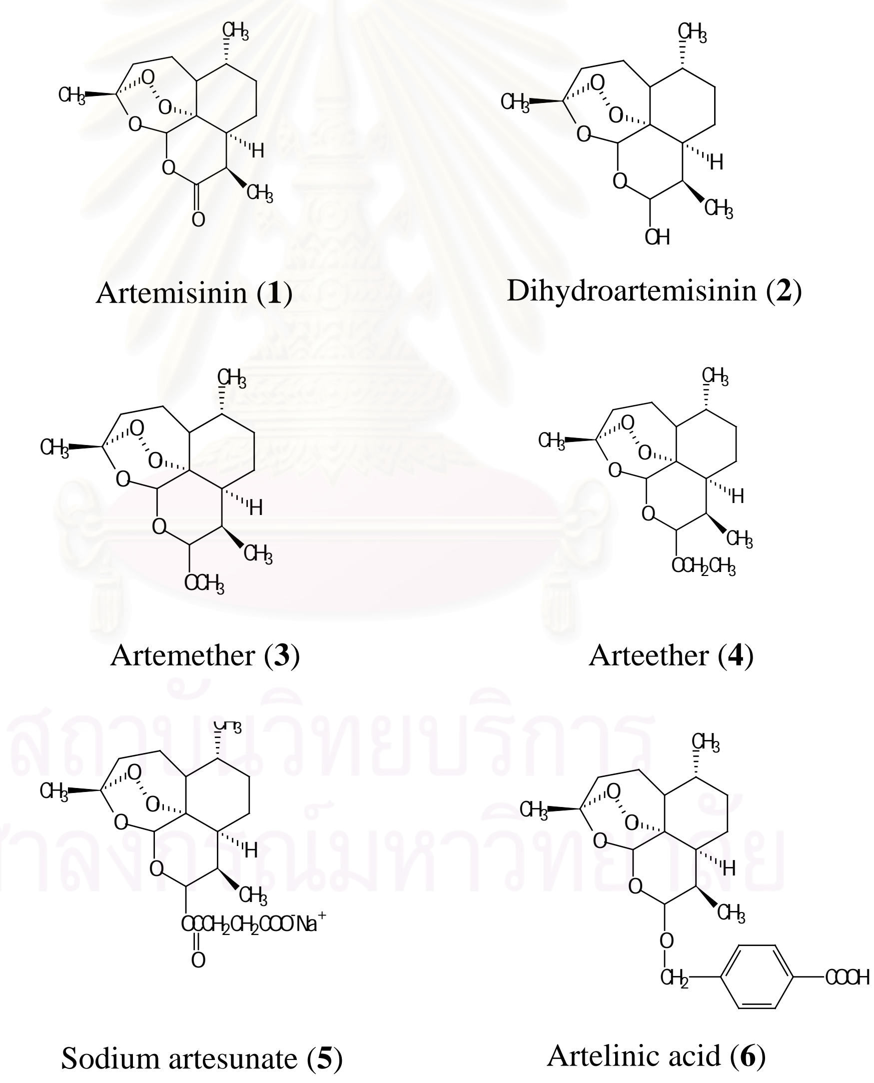
2.2. Anti-Malarial Drugs

Quinine is known to be the first antimalarial drug it was first extracted from Cinchona for longer than 350 years ago. The compound became the major treatment for malaria because quinine is quickly lethal to plasmodia. Due to its serious side effects, quinine has been replaced by a new synthetic drug, chloroquine which is more effective and less toxic than quinine (Gamey 2002). This compound used to prevent and cure *P. falciparum* completely, and can also suppress the other three malarial species. Subsequently, the resistance to chloroquine of the parasites occered by the mechanism associated with reduced drug concentrations within the parasites due to reduced ingress or increased influx (Phillips 2001). Due to an increased treatment failure, chloroquine is now replaced by pyrimethamine/sulphodoxine. The antifolates proquanil and pyrimethamine are usually used in combination with sulphonamides. Mefloquine has been used widely in Southest Asia and South America (Phillips 2001). However, resistance to the compound has been observed in treatment of infections caused by *P. falciparum* malaria in Sountheast Asia. Primaquine 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). Artemisinin has been used to treat the malarial parasites. It has low acute toxicity after oral or subcutaneous administration (Klayman 1985). Another pharmacuetical strategy for malaria control is the development of a vaccine regimen for endemic areas. Unfortunately, despite of the strong immune response stimulated by malarial infections, repeated attempts to develop an efficacious vaccine for use in human have failed (Wheeler and Berkly 2001).

2.3 Artemisinin

2.3.1 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-benzodioxepin-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 2 (**1**). Artemisinin has no any UV or fluorescent chromophore. It is soluble in apotic solution and slightly in oil.



Artemisinin (**1**) Dihydroartemisinin (**2**)

Artemether (**3**) Arteether (**4**)

Sodium artesunate (**5**) Artelinic acid (**6**)

Figure 2. The chemical structures of artemisinin and its derivatives.

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2.3.2 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 2). 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 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).

2.3.3 Extraction of Artemisinin from *A. annua*

A vast array of isolation procedures has been used to extract the active ingredient from the plant *A. annua*. Hot-water extracts have failed to show antimalarial activity (Klayman 1985). Low-temperature extraction with solvent has been introduced the ethylether extract showed antimalarial activity against *P. berghei* in mice (Acton and Klayman 1985). After that, a large-scale extraction of artemisinin from the plant has been reported. Petroleum extraction has been performed with died leaves and fractionated the extract on a silica column (Klayman 1985). 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). Three years latter, an isolation method has been developed for a large-scale production of artemisinin (Charles *et al.,* 1990). The method involves hexane extraction of the unground dry 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 artremisinin 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, have been obtained from samples of *A. annua* collected in the U.S.A.. Yields of extracted artemisinin from the aboveground portions of the plant have ranged from 0.01 % to 0.5 % (w/w) in China (Singh *et al.,* 1988).

2.4 *Artemisia annua*

2.4.1 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 3).

Although *A. annua* is traditional herb antimalarial drug in China, where it is widely distributed as a weed mostly in temperature regions. The plant can be grown to 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, Singapo and Thailand).

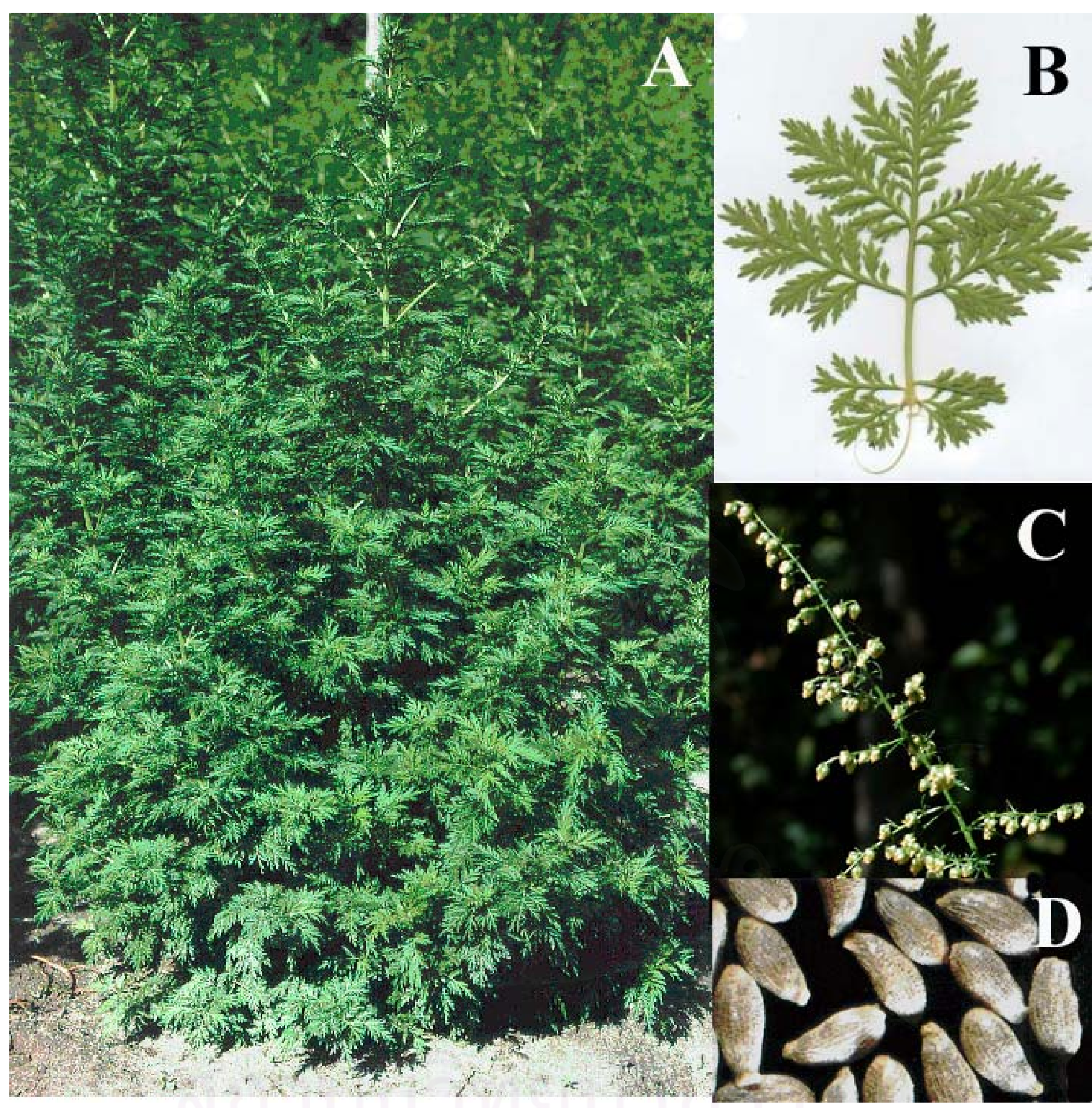

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Figure 3. The plant of *Artemisia annua* L. (A) with the part of Leaves (B), Inflorescences (C) and Seeds (D)

2.4.2 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 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) has 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 artimisinin 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 very 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 weight.

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 ^{o}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.

In addition, the effect of plant growth regulators has been studies. 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).

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;).

2.5 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). Beside, 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.

Thin-layer chromatography 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, the techniques give poor staining characteristics of the intact molecule and interference with contaminating constituents of the plant, this method is not, therefore, very reliable.

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 Q$_{292}$ Q$_{260}$

Figure 4. The reaction of pre-column artemisinin derivatization for HPLC-UV analysis of artemisinin (Zhao and Zeng 1986).

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 4). In addition, KOH was used to decomposition of the compound and then monitoring at 286 nm by HPLC-UV (Edlund *et al.,* 1984). Alternatively, electrochemical HPLC detection procedure has been developed that can be used for determination of artemisinin in crude plant extracts (Acton *et al.,* 1985; Charles *et al.,* 1990). 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).

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Figure 5. 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 (Avery *et al.,* 1999)

Gas chromatography (GC) method for the analysis of the artemisinin compound at nanogram levels has been proposed (Sipahimalani *et al.,* 1991). 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.

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. 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.

2.6 Artemisinin Biosynthesis

Although a complete biosynthetic pathway of artemisinin has not been established, some biotransformation steps have been elucidated *in vitro* and *in vivo* (Geldre *et al.* 1997). In 1986, El-Feraly *et al*. 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. 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. 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 biosynthesis of artemisinin. In addition, Acton and Klayman (1986) have found the conversion of artemisinin to two new compounds, iso-artemisitene and 9-epi-artemisinin, which might not be involved in artemisinin biosynthesis. In 1987, Roth and Acton have reported the isolation of artemisinic acid and epi-deoxyarteanniun B from *A. annua*. Subsequently, El-feraly *et al.,* (1989) has shown a sequence of by-product formation from the oxidizing artemisinic acid to epi-deoxyarteanniun B and then to 6,7-dehydroartemisinic acid. These experiments have suggested that the biosynthesis of artemisinin involves the change of the oxidation stage in the pathway. Moreover, Roth and Acton (1989) have shown that the conversion of artemisinic acid into artemisinin involved 2 steps of reaction via a reduction of exocyclic ethylene group and photoxidation of the resulting dihydroartemisinic acid. In addition, June *et al.,* (1990) have proposed a biosynthetic pathway from artemisinic acid to artemisinin and arteannuin B via the intermediate of allylic hydorperoxide.

A complete biosynthetic pathway of artemisinin, starting from mevalonic acid and isopentenylpyrophosphate (IPP) has been proposed by Akhila *et al.,* (1987, 1990). The biosynthetic sequence includes the intermediates of farnesyl pyrophosphate (FPP), germacrane skeleton, dihydrocostunolide, cadinanolide, arteannuin B and artemisinin. However, Akhila's proposal did not include artemisinic acid as a precursor for artemisinin whereas others (El-Feraly *et a*l., 1986; June *et al.,*

1990; Roth and Acton 1987) consider artemisinic acid to be a possible biosynthetic precursor for both arteannuin B and artemisinin, either sequentially or independently. Subsequently, Sangwan *et al.,* (1993) have shown that [^{14}C] artemisinic acid can be incorporated into arteannuin B as well as artemisinin, both *in vivo* and in cell-free systems. The results have suggested that artemisinic acid might be a common precursor for arteannuin B and artemisinin synthesis. Beside, cell-free leaf homogenate of *A. annua* leaves have been shown to convert arteannuin B into artemisinin (Nair and Basile, 1993). In 1994, a novel secocadinane and a dihydroxyavdinanolide have been isolated from the aerial parts of *A. annua*. This has led to biosynthetic mechanism that involves the tranformation of arteannuin B into artemisinin by way of this dihydroxycadonanolide and the enol tautomer of 4,5-seconcadinane. In addition, a new substrate, dihydroarteannuin B has synthezided that can easily be radiolabeled with high specific activity. It has been utlized by the enzyme system and converted to artemisinin with the same efficiency as the natural substrates (Bharcl *et al.,* 1998). However, dihydroarteannuin B has not been isolated as a naturally occurring chemical constituent of the plants. This suggested that the compound might not probably be existed *in vivo*, on it might be getting converted to artemisinin very fast.

In 1999, a dihydroartemisinic acid hydroperoxide has been isolated for the first time as natural product from the plant *A. annua*. The compound is known as an intermediate of the photochemical oxidation of dihydroartemisinic acid leading to artemisinin (Wallaart *et al.,* 1999). The presences of dihydroartemisinic acid and dihydroartemisinic acid hydroperoxide are in the plant and the attacking singlet oxygen ($^{1}O_2$) conditions under, which dihydroartemisinic acid can be converted into dihydroartemisinic acid hydroperoxide. This compound can very easily be oxidized to artemisinin and has thus provided evidence for a nonenzymatic reaction but the reaction converted dihydroartemisinic acid into artemisinin by photochemical reaction *in vivo* and in plant (Wallaart *et al.,* 1999a, 1999b). Simultaneously, Bouwmeester *et al.,* (1999) have reported the presence of amorpha-4,11-diene in pentane extracts of *A. annua* leaves. The finding have proposed that amorpha-4,11-diene synthase is the enzyme catalyzing the first step of artemisinin biosynthesis, the enzyme converts farnesyl pyrophosphate into amorpha-4,11-diene (Figure 6). The enzyme has been partial purified and shows same typical characteristics of sesquiterpene synthase.

ADS

Farnesyl pyrophosphate

Amorpha-4,11-diene

13-hydroxy-amorpha-4,11-diene

Amorpha-4,11-diethyl-13-aldehyde

Artemisinic acid

Dihydroartemisinic acid

Dihydroartemisinic acid hydroperoxide

Artemisinin

Figure 6. The propose biosynthesis pathway of artemisinin. ADS) Amorpha-4,11-diene synthase

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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 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).

Very 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. In addition, the tranformation of the gene into tobacco (*Nicotiana tabacum* L.) has resulted in the expression of an active enzyme and the accumulation of amorpha-4,11-diene ranging from 0.2 to 1.7 ng per gram fresh weight.

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 1.

Table 1 Chemical constituent in *A. annua*

Chemical group	Chemical substance	Chemical structure	Reference
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		

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Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
Sesquiterpene	β-caryophyllene		Simon *et al.,* 1990; Bouwmeester *et al.,*1999
	Trans-β-farnesene		Bouwmeester *et al.,*1999
	α-humulene		
	β-caryophyllene		
	α-copaene		
	Selina-4,11-diene		

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Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
sesquiterpene	Biocyclogerma-crene		Bouwmeester *et al.*,1999
	Germacrene A		
	Germacrene D		
	γ-cadinene		
	Muurola-4,11-diene		
	Amorpha-4,11-diene		

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Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
sesquiterpene	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
	6,7-dehydroartemisinic acid		El-Feraly *et al.,*1989

Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
sesquiterpene	Dihydroartemisinic acid hydroperoxide		Wallaart *et al.*,1999a
	secocadinane		Browm 1994
	artemisinin		Tu *et al.*, 1982; Klayman 1985
	artemisitene		Acton and Klayman 1986
	isoartemisitene		
	9-epi-artemisinin		

Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
sesqiuterpene	Dihydroxycadia-nolide	HO OH O O	Browm 1994
	Epi-deoxyartean-nuin B	H H O O	Roth and Acton 1987 El-Feraly *et al.,* 1989
	deoxyartemisinin	H H O O H H O O	Tu *et al.,* 1982
	Arteannuin A	H O O	
	Arteannuin B	H O H O O	
	Hydrodeoxyartemi-sinin	HO H O O H H O O	

Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
sesquiterpene	Dihydro-epi-deoxyarteannuin B		Wallaart *et al.*, 1999
	Arteannuin C		Tu *et al.*, 1982
	Arteannuin E		
	Arteannuin F		
flovonoid	5,4'-dihydroxy-3,6,7,3'-tetramethoxy-flavone		
	chrysosphenol		

Table 1 Chemical constituent in *A. annua* (continued)

Chemical group	Chemical substance	Chemical structure	Reference
flovonoid	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		

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Table 1 Chemical constituent in *A. annua* (continued)

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

CHAPTER II

MATERIALS AND METHODS

1. Chemicals and Equipments

Authentic artemisinin was obtained from the Plant Biochemistry and Plant Physiobiochemistry Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

[1-3H(N)]Farnesyl pyrophosphate triammonium salt, (specific activity = 16.1 Ci/ mmol, 0.5 mCi/ml) was purchased from Perkin Eler Life Sciences Inc., Boston

3-(N-morpholino)-propanesulfonic acid (Mops), ascorbic acid, glycerol, bovine serum albumin (BSA), β-nicotinamine dinucleotide phosphate (reduced form), tetrasodium salt (NADPH), *dl*-dithiothreitol (DTT) were purchased from Sigma Chemical Company, St Loius, Mo., USA. Dihydrogen orthophosphate ($Na_2HPO_4.12H_2O$) and sodium dihydrogen ortophosphate ($NaH_2PO_4.H_2O$), magnesium chloride ($MgCl_2$), and sodium molipdate (MoO_4) were obtained from Merck, Damstadt, Germany. Coomassie brilliant G-250 (for protein analysis) was purchased from Bio-Rad Laboratories, Richomo, Ca., USA. Liquid nitrogen and nitrogen gas were purchased from Thailand Industrial Gases, Samutprakan, Thailand.

Organic solvents were all reagent grade or better from LAB-SCAN Analytical Scinces, Dublin, Ireland and Merck, Damstadt, Germany. Water was triple deionized.

Thin-layer chromatography plates, Silica gel POLYGRAM® SIL G/UV254 was purchased from MACHEREY-NAGEL GmbH& Co. KG, Germany.

Detection of Radioactive was performed by using TLC Linear analyzer LB 284/285, Berthold, Germany.

Densitometric technique was performed by using Shimadzu Dual Wavelength TLC scanner Model CS-930

High performance liquid chromatography (HPLC) apparatus consisted of a Varian Model 9010 ternary solvent delivery system attached to Model 9050 variable-wavelength UV-VIS detector. Samples were automatically injected by

Varian Model 9095 autosampler and the data were recorded by Varian Star Version 6.0 program in personal computer on Window 98. Analytical separation was performed by using a reverse phase C-18 stainless-steel column (Varian SP-C18, 15 cm x 4 mm I.D., particle size 5 μm)

2. Plant Material

2.1 The seeds of *Artemisia annua*

The seeds of *Artemisia annua* were obtained from Vietnam. The seeds were surface-sterilized in 10 % Cholrox®(0.35 % sodium hypochlorite active ingredient) for 20 min and in 5 % Cholrox® for 10 min. After being washed three times with sterilized water the seeds were propagated in MS medium (Table 2) containing 3 % sugar and 0.8 % agar. The germinated seedling cultures were kept in 40 $\mu mol.m^{-2}.s^{-1}$ of fluorescence light for 2 months. The plantlets were then subcultured five times before being subjected to γ-ray irradiation. Practically, the shoots were removed from the plantlets, cut to the sized of 0.5 cm long on to the same MS medium. Two hundred obtained shoots were irradiated with γ-ray 650 of Cobalt-60 (dose rate 856 $rad.min^{-1}$) in three doses as 0, 500, and 800 rad, cordially coorperated by the Office of Atomic Energy for Peace (OAEP). The exposed shoots were then transfered to the fresh MS medium with the same ingradients. The shoots were subcultured every six weeks for three times. In the forth subculturing, the plants were propagated to many clones for selecting heat tolerance strains and then transferred to the bottom culture which has filter on the cover. The heat tolerance strains were selected by growth the plant in the medium under the temperature as 25°C and 30°C for six weeks and then picked up the serviced clone. After the selection, the leaves were collected from the plants and extracted with hexane. The hexane extracts were then concentrated before being tested for antimalarial activity. The plant which has high antimalarial activity under temperature as 30°C were selected and there leaves of 200 clones in each irradiation dose for determination of artemisinin and test antimalarial again.

A. annua mutants were grow with plant tissue culture technique at the Plant Biochemistry and Plant Physiso-Biochemistry Laboratory, National Center for Genetic Engineering and Biotecnology, Thailand.

Table 2 MS media (Murashige and Skoog, 1962)

Macronutrients	mg/l
NH_4NO_3	1,650
KNO_3	1,900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	mg/l
H_3BO_3	6.20
$MnSO_4.2H_2O$	6.90
$ZnSO_4.H_20$	6.14
KI	0.83
$Na_2MoO_4.H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron	mg/l
Sodium EDTA	37.25
$FeSO_4.7H_2O$	27.85
Organic components	mg/l
Glycine	2.0
Nicothinic acid	0.5
Pyridoxine	0.5
Thiamin	0.1
Sucrose	30 g/l
pH	5.5

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3. Structural Conversion of Artemisinin Molecule on Silica Gel Plate

A solution of artemisinin (0.4 µg/ml) was spotted (10 µl) onto a precoated silica gel plate (POLYGRAM® SIL G/UV_{254}, 0.25 mm thinness) and developed by the solvent system of hexane : ethylacetate : acetone, 80:5:5. The TLC plates were then exposed with saturated ammonia under various temperatures: room temperature, 50, 75, 100 and 125°C for 2 h or under various time intervals of 20, 40, 60, 80, 100, 120, 160 and 180 min at 100°C. After that, the TLC plate was scanned by TLC-densitometer (Shimadzu TLC-scanner Model CS-930) to obtain both an optimum wavelength and optimum condition for subsequent artemisinin analysis.

4. Structure Elucidation of NH_3-treated Artemisinin on a TLC plate

The solution of standard artemisinin (2 µg/ml) was spotted (1000 µl) onto a precoated silica gel plate (POLYGRAM® SIL G/UV_{254}, 0.25 mm thinness) and developed by the solvent system of hexane: ethylacetate: acetone, 80:5:5. The TLC plate was then exposed with saturated ammonia at 100°C for 2 hours. The modified artemisinin band was then scraped from the plate and elucidated with absolute ethanol and filtered through Wachtman filter paper number 40. The obtained product was purified one more time by TLC using the solvent system hexan:acetone:methanol, 10:3:1. The compound with (Rf 0.45) was eluted with absolute ethanol and dried with introgen gas. The chemical structure of determined by NMR techniques. The compound was resolubled in CD_3Cl and scanned ^{1}H, ^{13}C-NMR spectrum by JNM-A500 NMR instrument.

5. Artemisinin Analysis by TLC-densitometric Method

Based on the method optimization, the developed TLC-densitometric method was used for artemisinin analysis. In practice, the leaves of *A. annua* harvested for 1.5 month after subcutured were dried in a hot air oven. The dried leaves from various samples of *A. annua* mutants were ground and extracted under reflux with 10 ml hexane for 4 hours in a 20 x 2.5 cm tube connected with a 15 cm condenser. After cooling and precipitating of the extracted powder, the clear solution of crude extract was spotted directly (20 µl) onto a precoated silica gel (POLYGRAM^{R} SIL G/UV_{254}, 0.25 mm thinness) and developed by the solvent system of hexane:ethylacetate: acetone, 80 : 5 : 5. The TLC plate was then exposed with saturated ammonia at

100^oC for 2 hours for chromophore development of artemisinin. The TLC plate was then scanned using TLC-densitometer (Shimadzu TLC-scanner Model CS-930) under wavelength of 320 nm.

6. Artemisinin Analysis by HPLC Method

6.1 Sample Preparation

The same samples of the crude extracts were spotted onto a precoated silica gel (POLYGRAM^R SIL G/UV$_{254}$, 0.25 mm thinness) and developed by the solvent system of hexane : ethylacetate : acetone,80 : 5 : 5. The spotted at the Rf 0.75 of each sample corresponding to the position of artemisinin was then scraped from the TLC plate extracted in each samples and extracted for artemisinin with dichromethane in a microtube. Each microtube was centrifuged to precipitate the silica gel pert followed by transferring the dichromethane part into another microtube. The dichromethane fraction was evaporated by speed Vac®. The resulting residue was each redissolved with 100 µl methanol before performing a pre-column derivativition. The pre-column reaction was carried out by mixing the sample solution with mixed 400 µl 0.2 % NaOH solution and warmed in water bath at 50^oC for 1 h. The resulting mixture was cooled in water and diluted to 1,000 µl with 0.1 M acetic acid in 20 % ethanol. The solution was then directly injected for HPLC analysis.

6.2 Chromatographic Conditions

The HPLC operation was carried out using Varian Model 9010 ternary solvent delivery system attached to a Model 9050 variable-wavelength UV-Vis detector. Samples were automatically injected by Varian Model 9095 autosampler and data were recorded by Varian Star Version 6.0 program in personal computer Window 98. A reverse phase C-18 stainless steel column (Varian SP-C18,15 cm x 4 mm I.D., partical size 5 µm) was used as a separate column. All of the solvent was passed through a membrane filter (0.45 micron porn size). The chromatographic condition used an isocratic solvent system of 0.01 M Na_2HPO_4-NaH_2PO_2 buffer pH 6.7 (water : methanol, 38 : 62, v/v) ; flow rate : 1.5 ml/min ; wavelength 260 nm.

7. Preparation of Crude Enzyme Extracts

Fresh leaves (5g) of *A. annua* were quick frozen in liquid nitrogen and ground in pre-cooled mortar. The resulting fine powder was added with 15 ml cold extraction buffer containing 5 mM Mops buffer, pH 7.0, 10%(v/v) glycerol, 1 mM ascorbic acid, 10 mM $MgCl_2$ and 2 mM DTT. After being stirred for 15 min, the suspension was passed through four layers of cheese-cloth and the filtrate was centrifuged 100,000x*g* at 4°C. The supernatant was then desalted on through a PD-10 column. The filtrate was used as "crude enzyme extract" and kept in –80°C refrigerator before using.

8. Enzyme Assay for Amorpha-4,11-Diene Synthase

For an enzyme assay of amorpha-4,11-diene synthase, a reaction mixture used contained [1-^{3}H(N)]FPP (100,000 cpm), 5mM Mops buffer, pH 7.0, 10%(v/v) glycerol, 10 mM ascorbic acid, 10 mM $MgCl_2$, 2 mM DTT and 10 mM Na_2MoO_4 in total volume of 70 µl. After being incubated at 30°C for 30 min, the reaction mixture was extracted with 1 ml hexane for 20 second using Vortex. The hexane fraction was then taken and evaporated (Speed Vac®) and spotted onto a TLC plate (POLYGRAM® SIL G/UV_{254}, 0.25 mm thinness). The resulting TLC plate was then developed in a solvent system of hexane:ethylacetate:acetic acid; 25:7:1 and then scanned to obtain radiochromatograms by TLC-radioscanner (Linear analyzer B284/285; Berthold, Germany).

9. Protein Determination

Various enzyme solutions were determined for their protein content by the method of Bradford (1976). In practice, each enzyme solution was taken160 µl to be mixed with 40 µl of the commercial available dye reagent concentrate (Bio-Rad protein assay) in a 96-well microtiter plate. The mixtures in the plate were incubated at room temperature for 5 min followed by reading their absorbance which was measured at the wavelength 595 nm using Bio-Rad Model 450 Microplate Reader. A calibration curve of BSA followed its linearity of the relationship between BSA (0-70 µg) and absorbance 595 nm (0-0.8). This calibration curve was used for converting the obtained absorbance values to their protein concentrations.

10. Analysis of antimalarial activity

Antimalarial assay used in this study was modified from method of Desjardins *et al*. (1979). A crude extract from each sample was initially dissolved in DMSO to obtain a final concentration of 10 mg/ml and the antimalarial activity was determined by using [^{3}H]-hypoxanthine incorporation method which was kindly performed by Dr. Sumalee Kamchonwongpaisan of BIOTEC, Thailand. Aliquots (25 μl each) of the extract solutions having different concentrations were dispensed in a 96-well plate containing 200 μl of 1.5% all suspension of parasitized erythrocytes of *Plasmodium faliparum* K1CB1 with the addition of 1-2% parasitenia. The mixtures were incubated in a CO_2 incubator at 37 °C. After 24 h of incubation, 25 μl of 0.25 μCi [^{3}H]-hypoxanthine was added to each well, and the parasite cultures were further incubated for 18-24 h prior to be harvested by Filtermate®. After being air-dried and 20 μl of liquid scintillation fluid were added to each filter and their radioactivity were then counted using 6-probe liquid scintillation counter. The concentration of inhibitor which inhibit 50% of the parasite growth (IC_{50}) was determined from sigmoid dose-response curve obtained by plotting the percentages of [^{3}H]-hypoxanthine incubation against the concentration of the extract solution used.

CHAPTER III

RESULTS

1. Development of a New Analytical Method for Determination of Artemisinin Content

1.1 Identification of the Modified Structure of Artemisinin

The structure of NH_3-treated artemisinin on the silica gel plate was first determined by NMR. The compound obtained by TLC was dissolved in CD_3Cl and analyzed using NMR techniques. The resulting ^{13}C-NMR spectrum (Figure 8), and ^{1}H-NMR spectrum (Figure 9) indicated that the artemisinin derivative obtained by NH_3 exposed was 10-azadesoxyartemisinin Figure 7. ^{1}H NMR δ 0.86 (3H, d, J=1), δ 1.06 (3H, d, J=1), δ 1.40 (3H, s), δ 1.93 (1H,ddd, J=13,5,5), δ 2.91 (1H, ddd, J=14,1,4), δ 5.06 (1H,d, J=3) (Figure 9). For ^{13}C NMR was shown in Figure 8 and Table 3.

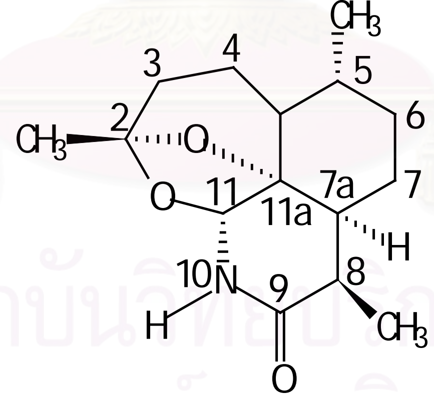

Figure 7 The structure of 10-azadesoxyartemisinin which is obtained from artemisinin with NH_3 on a silica gel plate

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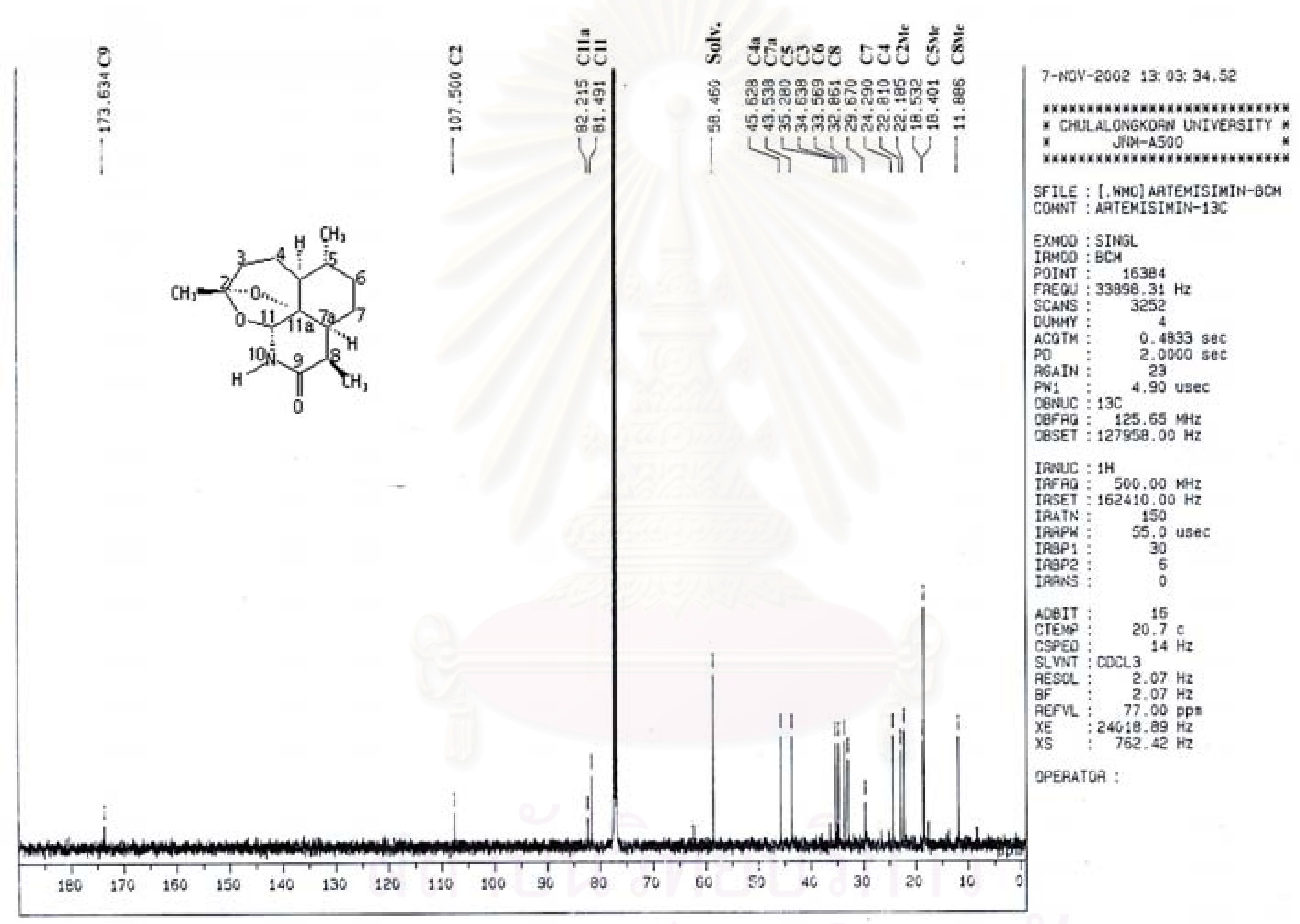

Figure 8 ^{13}C-NMR spectrum of the part of artemisinin after being exposed on a TLC plate with saturated ammonia at 100°C for 2 h.

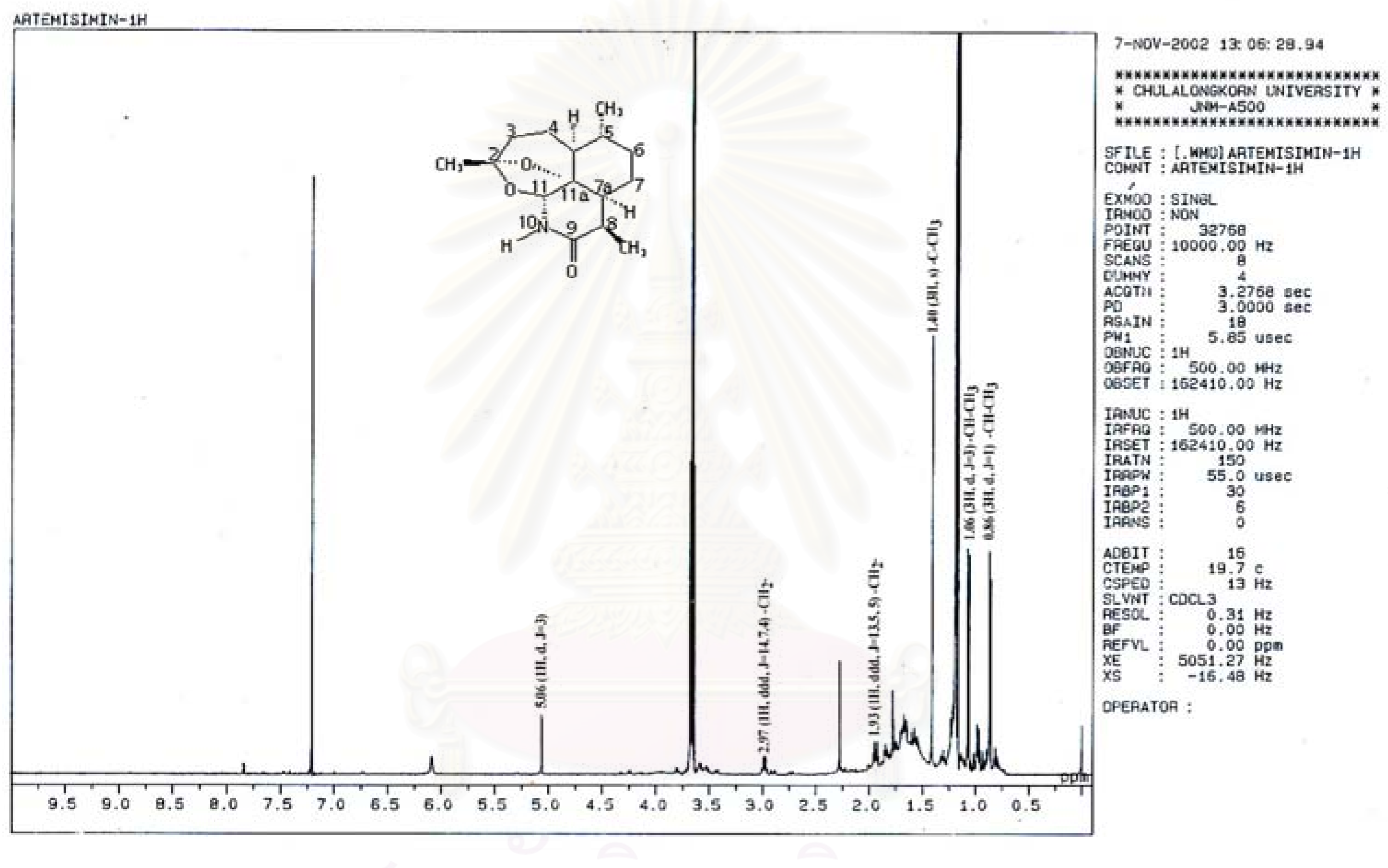

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Figure 9 ^{1}H-NMR spectrum of the product of artemisinin after being exposed on a TLC plate with saturated ammonia at 100°C for 2 h.

Table 3 Summary of ^{13}C NMR data and assignments for 10-Azadesoxyartemisinin reported previously (Torox *et al.,* 1995) and of the NH_3-treated artemisinin product obtained from this study.

Carbon	10-Azadesoxyartemisinin	NH_3-Artemisinin derivative
2	107.5	107.5
3	34.7	34.63
4	22.8	22.81
4a	45.6	45.62
5	35.2	35.28
6	33.6	33.57
7	23	24.29
7a	43.6	43.54
8	32.8	32.86
9	173.8	173.63
11	81.5	81.49
11a	82.2	82.21
2Me	22.2	22.19
5Me	18.5	18.53
8Me	11.9	11.89

1.2 Determination of Optimum Wavelength for TLC-densitometric Analysis of 10-azadesoxyartemisinin

In order to select an optimum wavelength for TLC-densitometric analysis of artemisinin after being converted to 10-azadesoxyartemisinin, an UV-scan for an absorbance spectrum of the compound on a TLC plate was performed. The absorbance was scanned by TLC-densitometer from 200 nm to 370 nm. As shown in Figure 10A the UV absorption spectrum appeared to have a single λ_{max} at 320 nm. It can be seen that this UV absorption characteristic was significantly different from that of artemisinin itself. Therefore, the wavelength of 320 nm was used throughout in this study for the analysis artemisinin in various *A. annua* mutant samples. Preliminary study on a TLC-densitometric scan of authentic artemisinin after TLC development and NH_3 treatment found that the spot of artemisinin was very sensitive

to the scaning by the wavelength of 320 nm as shown by the densitometric chromatogram in Figure 10B.

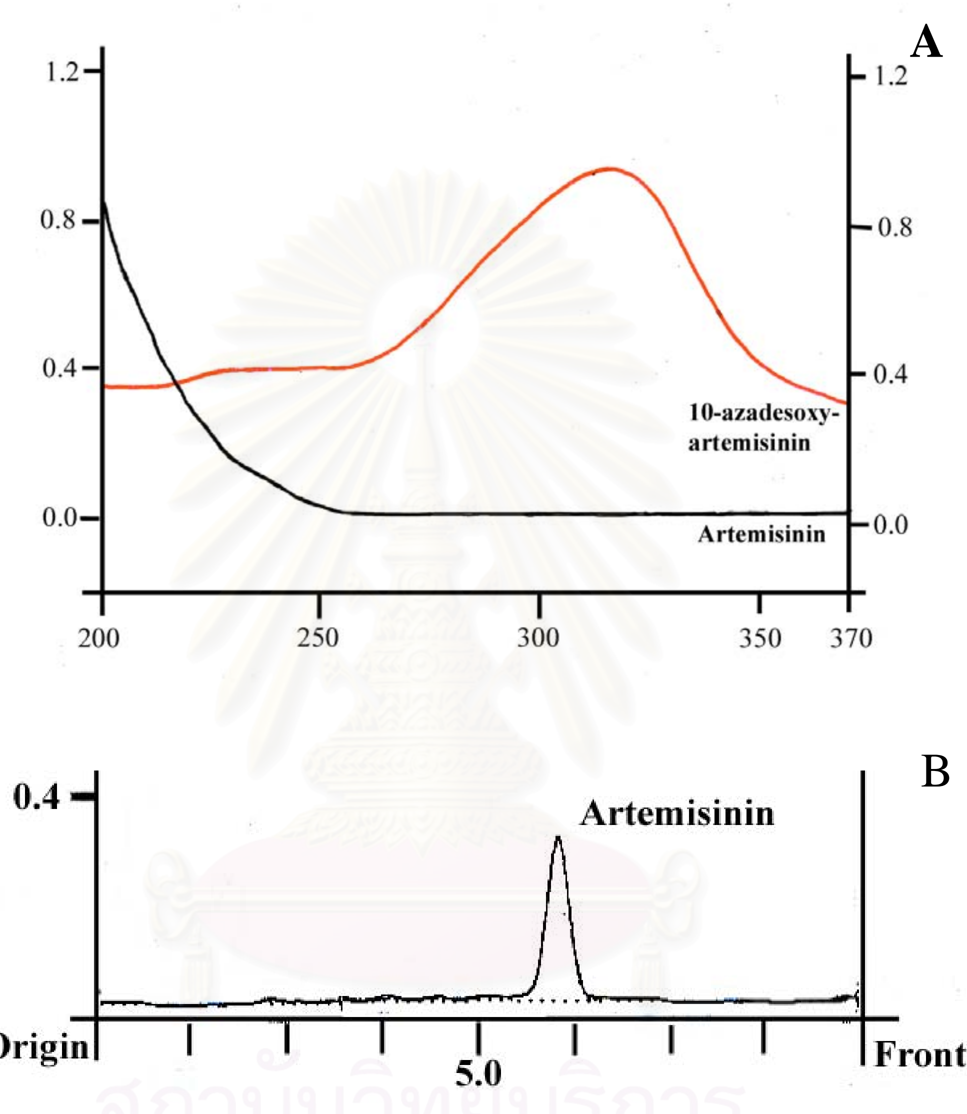

Figure 10 A) Absorption spectra obtainted from of TLC-densitometric scan of authentic artemisinin and of NH_3-treated artemisinin product. B) TLC-densitometric scan of NH_3-treated artemisinin product after TLC development of authentic artemisinin using a solvent system of hexane:ethylacetate:acetone, 80:5:5 followed by NH_3-exposure of the plate

1.3 Condition Optimization of NH_3-treated Artemisinin Conversion on a TLC-plate

Since a complete NH_3-treated artemisinin conversion a TLC-plate is important for consistent analysis of the compound, the effects of temperature and NH_3-exposure time were investigated. For studying the effect of temperature, a fixed time of 2 h were used and temperatures of 50, 75, 100 and 125°C were varied. It was found that the amount of 10-azadesoxyartemisinin was increased from the room temperature to reach highest content at 75°C (Figure 11). The temperatures of 100°C and 125°C shown no further increase of the artemisinin product. In terms of time-course study, an exposure of artemisinin with NH_3 at 100°C shown an continuous increase of 10-azadesoxyartemisinin content from 20 min to 120 min. There after, there was no further derivatization was observed (Figure 12). Therefore, the artemisinin conversion a silica gel plate was completed at 75°C for 2 h. However, for completed reaction the experiment was used 100°C for 2 h to artemisinin analysis.

1.4 TLC-Separation of Artemisinin from the Crude Extracts of *A. annua*

TLC-separation of artemisinin from the crude extracted of *A. annua* was studied in a few solvent systems. These include carbontetrachloride :hexane:ethylacetate:acetic acid, 22:46:6:2, hexane:ethylacetate:acetone: acetic acid, 60:6:3:1, hexane:dichlormethane:ethylacetate:ethnol, 60:1:10:6, hexane:ethylacetate:acetic acid, 25:7:1, hexane: ethylacetate:acetone:acetic acid, 80:10:5:1 and hexane: ethylacetate: acetone, 80:5:5.

. The results of the chromatograms obtained from the scanning using λ_{max} 320 nm are shown in Figure 10B. As shown in Figure 13 the trial of various solvent systems could not separate artemisinin from the crude extracts. Repeat development with the same solvent system could not get good result as well (Figure 14). Eventually, it was found that the solvent system of hexane:ethylacetate:acetone, 80:5:5 could completely separation of the artemisinin compound from the other constituents in *A. annua* crude extracts (Figure 15). Therefore, the solvent system has been used to artemisinin analysis.

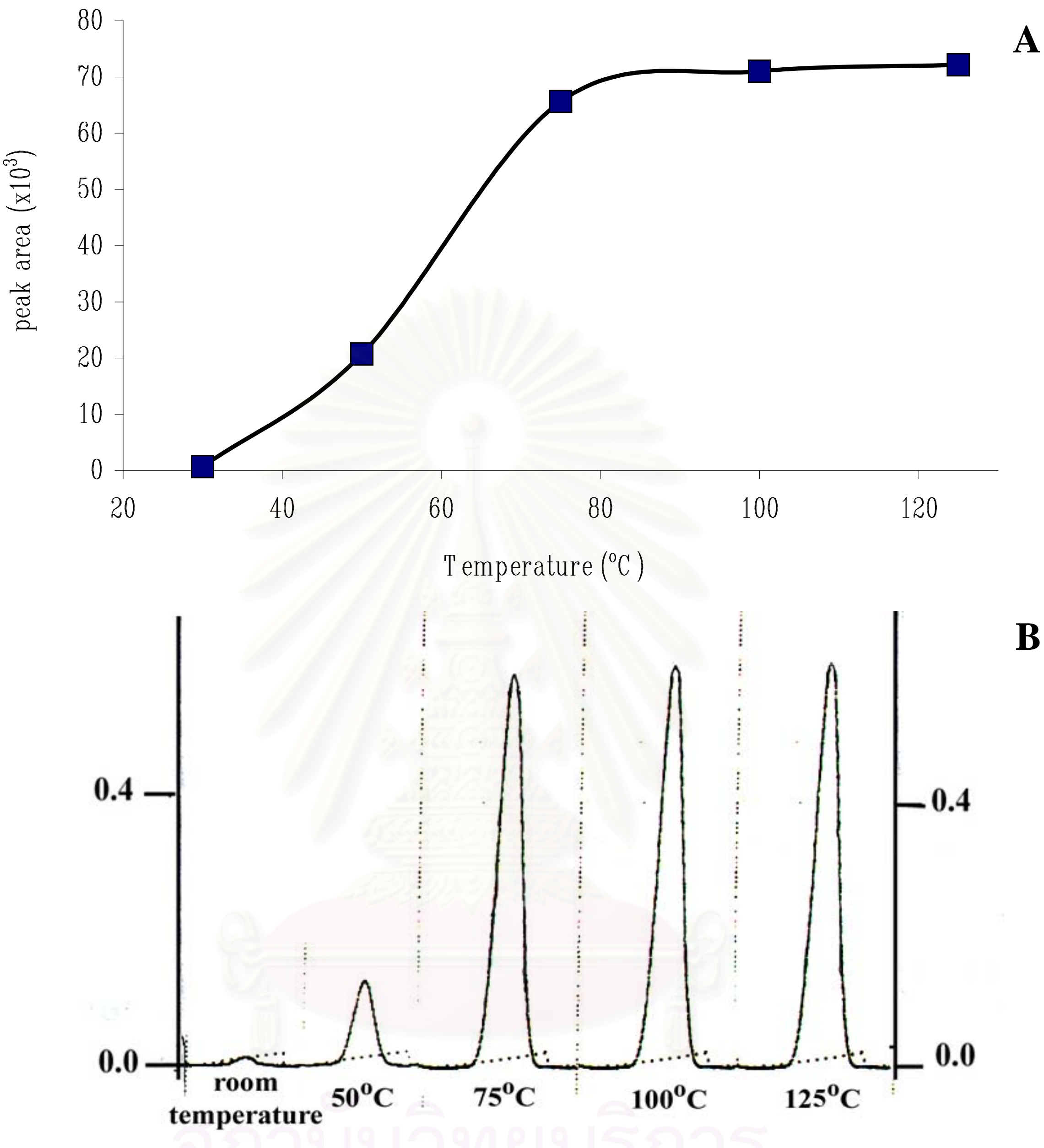

Figure 11 Effect of temperature on the ammonia treated of artemisinin conversion. A) The graph showing area-temperature relationship of the NH_3-treated artemisinin conversion. B) TLC-desitometric chromatograms of the conversion of various temperatures.

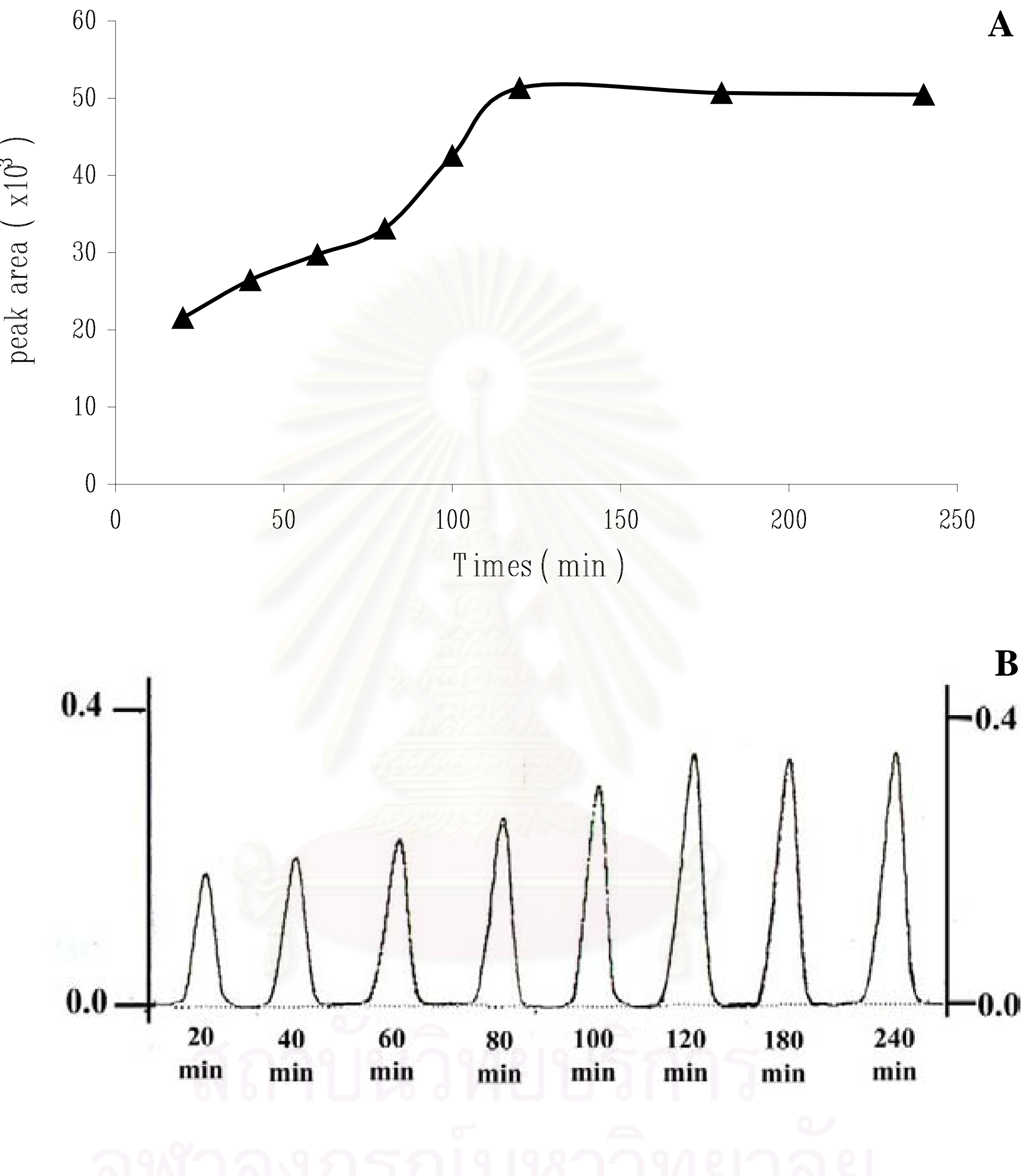

Figure 12 Times-course of ammonium derivatization of artemisinin. A) The graph showing peak area exposure time relationship of the NH_3-treated artemisinin conversion. B) TLC-densitometric chromatograms of the conversion at various time intervals.

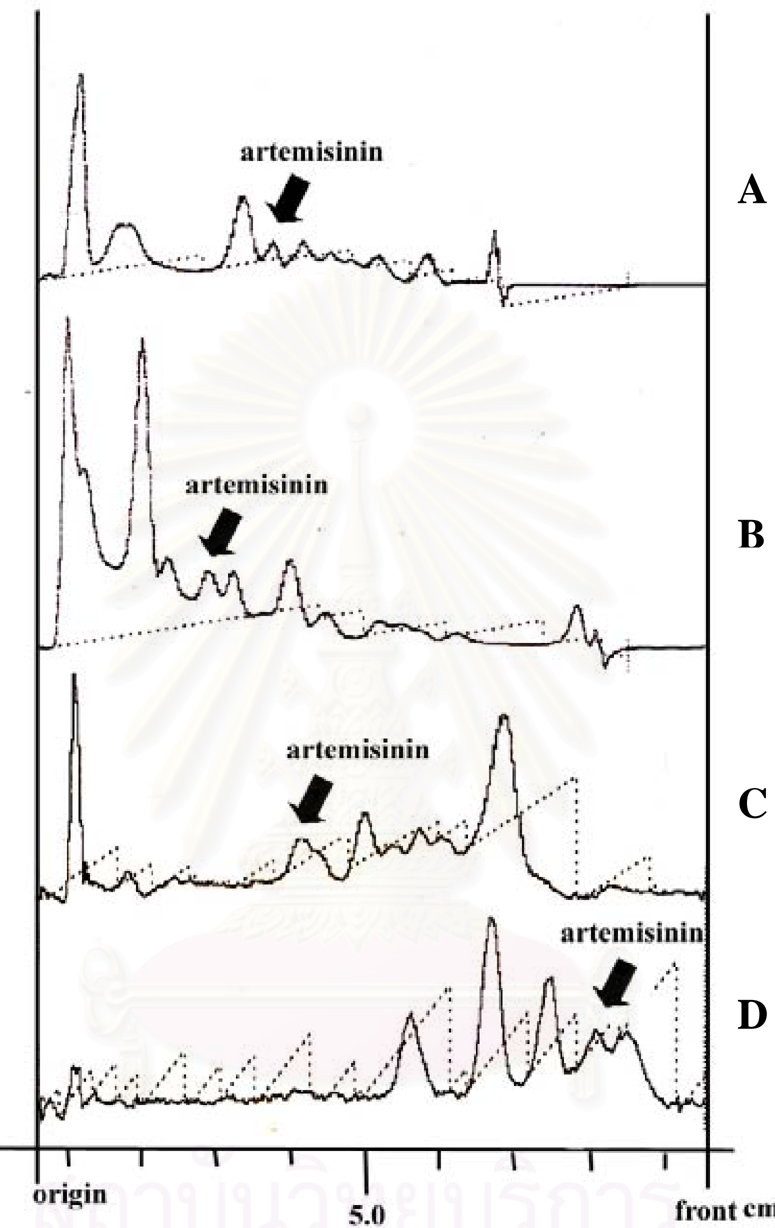

Figure 13. TLC-desitometric chromatograms of crude extracted from some *A. annua* mutants in each solvent. A) carbontetrachloride:hexane:ethylacetate: acetic acid, 22:46:6:2, B) hexane: ethylacetate: acetone:acetic acid, 60:6: 3:1, C) hexane:dichlormethane:ethylacetate:ethnol, 60:1:10:6, D) hexane: ethylacetate:acetic acid, 25:7:1.

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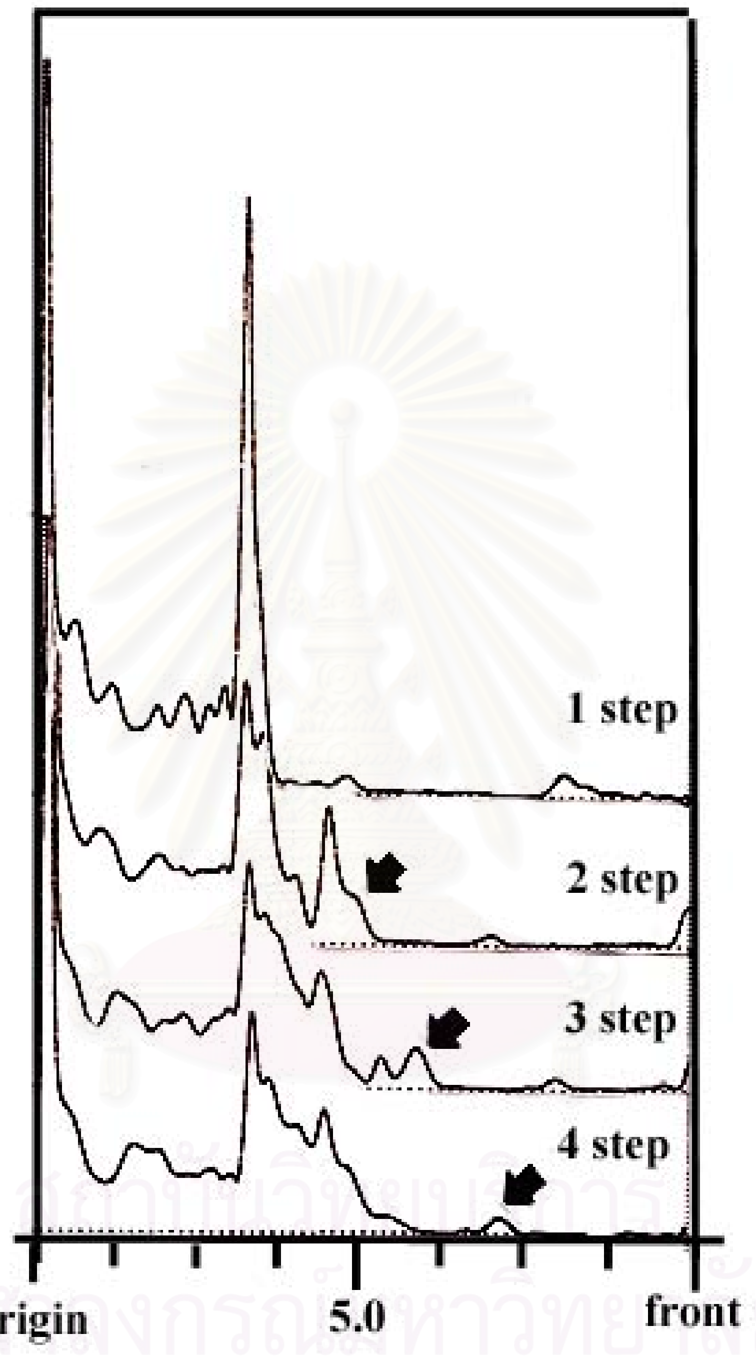

Figure 14 TLC-desitometric chromatograms of crude extracted from some *A. annua* mutants using solvent system of hexane: ethylacetate:acetone:acetic acid, 80:10:5:1 in four developing steps.

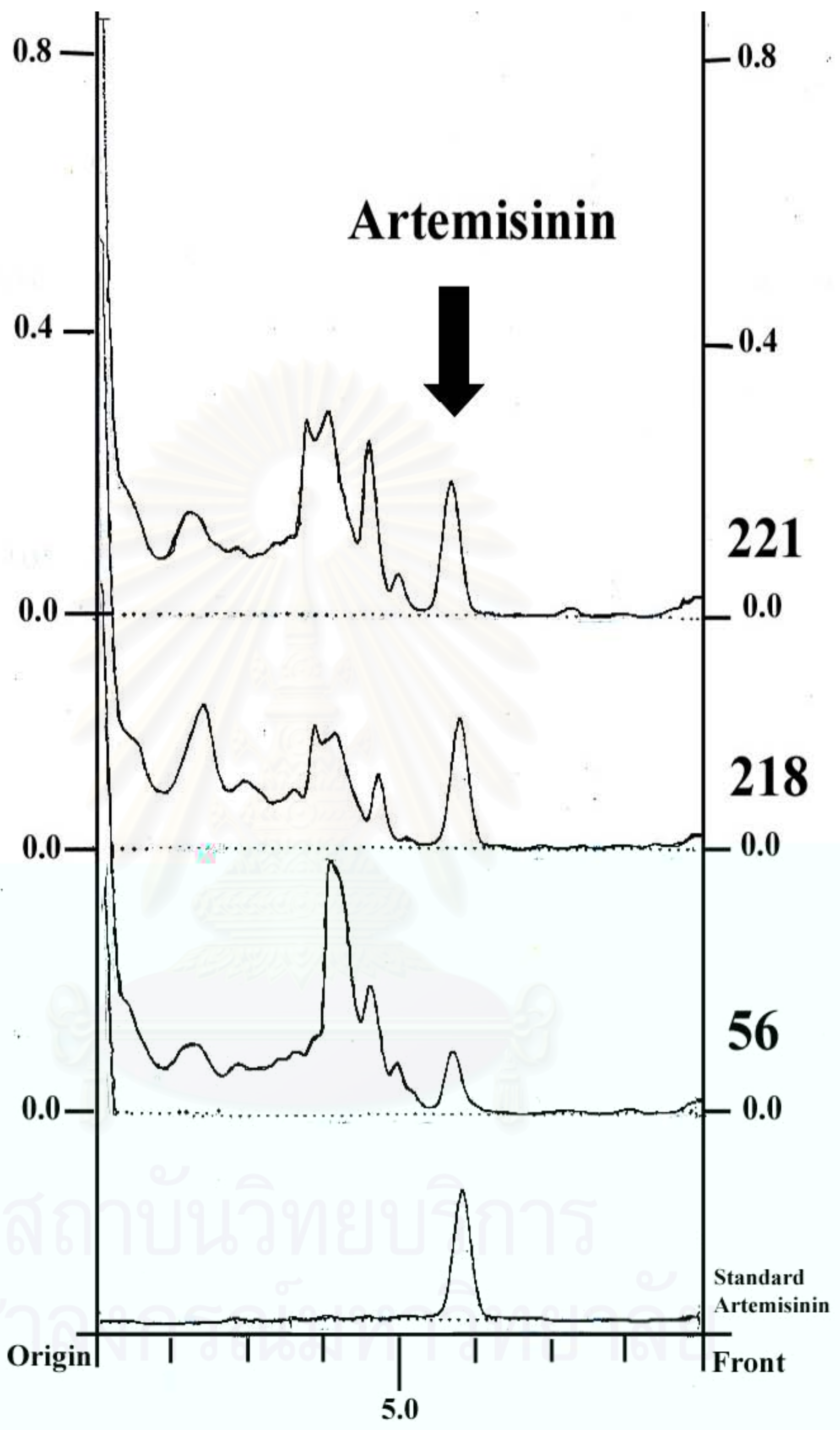

Figure 15 TLC-densitometric chromatograms of crude extracted from some *A. annua* mutants using the solvent system of hexane: ethylacetate: acetone, 80:5:5.

1.5 Method Validation of TLC-Densitometric Analysis

In terms of accuracy and precition, the developed TLC densitometric method was compared with previous report of HPLC (Zhao and Zeng 1986) method. It was found that both methods showed linearity of the artemisinin content in range of 0.25 – 12 μg with the correlation coefficients (r) of 0.9976 for HPLC method and 0.9951 for TLC densitometric method (Figure 16). The regression equations of standard curves are summerized as follow:

For HPLC method: $y = 960837x-41427$ $r^2=0.9998$

For TLC densitometric method: $y = 15743x+3195$ $r^2=0.9938$

By determining artemisinin content of some plant samples using both the TLC-desitometric and HPLC method, if was found that the values of artemisinin contents of various leaf samples determined by TLC densitometry was similar to those determined by the HPLC method (Table 4). Since there were more than 200 mutant samples to be analyzed for their artemisinin content, TLC densitometric method was used for the rapid screening of high-artemisinin producing mutants of *A. annua*.

Table 4 Comparison of artemisinin content by TLC-densitometric method and HPLC method

Sample	Artemisinin content (%w/w crude extract)	
	TLC-densitometric method	HPLC method
1	1.55±0.143	1.23±0.085
2	1.27±0.271	1.30±0.081
3	1.23±0.207	1.24±0.053
4	2.66±0.263	2.62±0.078
5	1.51±0.09	1.57±0.011
6	0.87±0.165	0.76±0.03

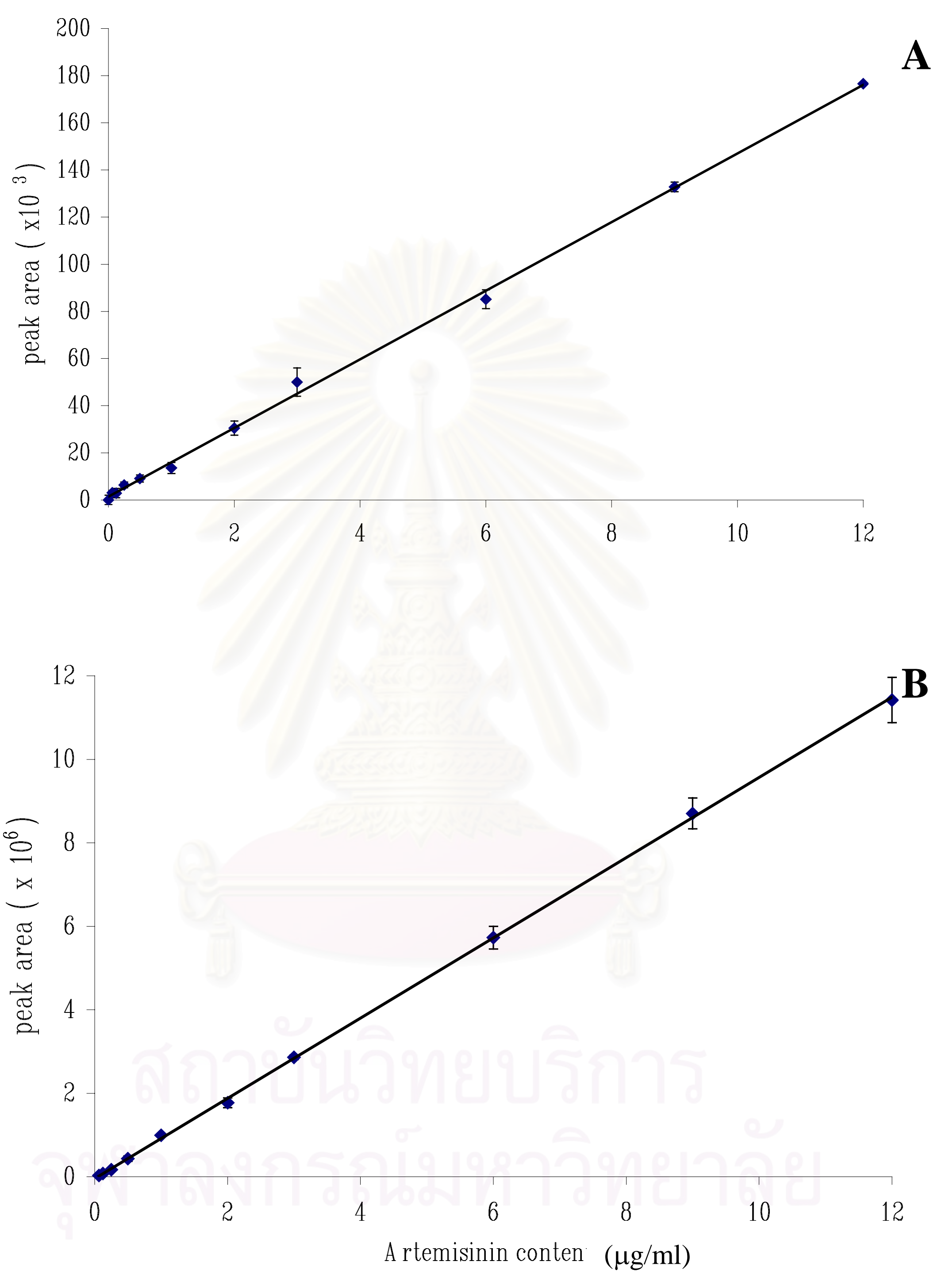

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Figure 16 Calibration curve of standard artemisinin determined by A) TLC-densitometric method. B) HPLC method.

2. Variation of Artemisinin Content is Various Mutants of *A. annua*

Various crude extracts of artemisinin used in this study were extracted as described in Materials and Methods by Miss Araya Choachai from Plant Biochemistry and Plant Physiology Laboratory, BIOTEC. In terms of artemisinin analysis, the crude extracts were each diluted to 8 µg / µl and spotted (10 µl) onto a TLC silica gel plate. After being developed with the solvent system hexane:ethylacetate:acetone, 80: 5 : 5, and exposed under saturated ammonia 100 ^{o}C for 2 hours, the plate could be visualized under UV_{254} the chemical patterns of various mutants with different intensities of the spot of artemisinin (Rf 0.75) (Figure 17). After being scanned by a TLC-densitometer to obtain the peak area of artemisinin, it was found that the artemisinin contents of various *A. annua* mutants were varied in from 0.06 to 3.4 % of the dry crude extract weight. The sample from the 500 rad γ-ray irradiation were found to have artemisinin content in the range from 0.02 to 3.34 % crude extract (Figure 18) and those from the 800 rad γ-ray irradiation were 0.14 to 3.46 % crude extract (Figure 19). In terms of distribution of percent artemisinin content, both 500 and 800 rad γ-ray irradiation samples showed similar major distributtion of the population in range from 0.5 to 1.0 and 1.0-1.5 % crude extract (Figure 20). Interestingly, some high artemisinin samples were found in a few samples of both treatments with the range of 2.5 - 3.0 and 3.0 - 3.5 % crude extract.

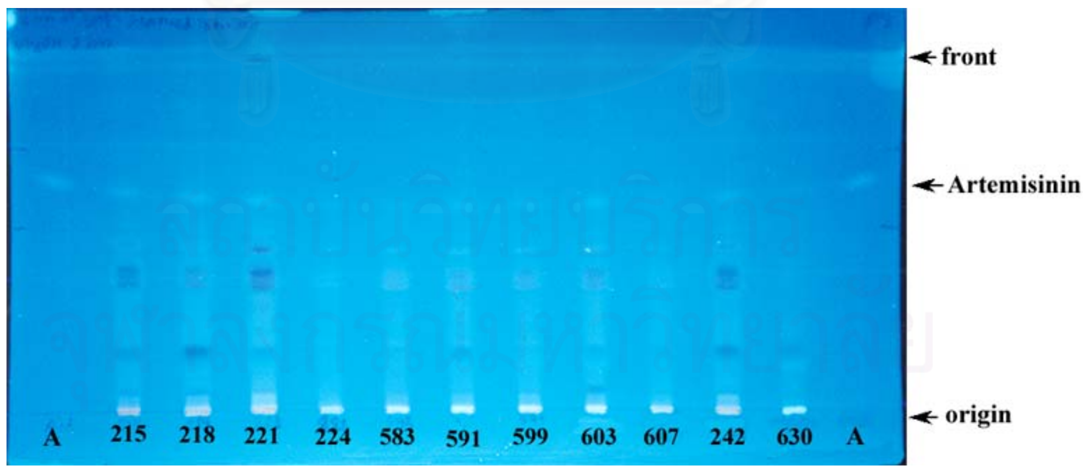

Figure 17 TLC-patterns observed under UV 360 nm of a some *A. annua* crude extracts were observed after exposed with ammonia at 100°C for 2 h. A) authentic artemisinin.

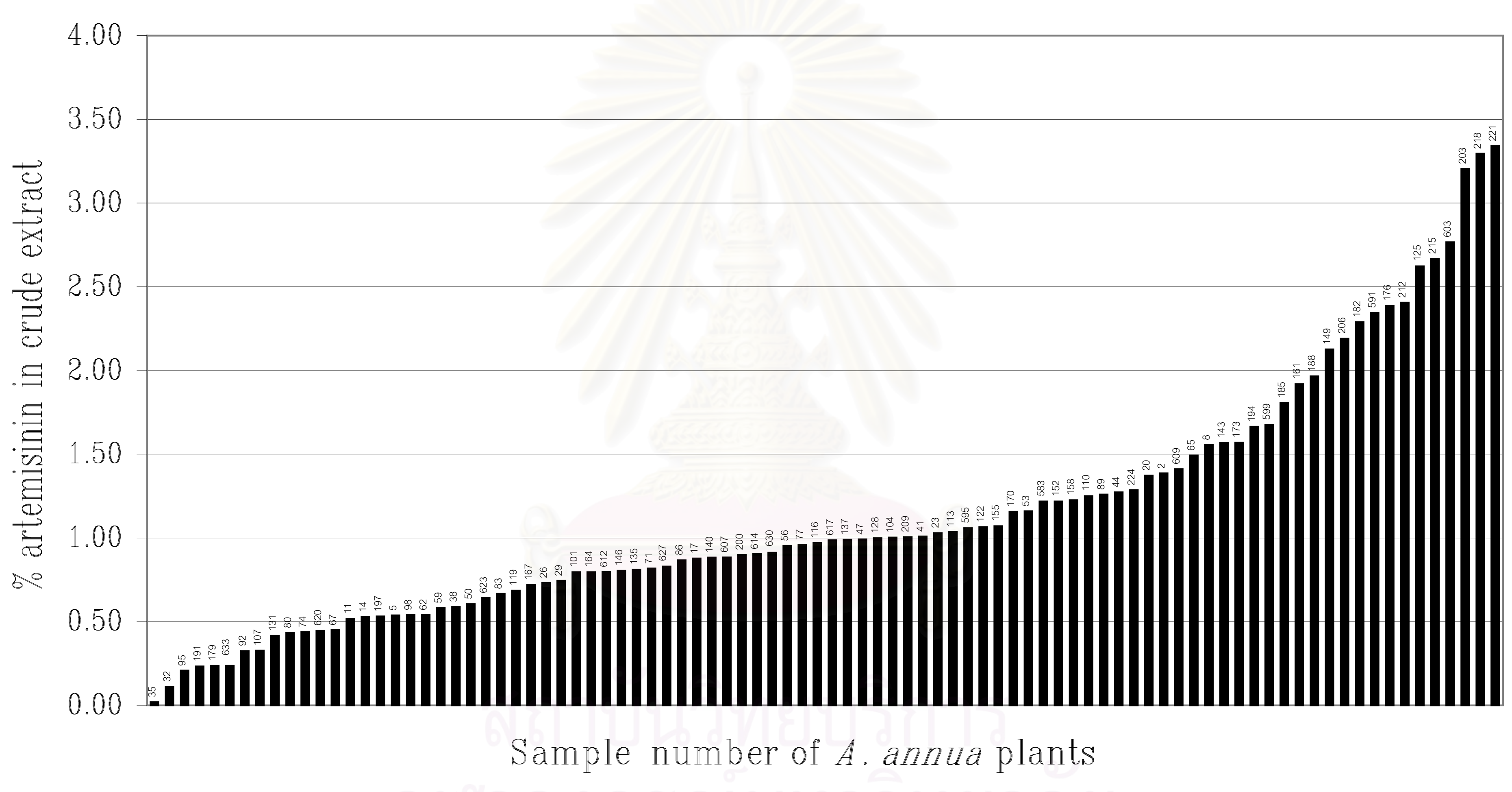

Figure 18 Artemisinin content in various *A. annua* mutants obtained after the plants were exposed with 500 rad γ-ray irradiation.

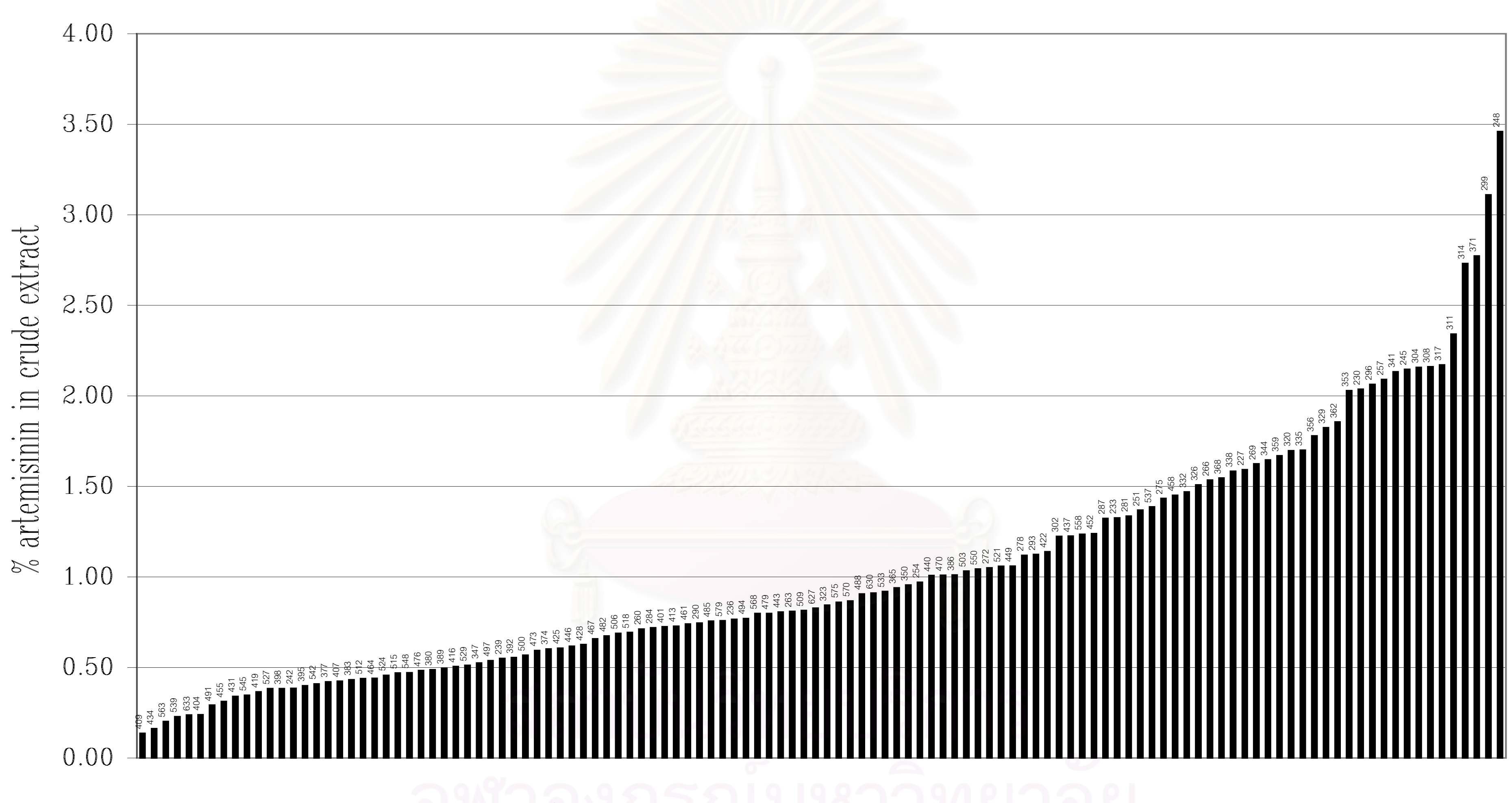

Figure 19 Artemisinin content in various *A. annua* mutants obtained after the plants were exposed with 800 rad γ-ray irradiation.

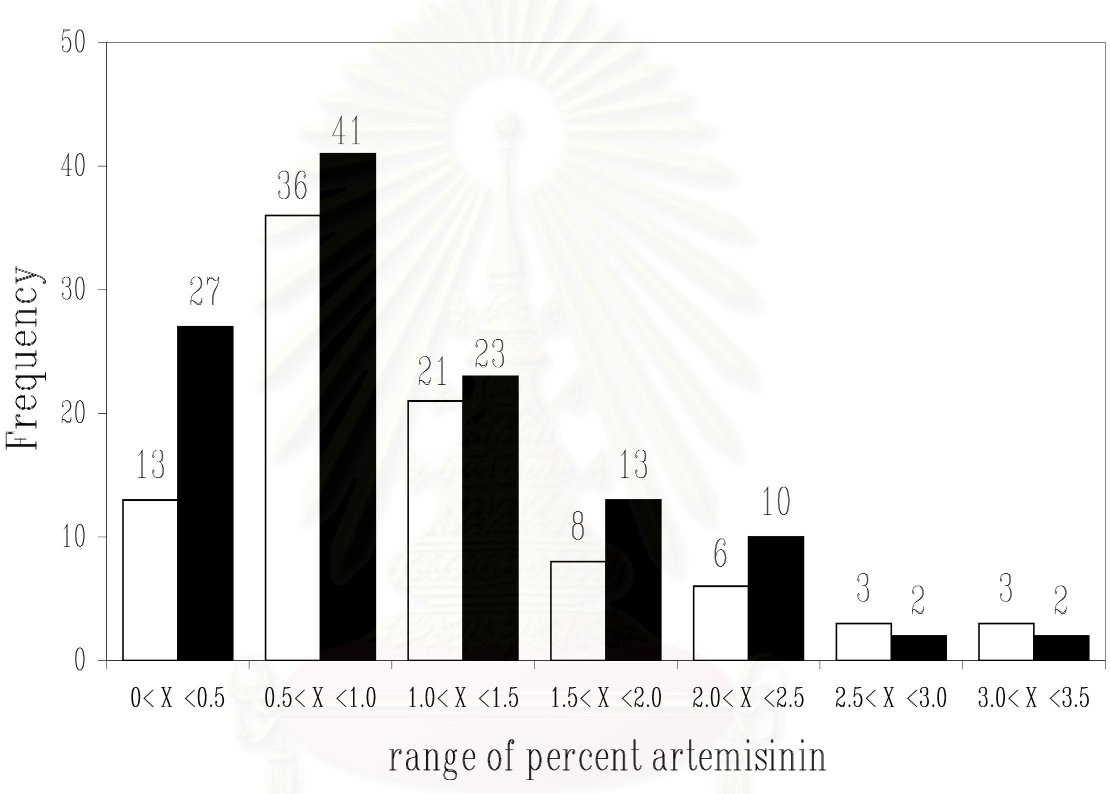

Figure 20 Distribution of artemisinin content in various *A. annua* plants after being exposed with 500 rad (□) and 800 rad (■) γ-ray irradiation.

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3. Development of the Enzyme Assay of Amorpha-4,11-Diene Synthase by TLC-Radioscanning

3.1 Detection of Enzyme Product of Amorpha-4,11-Diene Synthase

An enzyme assay of amorpha-4,11-diene synthase has been reported previously by Bouwmeeteer *et al.,* (1999). This method used radioactivity labeled [1-3H(N)]FPP as substrate and detected the formation of the radioactive product by radiocounting using a liquid scientillation counter. In this study, the assay condition used in the reaction mixture was similar to that of Bouwmeesteer *et al.,* (1999), except the step of the detection of radioactively labeled product was detected by TLC-radioscan which detected directly the availability of the enzymatic product. In practice, the non-polar enzyme product was separated from the polar substrate by extracting the reaction mixture with 1 ml hexane. The hexane fraction was then evaporated to a small volume and spotted onto a silica gel plate. After that, the plate was developed under the solvent system of hexane : ethylacetate : acetic acid, 25:7:1 to separate the enzyme product from other compounds. The enzyme activity was then detected by TLC-radioscanner. Figure 21 shows typical TLC-radiochromatograms both of an enzyme-catalyzed reaction and a boiled control of an enzyme extract prepared from a *A. annua* plant without being exposed with γ-ray irradiation. It can be seen that, the enzyme substrate of [1-^{3}H(N)] FPP did not appear in the TLC plate because of the polar property of the substrate which is not soluble in hexane while the enzyme product, amorpha-4,11-diene was detected clearly at the Rf value of 0.4. The boiled control that had no enzyme activity showed no conversion of [1-^{3}H(N)] FPP to the enzyme product.

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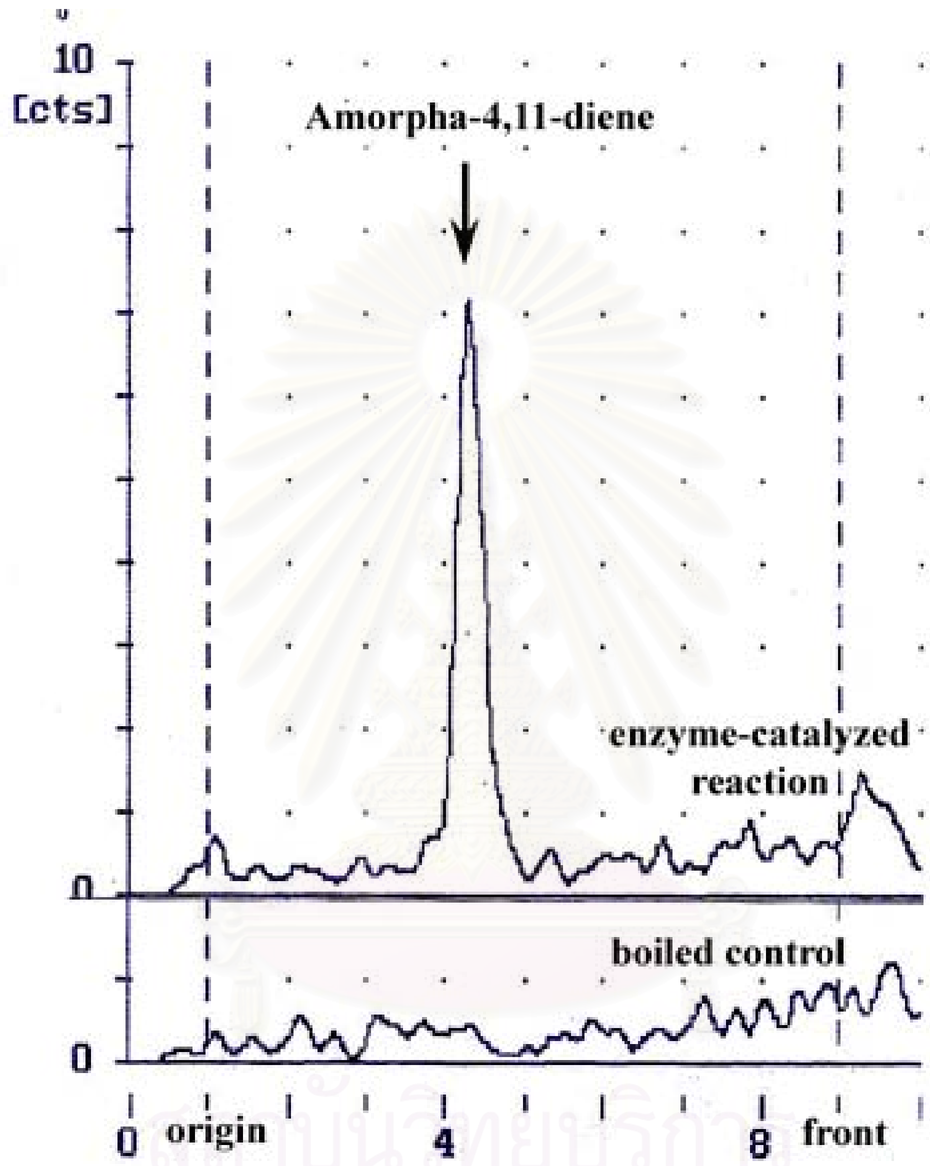

Figure 21 The TLC-radiochromatograms of the enzyme assay of amorpha-4,11-diene synthase from the enzyme extract of *A. annua*

3.2 Time-course study on the conversion of [1-^{3}H(N)] FPP to amorpha-4,11-diene

In order to confirm that the observed radioactive peak in Figure 21 was the enzymatic product of FPP, which is catalyzed by amorpha-4,11-diene synthase, a time-course of the enzyme catalyzed reaction was studied. The enzyme crude extract was incubated with radioactively labeled [1-^{3}H(N)] FPP under same reaction mixture. After the times 0, 5, 10, 20, 40, 80, 120, and 180 min, various reaction mixtures were extracted from their enzyme product with hexane for determined the formation of the radioactive product by TLC-radioscanner. As shown in Figure 22 and Figure 23 the enzyme product was immediately detected from 5 min to 40 min and slidely increased from 40 min to 80 min and stabled until 180 min.

3.3 Coenzyme requirement in the enzyme catalyzed reaction

Incubation of the reaction mixture was also carried out in the presence and absence of NADPH for 20 and 120 min. The enzyme activity showed no difference in their radiochromatograms in both conditions.

4. Correlation between Artemisinin Content and Enzyme Activity of Amorpha-4,11-Diene Synthase in various *A. aanua* Mutants

The results from the variation of artyemisinin content in various *A. annua* mutants (Figures 24, 25, 26 and 27) raised a question on the possible site of gene mutation occuring in the biosynthesis pathway of artemisinin in *A. annua*.

Recently, the first probable step in artemisinin biosynthesis has been proposed to be amorpha-4,11-diene synthase (Bounmester *et al.,* 1999). It was, therefore, interesting to investigate in this study the relationship between the activity and the artemisinin content in the generated mutants. If the artemisinin content was parallely increased or decreased with the enzyme activity, of amorpha-4,11-diene synthase, it would indicate that the mutation might occur at the gene of amorpha-4,11-diene synthase and thus be important for the production of the compound in the plant.

In this experiment, some selected mutants of *A. annua* were subjected to both the analysis of artemisinin content and enzyme assay using the methods developed in this study. For the artemisinin determination, it was found that the content of artemisinin was detected in range from 0.45 to 2.73 % dry leave (Figure 24).

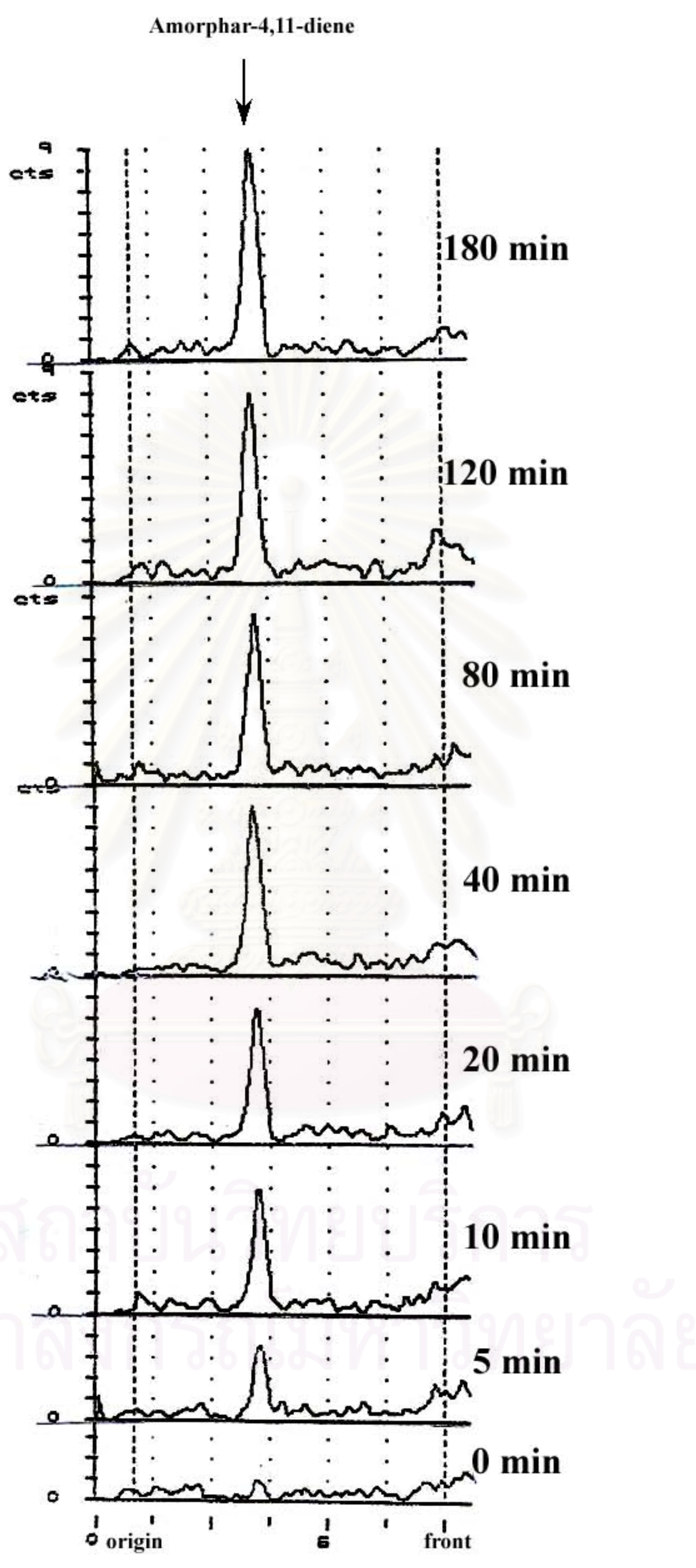

Figure 22 TLC-radiochromatograms showing the time-course of the conversion of [1-^{3}H(N)]FPP to a radioactively labelled amorpha-4,11-diene at 30°C the enzyme amorpha-4,11-diene synthase.

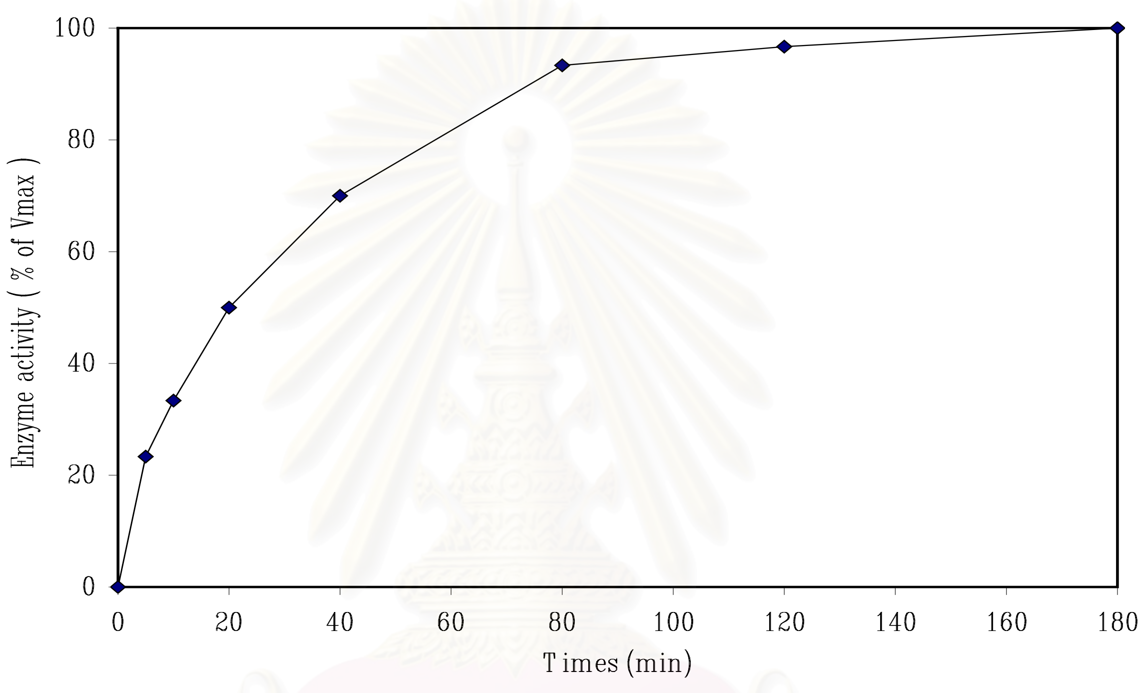

Figure 23 Time-course of the enzyme activity of amorpha-4,11-diene synthase in the crude enzyme extract prepared from *A. annua*.

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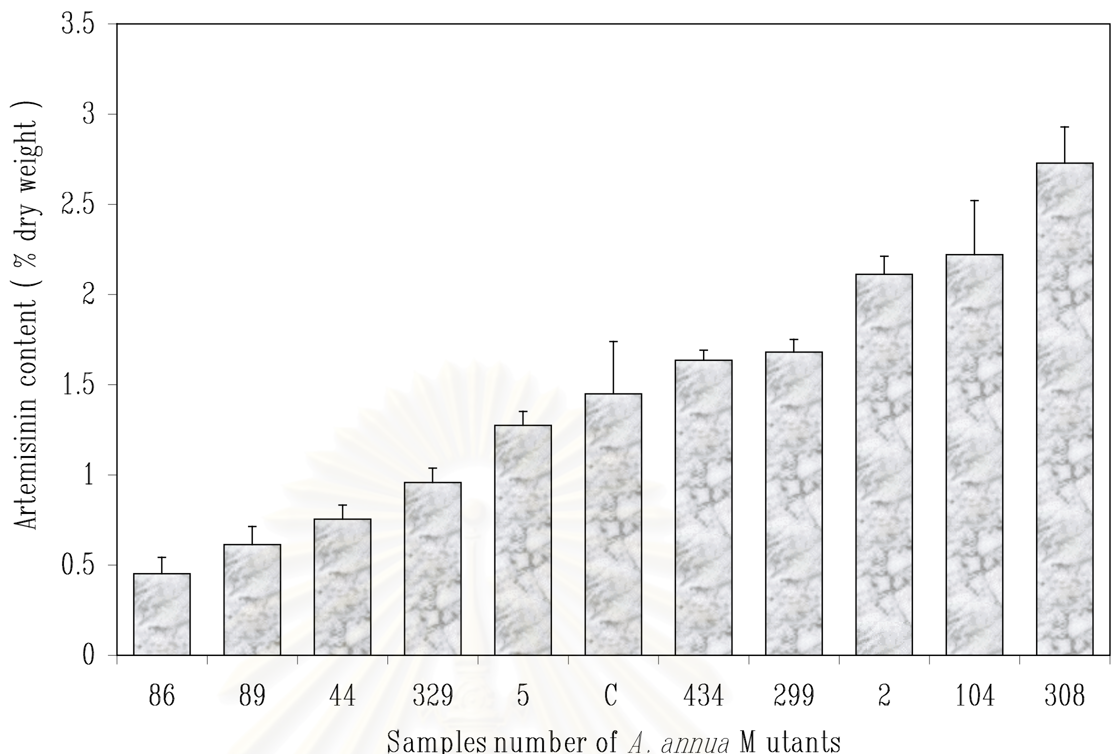

Figure 24 The artemisinin content in a some *A. annua* mutants for assay the enzyme activity of amorpha-4,11-diene synthase

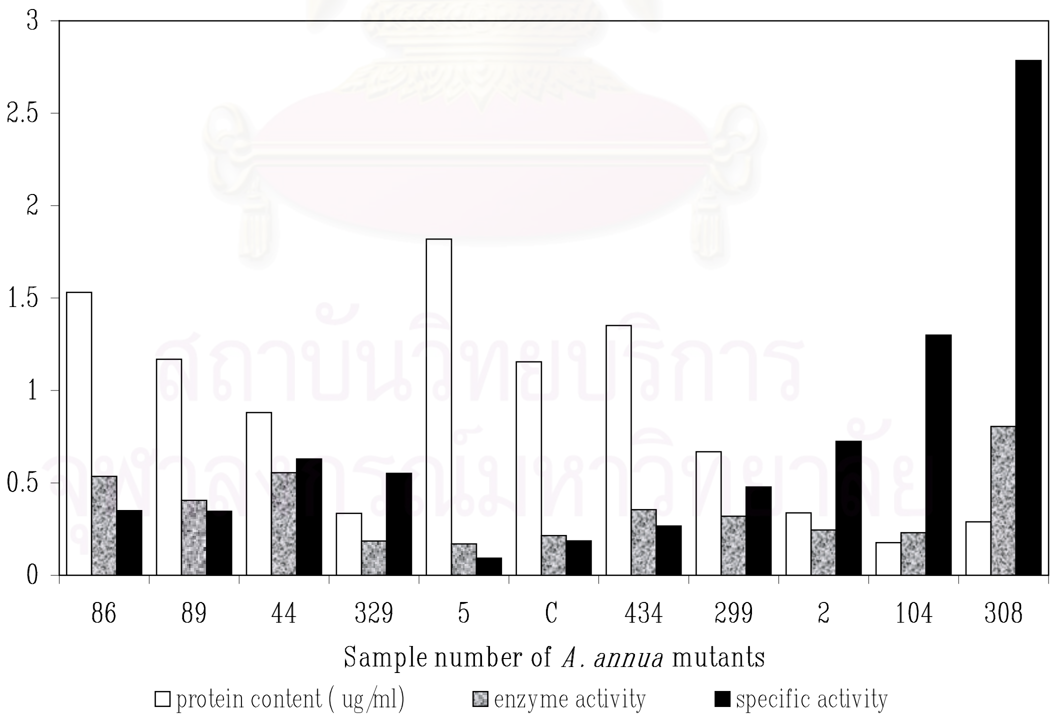

Figure 25 Comparison of the total protein, enzyme activity and specific enzyme activity of a some enzyme crude extract from *A. annua* mutants.

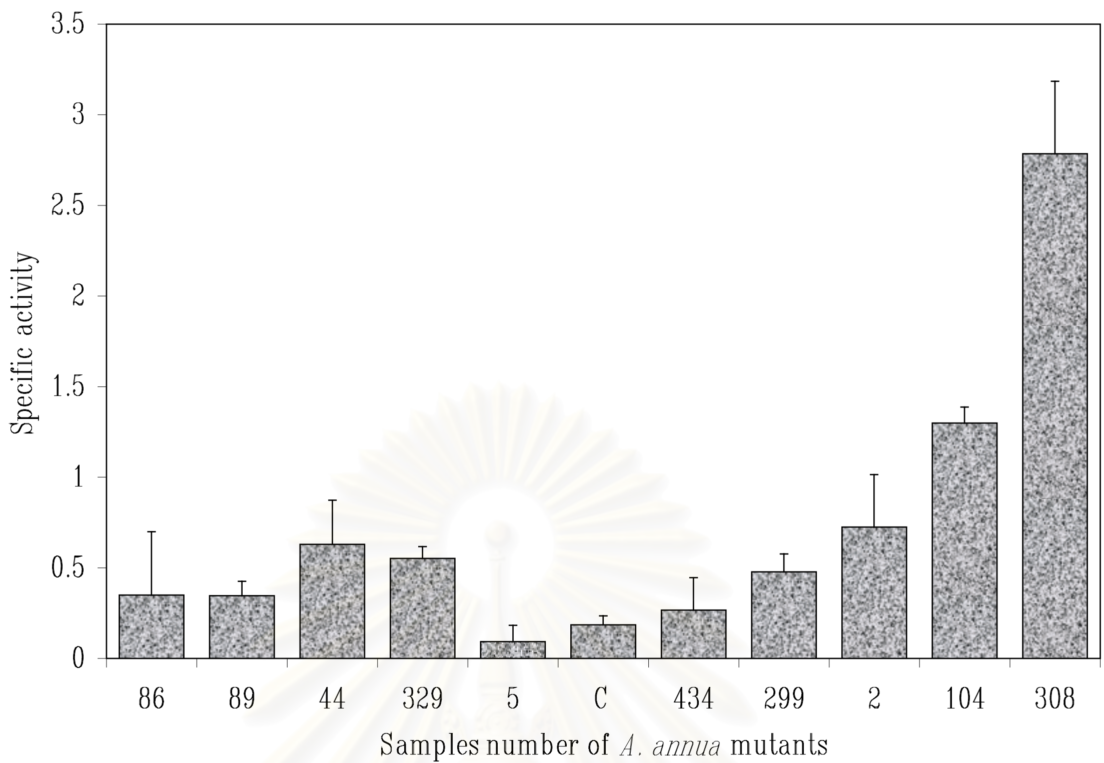

Figure 26 Specific activity of an amorpha-4,11-diene synthase in a some enzyme extract from *A. annua* mutants.

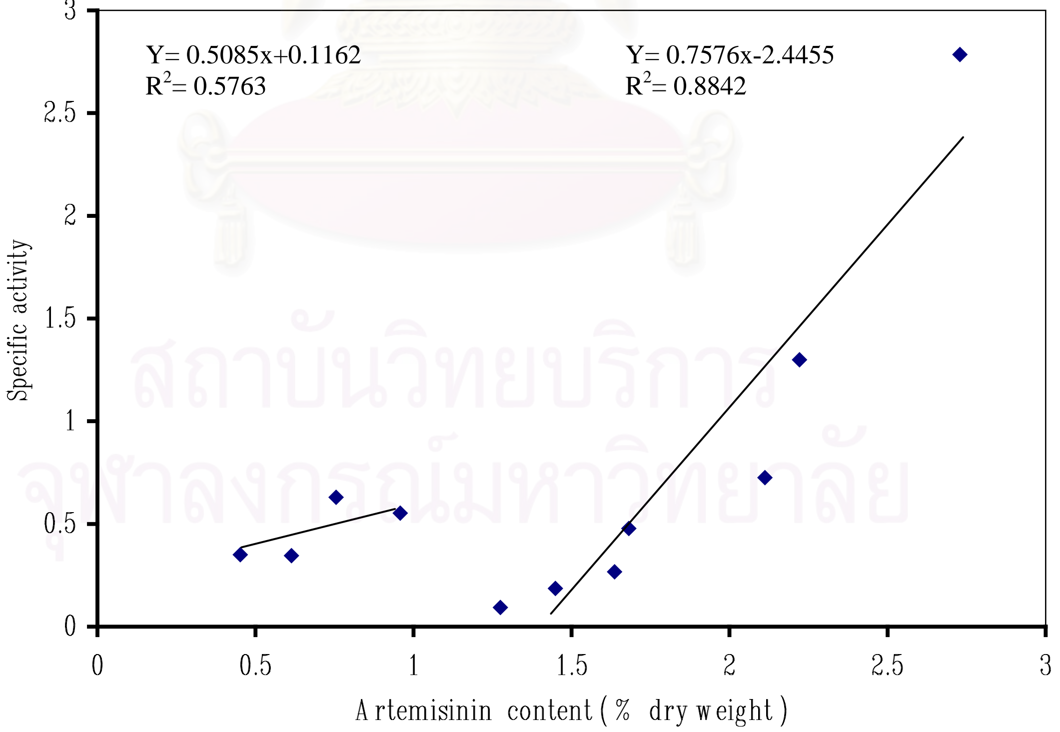

Figure 27 Correlation between an enzyme activity of amorpha-4,11-diene synthase and the content of artemisinin in some *A. annua* mutants

For the enzyme activity, it was found that amorpha-4,11-diene synthase extracted from the same selected *A. annua* mutants also showed variation in its enzyme activity (Figure 26). Interestingly, the mutants also showed a content. By comparing both parameters, it was found that the mutants could be divided into two groups. One group showed a clear linear relationship between the compound content and the enzyme activity. These included most of high artemisinin producing mutants of numbers 5, 434, 299, 2, 104 and 308 (1.25-2.50 % dry weight). The other group of mutants, including the numbers 86, 89, 44, and 329 were observed to be the low-artemisinin producing mutants (0.45-0.96 % dry weight).

5. Study on the Antimalarial Activity of some selected *A. annua* Mutants

In this study crude extracts and prepared from the dry leaves of various *A. annua* mutants were each diluted with DMSO to obtain a concentation of 10 μg/ml tested for their antimalarial activity against *Plamodium falipalum*. The antimalarial activity of each sample was exposed as a unit of the extract concentration that could decrease *Plasmodium* parasite growth by 50% (IC_{50}). The results are shown in Figure 28. It can be seen that the antimalarial activities of all clones were higher than the control parent plant. Among their clones, the numbers 299 and 89 showed highest antimalarial activity with their IC_{50} values of 0.015 μg/ml and 0.025 μg/ml, respectively. The results also showed that there were some degrees of the correlation between artemisinin content and antimalarial activity except the clone number 89. On the other hand, the highest antimalarial activities of clone number 299 showed good correlation with artemisinin content.

For the clone number 89, which had high antimalarial antivity but low artemisinin content, if was found that its crude extract had some extracompound appearing on a TLC plate (Figure 29). These spots of compounds were not found in the other clones. It is possible that the compound may exhibit the observed high antimalarial activity in this clone. It would be interesting to study the chemical structure and the antimalarial of such a compound.

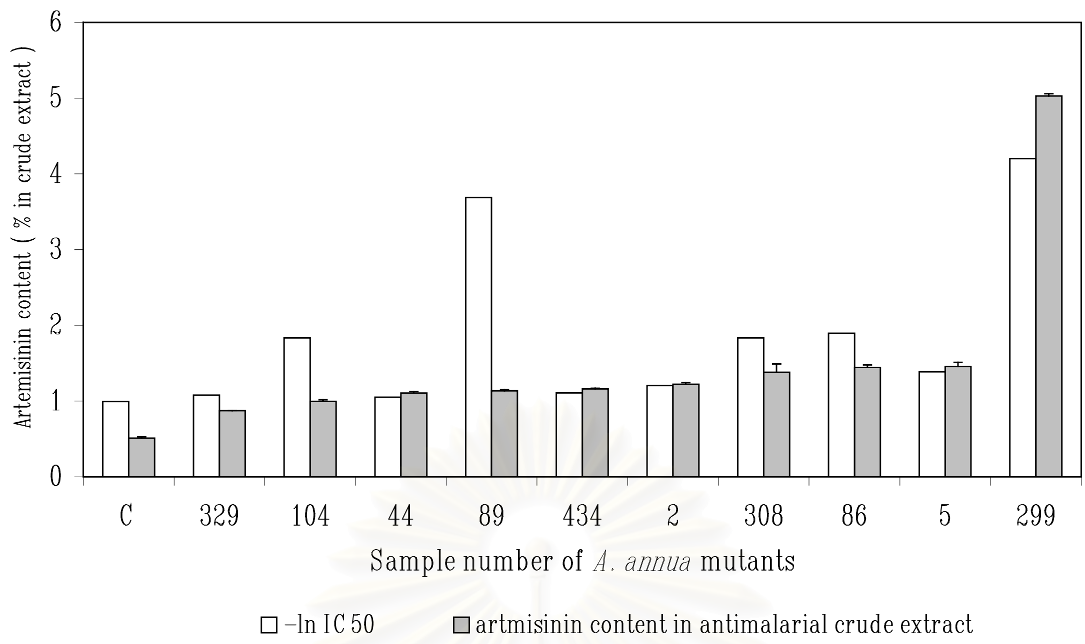

Figure 28 Correlation of an artemisinin content in antimalarial crude extract and $-\ln IC_{50}$

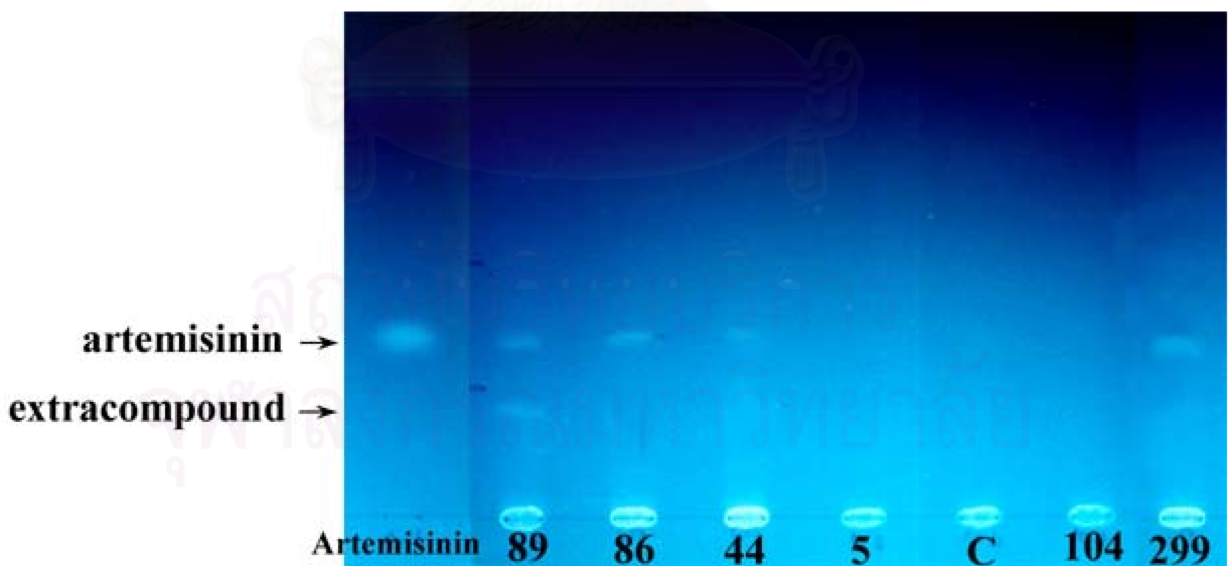

Figure 29 TLC pattern of the antimalarial crude extract after exposed with ammonia at 100°C for 2 h.

CHAPTER IV

DISCUSSIONS

1. Determenation of TLC-Densitometric Method for Determination of Artemisinin Content

Although artemisinin has been isolated from *Artemisia annua* plant since 1985 (Klayman 1985), the content of artemisinin in the leaves of this plant is still present in relatively low level. There have been several attempts from many laboratories to increase the production of artemisinin (for review, see Geldre *et al.,* 1997). However, one major obstacle on the selection of high-artemisinin producing plants is the determination of the compound in various variants of the plant samples. This is due to the absence of an UV or fluorescence chromophore in the molecule of artemisinin. So far, some techniques have been developed for the analysis of artemisinin. An enzyme-linked immunosorbent assay (ELISA) has been reported for the analysis and screening of artemisinin content in *A. annua* plants (Jaziri *et al.,* 1993; Ferreira and Janick 1996) but the antibody produced have cross-reactivities between artemisinin and its derivatives. Gas chromatography (GC) has been used for artemisinin analysis (Sipahimalani *et al.,* 1991) but thermal decomposition of artemisinin in the column causes the appearance of two peaks of degraded products. High performance liquid chromatography (HPLC) with UV monitoring at 210 nm has also been used but the presence of the other constituents that also absorb strongly at 210 nm interferes easily the peak of artemisinin (Acton and Klayman 1985; Liesh *et al*., 1986; Singh *et al.,* 1988). HPLC with electrochemical detector (HPLC-EC) has been developed successfully to detect artemisinin (Acton 1985). However, this type of detector is not commonly available in laboratories and the reductive electrochemical detection of artemisinin requires very special precautions as the peroxide oxygens of the compound can be reduced at low cathodic potential (Siphamalani *et al.,* 1991). In addition, the analysis of artemisinin employing HPLC with chemiluminasence (CL) detection in the absence of hydrogen peroxides (Green *et al.,* 1995) and with light

scattering (LS) detector (Avery *et al.,* 1999) have been developed. Both techniques of HPLC-CE and HPLC-LS, however, require complicated procedure for the operation. HPLC with pre-column NaOH derivatization and UV detection at 260 nm has also been used (Zhao and Zeng 1986). This method is relatively sensitive but required pre-column derivatization for the analysis. Finally, thin-layer chromatography (TLC) has been developed to detect artemisinin (Tu *et al.,* 1982; Tawfiq *et al.,* 1989) but presented the spot of artemisinin is poor staining and overlaped with other constituents in the crude extract.

In this study, a simple and accurate TLC-densitometric analysis of artemisinin was developed for screening high-artemisinin producing plants of *A. annua*. The developed TLC-densitometric method was first designed to first separate artemisinin on a TLC plate from other crude extract constituents. The spot of artemisinin was then converted by NH_3 vapor to form a chromophore-containing compound that is allowed for UV detection and scanning to generate a chromatogram. The reason of using NH_3 vapor in instead of NaOH solution in the structure conversion because NaOH is not volatlile and will remain on the TLC plate and would interfere the scanning of TLC-densitometer.

Preliminary studies on the conversion of artemisinin spot on a silica gel plate by exposure with saturated ammonia vapor (at room temperature for overnight) should that the chromophore-forming compound had its maximum UV absorption at 320 nm. However, although ammonia has alkaline property as sodium hydroxide, the chromophore formed obtained from using in the saturated NH_3 vapor is different from that the NaOH condition. Structure elucidation of the NH_3-forming produce showed that the compound was 10-azadesoxyartemisinin. This compound has been reported to form in two steps of reaction sequences (Torok and Ziffer 1995). First, artemisinin reacts with methanolic ammonia followed by amberlyt 15 treatment to form 11-azaartemisinin. Second, the product is reacted in a mixture of sulfuric acid/silica gel to form both 10-azadesoxyartemisinin and 11-azaartemisinin in 15 % and 45 % yield, respectively (Torok and Ziffer 1995). In that report, the amount of standard artemisinin used was as much as 564 mg and thus the reaction might not be converted completely from artemisinin to 10-azadesoxyartemisinin because of a stearic effect of artemisinin itself and thus led to low amount of 10-azadesoxyartemisinin. In our study, however, very low amount of artemisinin (14 $\mu g/mm^2$ on silica gel plate) was

used and the TLC plate was developed by a solvent system that made even distribution of artemisinin on the silica gel plate. A complete conversion of artemisinin to 10-azadesoxyartemisinin was likely to occur after being incubated with

Artemisinin

saturated NH_3
Silica gel

H^+

10-Azadesoxyartemisinin

Figure 30 Propose conversion of 10-azadesoxyartemisinin from artemisinin by exposed under saturated ammonia at 100 ^{o}C for 2 h on pre-coated silica gel plate.

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saturated ammonia vapor. In terms of mechanism, Yagen *et al.,* (1994) has proposed the effect of silica gel-catalyzed rearrangement and subsequent Baeyer-Villiger reaction of artemisinin derivatization. The result of the catalytic reaction has been shown for the rearrangement of dexoartemisinin from artemisinin. Similarly, the reaction occuring to artemisinin on a silica gel plate may be the rearrangement of artemisinin to form an intermediate that can be reacted with ammonia molecule and converted to the final product of 10-azadesoxyartemisinin (Figure 30). Therefore, on the silica gel plate of our TLC-system, the conversion of artemisinin to 10-azadesoxyartemisinin can occur in one step. The product is quite stable since the value of peak area of 10-azadesoxyartemisinin obtained from its densitometric chromotogram appeared to be no change for least than 1 month after the structure conversion.

Another point of the difference between our TLC-desitometric method and the precolumn derivatized HPLC-UV method is on the step of artemisinin separation. The TLC method separates artemisinin compound from the other constituents first followed by the conversion of artemisinin to 10-azadesoxyartemisinin. On the other hand, the HPLC-UV method converte artemisinin to the compounds with Q_{292} and Q_{260} in crude mixture of artemisinin before the products are separated by HPLC. This complication of the reaction can interfere the conversion of artemisinin and affect the accuracy of artemisinin determination. Therefore, our TLC method is based on a complete artemisinin separation from the crude extract followed by a complete artemisinin conversion to 10-azadesoxyartemisinin in only one step reaction.

For the reliability in using the TLC-densitometric method to analyze artemisinin content, the optimum conditions for complete artemisinin conversion were subsequently studied. It was found that the optimum temperature (Figure 14) and time (Figure 15) were found to be 75°C and 2 h, respectively. However, to be sure on the complete reaction, the reaction conditions were used at 100°C for 2 h throughout the study.

In consideration of TLC-separation of artemisinin, because artemisinin is relatively non-polar (Bown, 2001) and the silica gel plate is polar, the main solvent systems were chosen to be non-polar for dissolving artemisinin during the TLC development. Other solvent composition should have modulate polarity for support

interaction of artemisinin with silica gel on the TLC plate. However, the crude extract of *A. annua* appeared to have many constituents, the suitable solvent system was, therefore subjected to trial and error for good results on artemisinin separation. As a result, the solvent system was found to be hexane:ethylacetate:acetone, 80:5:5. This solvent system clearly separated artemisinin from other constituents on the TLC plate, as shown by the TLC-densitometric chromatograms (Figure 15).

With respect to accuracy and sensitivity, the developed TLC method was compared with the well-established HPLC-UV method (Zhao and Zean 1986). Both methods shown their artemisinin calibration curves with linearity in the range from 0 to 12 μg/ml of artremisinin concentration. However, the HPLC method requires sample preparation that may interfere the reaction of artemisinin conversion whereas the TLC separation technique removes other constituents from crude extract before artemisinin conversion. Therefore, the TLC-densitometric method is clearly accurate, sensitive and reliable for artemisinin analysis.

2. Variation of Artemisinin Content in Various *A. annua* Mutants

Using the TLC-densitometric method, the selection of high artemisinin yield from *A. annua* mutants was performed. In this study, the mutants were generated by gamma ray irradiation and propagated by plant tissue culture technique. The results of artemisinin analysis showed a wide range of the compound from 0.02 to 3.46 % dry crude extract. Distribution of sample population in each artemisinin range showed that there were a few clones with highest artemisinin content in the range 2.5-3.0 and 3.0-3.5 % crude extract. In the literature, Cheewasakulyong (2001) has studied artemisinin production in *A. annua* mutants which are generated by gamma ray irradiation and propagated in MS medium. The artemisinin content in the mutant appears to be higher than the parent control plant. In our study, however, both higher and lower values than the parent control plants of artemisinin content were observed.

In principle, gamma ray is a mutagen with high radiation energy which can lead to damage to covalent bonding, hydrogen bonding and/or other molecular bondings of biomolecules in the cell and thus causing chromosome damage, gene damage (mutation), and eventually cell death. Generally, the damage of protein and RNA molecules can be quickly replaced using information encoded in the DNA but

the DNA molecules themselves are irreplaceable. Therefore, maintaining the integrity of the information of DNA is a cellular imperative and supported by elaborate set of DNA system. Although most such lesions are normally repairable through the action of intracelluar DNA repair process, those that remain unpaired or are misrepaired may give rise to permanent changes in the affected gene (mutation) or in the chromosomes on which the genes are carried. The cellular response of this change includes a wide range of the enzymatic systems that catalyze some of the most interesting chemical transformations in DNA metabolism and the other cellular metabolite both primary and secondary metabolite (Alberts *et al.,* 2002).

In practice, choice of suitable does for mutant generation has to be compromised between the mutagenic effects and damaging effects of the radiation. As the number of mutation increase, so also does the extent of damage to the plants (Mantell *et al.,* 1985; Gerates *et al.,* 1991). According to the report of Cheewasakulyong (2001) five does of gamma ray irradiation including 300, 500, 800, 1000 and 1500 rad were used. The results showed that artemisinin contents present in highest content in each does of radiation were 37.86, 34.49, 52.98, 47.34 and 26.92 $mg.g^{-1}$ dried weight, respectively. On the other hand, our study used the irradiated gamma ray in two does, 500 and 800 rad causing artemisinin content to be increased significantly to 3.34 and 3.46 % crude extract (Figures 18, 19).

Several reports have shown that gamma irradiation can be effective to induce mutation in plant species. Hasegawa *et al.,*(1995) have reported that the appearing of small pale green spots in leaves of tobacco plants derived from gamma irradiated anther. In peppermint and spearmint, the mutants obtained from irradiation with gamma ray have been shown to be resistant to witting disease (Johnson and Cumming 2000). In ornamental business, gamma irradiated canna plants have been used to generated spots, streaks, sector or even changing the color of ornamental (Nakornthop 1965).

3. Correlation Between Artemisinin Content and Enzyme Activity of Amorpha-4,11-Diene Synthase in Various *A. annua* Mutants

An enzyme assay of amorpha-4,11-diene synthase has been first reported by Bouwmeester *et al.,*(1999). The report has proposed that amorpha-4,11-diene synthase is the enzyme catalyzing the first step of artemisinin biosynthesis. The enzyme catalyzes the conversion of farnesyl pyrophosphate into amorpha-4,11-diene (Figure 6). Therefore, it is interesting to investigate in this study the relationship between the enzyme activity and artemisinin content in the generated mutants. If the artemisinin content changes paralleley with the enzyme activity of amorpha-4,11-diene synthase, it would indicate that the mutation may occur at the gene of amorpha-4,11-diene synthase and thus be important for the production of the compound in the plant.

In the step of enzyme preparation, the extraction buffer used in this study was modified from that reported by Bouwmeester *et al.,* (1999) which included 5 mM Mops buffer, pH 7.0, 10%(v/v) glycerol, 1 mM ascorbic acid, 10 mM $MgCl_2$ and 2 mM DTT. It can be seen that the buffer contains a number of chemicals for enzyme protection. These include thiol protective products (DTT), antioxidant agent (ascorbic acid), polyhydric alcohol (glycerol) and ionic stabilizer ($MgCl_2$). Since the enzyme activity has been reported to be present in a soluble fraction (Bouwmeester *et al.,* 1999). The enzyme preparation used in this study was subjected to 100,000*g* centrifugation for removal of other particulate components. The resulting supernatant was then desalted using a PD-10 column before being used.

In the step of enzyme assay, the method reported by Bouwmeester *et al.,* (1999) has used radioactivity labeled [1-^{3}H(N)]FPP as substrate and detected the formation of the radioactive product by radiocounting using radio-GC. The product of amorpha-4,11-diene appears 14 min retention time together with the product of farnesol at 28 min retention time. Farnesol can be formed from farnesyl pyrophosphate by phosphohydrolase activity, and thus in this study, molypdate salt was added to the enzyme assay solution to inhibite the phosphohydrolase activity. Using TLC-radioscanner for detection of radioactive amorpha-4,11-diene, the resulting radio peak was found at Rf 0.45 (Figure 21) confirming the presence of the

amorpha-4,11-diene synthase activity in the enzyme extraction. Under the assay condition, no farnesol peak was detected. Time course study (Figure 23) showed clearly the catalization of amorpha-4,11-diene synthase was time dependent under the temperature at 30°C. Incubation of the reaction mixture in the presence and absence of NADPH for 20 and 120 min showed no different in the enzyme activity. This suggested the reaction of the enzyme requires, no-electron donor. It is possible that the cyclization catalyzed by the enzyme can be carried out by high-energy bond of pyrophosphate from farnesyl pyrophasphate compound.

In terms of the relationship between enzyme activity and artemisinin content, this study considered that enzyme amorpha-4,11-diene synthase might be the rate limiting step of the pathway and thus might affect the artemisinin content of *A. annua* mutants. The result obtained showed that there were a number of mutants that had a close correlation between the two parameters (Figure 27). However, a few clones showed no such a correlation. The appearance of the correlation between the enzyme activity and the content of artemisinin may be explained by the directed effect of the gamma ray irradiation at a gene which encodes to the amorpha-4,11-diene synthase enzyme and thus leading to increase of the enzyme activity. In contrast, the non-correlated clones of the plant may be the results of either the effect on other enzymes in the pathway or negative effect on the amorpha-4,11-diene synthase to decease the enzyme function. As described previously, the effect of the gamma ray irradiation could affect living cells at the chromosome and gene levels to alter functions that may increase, decease or to talk loose their functions (Alberts *et al.,* 2002). If the irradiation lead to a balance of cellular function, the clones will survive and may generate alternative functions. It if leads to an unbalance of cellular functions, cell death will occur. In this study, some clones of the *A. annua* plant showed different morphology and some clones died after sequence of subculturing. As reported previously, (Cheewasakulyong 2001) some clones of *A. annua* treated with gamma irradiation appeared to have dark-green leaves with thickness and fully expand than those from controls.

For the significance of amorpha-4,11-diene synthase, there have been many reports confirming that it is the enzyme catalyzing the a first step in artemisinin biosynthesis. Bouwmeester *et al.,* (1999) proposed for the first time the presence of amorpha-4,11-diene in pentane extraction from *A. annua* as well as the assay system for amorpha-4,11-diene synthase. In addition, a cDNA encoding the enzyme has been

reported (Mercke *et al.,* 2000; Chang *et al.,* 2000; Wallaart *et al.,* 2001). The transformation of amorpha-4,11-diene synthase into tobacco (*Nicotiana tabacum* L.) has resulted in the expression of an active enzyme and the accumulation of amorpha-4,11-diene in range 0.2 to 1.7 $ng.g^{-1}$ fresh weight. However, there has been a report that give emphasis on the enzyme farnesyl pyrophosphate synthase (FPP) to be important for artemisinin synthesis. Chen *et al.,* (1999) have transferred a cDNA encoding farnesyl pyrophosphate synthase and placed under a CaMV 35S promoter into *A. annua* hairy roots. Analysis of artemisinin has shown that artemisinin content increase about 3-4 times from the control hairy root.

Biosynthetically, both farnesyl pyrophosphate synthase and amorpha-4,11-diene synthase are important enzyme in the biosynthesis of artemisinin. Farnesyl pyrophosphate is a central intermediate in many secondary metabolic pathways for example sesquiterpene including artemisinin and diterpene biosynthesis. In this study, however, we have shown clearly the enzyme activity of the amorpha-4,11-diene synthase correlated to the artemisinin formation in the *A. annua* plant and have thus confirmed the role of this enzyme in catalyzing the first step of the artemisinin biosynthesis in *A. annua*.

CHAPTER V

CONCLUSIONS

From this research work, the following conclusions can be drown.

1. The NH_3-treated artemisinin product after being exposed with ammonia on a silica gel plate appeared to be 10-azadesoxyartemisinin. Under the conditions of saturated ammonia at 75°C for 2 h, the structure conversion of artemisinin was complete. This chromophore which presented the containg product allows the detection by UV at the wavelength of 320 nm.
2. A new TLC-densitometric analysis for artemisinin determiniation has been developed successfully by using the solvent system of hexane; ethylacetate; acetone, 80:5:5. The plate is exposed with saturated ammonia at 100°C for 2 h for converted of artemisinin to 10-azadesoxyartemisinin and then scanned using the TLC-densitometer under the wavelength of 320 nm.
3. The developed TLC-densitometric method for the artemisinin analysis is simple, effective, accurate and sensitive for both qualitative and quantitative analysis of artemisinin in the plant crude extracts.
4. The crude extracts of the 205 mutant samples of *A. annua* have been determined the artemisinin content. It was found that *A. annua* samples contianed artemisinin in the range 0.02 to 3.46 % of dry crude extract.
5. The enzyme assay of the amorpha-4,11-diene synthase have been detected in the reaction mixture of [1-^{3}H(N)]FPP (100,000 cpm), 5mM Mops buffer, pH 7.0, 10% (v/v) glycerol, 10 mM ascorbic acid, 10 mM $MgCl_2$, 2 mM DTT and 10 mM Na_2MoO_4 at 30 ^{o}C for 30 min. The TLC-radioscan have been developed for detected enzyme activity of amorpha-4,11-diene synthas.
6. The results showed a correlation the enzyme activity of the amorpha-4,11-diene synthase and the artemisinin content of *A. annua* mutants and the antimalarial activity presented the correlated with the artemisinin content in the crude extract excepted the sample number 86, present the high antimalarial activity but contained a low artemisinin content.

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