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## Effect of Terbinafin and DMSO on The Gene Expression Level of Squalene Synthase (Sqs) and Amorpha-4,11-Diene Synthase (Ads) in *Artemisia Annua* L

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### Abstract

Artemisinin, a secondary metabolite from *Artemisia annua* L. is a sesquiterpene lactone that has antimalaria activity but produced at low quantities by the plant. Low levels of artemisinin in the plant is related to the biosynthetic pathways influenced by specific enzymes that play role in the formation of artemisinin. Farnesyl diphosphate (FDP), which is the main precursor of artemisinin, also known as the precursor for the formation of sterols. Compared with the other compounds, sterol biosynthetic pathway is the biggest competitor of the artemisinin production since sterols are needed by plants to regulate membrane fluidity and permeability. This research aimed to study the effect of terbinafin and DMSO as sterol synthesis inhibitors on the regulation of the artemisinin biosynthetic pathway by analyzing the expression level of two genes linked, squalene synthase (SQS) and amorpha-4,11-diene synthase (ADS) using quantitative PCR (qPCR) and the amount of artemisinin is determined using high performance liquid chromatography (HPLC). The results showed that at mRNA levels, terbinafin 30  $\mu$ M had no significant effect on SQS and ADS expression levels, but it increased the amount of artemisinin at 50 hours incubation up to 1.36 times higher than control. DMSO increased the expression level of ADS up to 3-4 times and increased the content of artemisinin up to 2.42 times higher than control at 50 hours incubation time

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**Keywords:** Artemisinin, *Artemisia annua*, Terbinafin, DMSO, Biosynthesis, Gene expression

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### 1. Introduction

Malaria is an infection disease which is still as a big problem in global area. The *Plasmodium sp.*-infect the

human and is easy to enter the blood circulation through its vector *Anopheles sp.* Based on the WHO data, in year 2010, there were over 216 million of malaria cases in the world with 655.000 death. In South East Asia region, there were 28 million cases and around 38.000 death<sup>1</sup>. Attempts to cure the malaria have been performed including using antimalarial quinine and artemisinin derivatives. Some resistance cases have been found when these medicines used. Since year 2000, the WHO has recommended artemisinin-based combination therapies (ACTs) which could reduce the death caused malaria around 5% every year<sup>1</sup>.

Artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L plant. has antimalarial activity. The plant belong to family of Asteraceae is distributed in Asia especially in China, Europe, North America and South America<sup>2</sup>. The plant, known as qinghao or sweet wormwood has been used in traditional Chinese medicines since long time ago as anti fever and malaria. Its active metabolite, artemisinin which is from its original known as *qinghaosu* has a broad schizontocidal and less toxic for the human. However, natural content of artemisinin in *A. annua* is low, about 0,1-1% dry weight produced in dried leaves and accumulated in trichome<sup>3</sup>.

Low content of artemisinin stimulate the researches in all aspects to enhance its production such as through the tissue cultures and biotechnology. Biosynthetic pathway of artemisinin production in plant has been established leading to the investigation of few key enzymes which have important role in the production of artemisinin. The key enzymes are farnesyl diphosphate synthase (*fps*), amorpha-4,11-diene synthase (*ads*), and cytochrome P<sub>450</sub> monooxygenase (*cyp71AV1*). The pathway leading to artemisinin production is actually one of branches for the biosynthesis others terpenoid compounds including sterol via mevalonic acid pathway (MVA) as well non mevalonic acid pathway (MEP). Farnesyl diphosphate (FDP) is a precursor of artemisinin as well as for terpenoid and steroid compound through different pathway. So there is competitive pathway using FDP as precursor between artemisinin, terpenoid and steroid production. The expression level of these enzyme will determine which compound will be dominantly produced<sup>4</sup>.

Stigmasterol is a steroid compound which is produced in plants as an important compound in membrane cell activities and regulation of membrane fluidity and permeability<sup>5</sup>. In biosynthetic pathway, sterol synthesis is a competitor for artemisinin production in using a substrate FDP as precursor. Sterol synthesis is catalyzed by *sqs*, while artemisinin synthesis is catalyzed by *ads* and other enzymes.

The regulation mechanism of sterol biosynthesis has not much known. In previous research, it has been known that the sterol biosynthesis was blocked by an antifungal compound. Terbinafine, an allylamine antifungal group has been investigated in tobacco as an inhibitor of sterol production. It specifically inhibit squalene epoxidase (SE), an enzyme in sterol biosynthesis after squalene is synthesized by *sqs*<sup>6</sup>. In attempt to enhance the production of artemisinin, this research was done using an inhibitor of sterol synthesis which is an antifungal terbinafine and dimethylsulfoxide (DMSO). If the biosynthetic pathway leading to sterol formation could be inhibited, so that FDP as precursor will be only used for artemisinin production.

## 2. Experiments

### 2.1. Material

Trizol (invitrogen), diethylpirocarbonate (DEPC)-treated water, aquadest, SsoFast™ Evagreen® Supermix, DreamTaq™ Green PCR Master Mix from Fermentas, primer of *ads*, primer of *sqs*, primer of actine, Murashige and Skoog (MS) medium, sodium nitrate, myoinositol, vitamin MS 5x, terbinafin, dimethylsulfoxide (DMSO), artemisinin, methanol, acetonitril, phosphate buffer (5 mM, pH 7), aquabidest, *A. annua* tissue culture

### 2.2. Instruments

Autoclaf, PCR Applied Biosystems 2720 thermal cycler, Elektroforesis Mupid®R-Exu, Ultrospec 2000 Pharmacia Biotech, Centrifuge Hettich Mikro 120, low tube strip white Bio-Rad, bunsen, laminar air flow, sonicator, pH meter, mortar and stamper, filter 0,45µm, HPLC

### 2.3. Preparation of *Artemisia annua* cultures

Two weeks old *A. annua* cultures were subcultured using liquid MS medium in erlenmeyer and incubated 1 week. After 1 week, cultures were fed using elicitor terbinafin which was dissolved in DMSO in concentration 30  $\mu$ M. Other culture were fed only with DMSO. As the control untreated cultures were used. Samples were harvested at 24 and 50 hours

### 2.4. RNA Isolation

Amount of 1 g sampel were put in aluminium foil and freezed in liquid nitrogen. Sampel were grinded then transferred to the tube and added 500  $\mu$ l trizol. To remove hydrofobic compounds from RNA solution, samples were added 300  $\mu$ l chloroform : isoamylalcohol (24:1), vortexed and incubated at room temperature in 3 min. The mixture were centrifugated for 12000 g in 15 min at 4°C. Supernatant were collected and added isopropanol for RNA precipitation then centrifugated again. Supernatant were removed and pellet was washed with 200  $\mu$ l ethanol 70%. Pellet were redissolved with 30  $\mu$ l deion-DEPC treated water. DNase was added to RNA for removing DNA contamination. Isolated total RNA was confirmed by agarose 1,5% electroforesis gel and measured quantitatively by UV Spectrofotometer. RNA sample was stored at -80°C.

### 2.5. cDNA Synthesis

cDNA synthesis was done using iScript cDNA synthesis kita (Bio-Rad). Total RNA were added 4  $\mu$ l iScript reaction mix, 1  $\mu$ l iScript reverse transcriptase and nuclease free water up to 20  $\mu$ l. The mixture were resuspended using micropipet for homogenization, then incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min using PCR. cDNA was stored at -20°C.

### 2.6. Analysis of gene expression level of *sqs* and *ads* using qPCR

To measure the level of gene expression, BioRad iCycler<sup>®</sup> CFX 96<sup>™</sup> Thermal Cycler with iQ<sup>™</sup>5 Real Time PCR Detection System were used. SsoFast<sup>™</sup> Evagreen<sup>®</sup> Supermix were used as reagent. Primer were designed based on the previous research<sup>7</sup> with sequences as in Tabel 1. All reagent were mixed in low tube strip white consists evagreen 10  $\mu$ l, forward primer 0,5  $\mu$ l, reverse primer 0,5  $\mu$ l, cDNA template 5  $\mu$ l, and nuclease-free water 4  $\mu$ l with total volume 20  $\mu$ l. Quantification was measured relatively using actin gene as housekeeping control geen. All experiment were done triplo following with negative control. Amplification condition of realtime PCR for ads were at 95°C in 30 s for denaturation, 60°C in 5 s for annealing, and 65-95°C for melting curve. The same condition was performed for *sqs* with annealingtemperature at 55°C in 5 s

Table 1. *Primer Sequences of Actin, ADS, dan SQS*

Primer	Forward	Reverse
Actin	5'- CCAGGCTGTTTCAGTCTCTGTAT-3'	5'-CGCTCGGTAAGGATCTTCATCA-3'
Ads	5'- GGGAGATCAGTTTCTCATCTATGAA-3'	5'-CTTTTAGTAGTTGCCGCACTTCT-3'
Sqs	5'- GACCAGTTCACCATGTTTCTACT-3'	5'-GCTTTGACAACCCTATTCCAACAAG-3'

Quantification results were analyze using relative quantification methods developed by Livak *et al*<sup>8</sup>. To calculate the relatvie change of gene expression compare to the control used the formula (fig 1). The calculation result with Ct (Threshold Cycle) value was analyzed using  $2^{-\Delta\Delta Ct}$  method.

$$\Delta\Delta Ct = (Ct_{GT} - Ct_{GA})_{uji} - (Ct_{GT} - Ct_{GA})_{kontrol}$$

$$\text{Rasio Ekspresi} = 2^{-\Delta\Delta Ct}$$

Figure 1 Formula  $\Delta\Delta Ct$  in relative quantification qPCR. Ct is threshold cycle,  $\Delta\Delta Ct$  is Ct value which is normalized with actin, GT is target gene, GA is actine<sup>8</sup>.

### 2.7. Sample Extraction

Treated and control cultures samples were dried at 45°C in 24 hours then grinded. Hundreds mg powdered samples were extracted with 1 ml methanol, sonicated during 30 min, centrifugated for 5 min with 4000 rpm. Supernatant was collected and transferred into new tube. Extraction was performed three time, and all extract were combined and evaporated

### 2.8. Derivatization and Analysis of Artemisinin Content

Crude extract were dissolved in 1,5 ml methanol. From 1,5 ml of extract, 100  $\mu$ l was derivatized using 400  $\mu$ l NaOH 0,05 N and incubated at 50°C in 30 min then added CH<sub>3</sub>COOH 0,2 N, incubated again in ice for 10 min finally added methanol until 1 ml. Derivatized solution were filtered using membran filter with size 0,45  $\mu$ m. Artemisinin content was measured using standdard addition. Prepared samples were injected to HPLC with coloum Hewlett-packard Hwallet RP-18 (100 mm x 4,6 ; particle size 5  $\mu$ m), developing solution were phosphat buffer (5mM, pH 7) : methanol : acetonitril (60:30:10), with gradient elution, flow 0,6 ml/min at 30°C. Detector was DAD (Diode Array Detector) with wavelength 260 nm

## 3. Results and Discussion

Gel electroforesis of total RNA in fig 2 showed two dominant bands which is RNA ribosomal (rRNA) 28S and 18S. Absorban ratio in wavelength 260.280 of RNA was 1.5-1.7 which indicated that RNA is not really pure. The ration for the pure RNA is between 1,7-2,2<sup>9</sup>. Therefore the purification of RNA using DNase for removing DNA contamination was succesfully increase the absorban ratio. cDNA was reverse transcribed using this RNA as a template, confirmed with PCR using DreamTaq Green PCR *Master Mix* and primer of actin at 95°C for 3 min of denaturation, 95°C for 30 s, 56°C for 30 s, 72°C for 30 s amplification, and final amplification during 7 min.

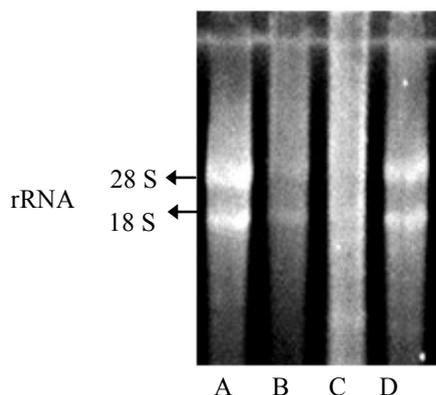


Figure 2. Electroferogram of total RNA, A and B isolated from sample treated with terbinafin 24 hours, C and D isolated from sample treated with terbinafin 24 hours

Inhibition of sterol biosynthesis with terbinafine which is an competitive inhibitor of squalene synthase. In sterol

biosynthetic pathway, squalene synthase together with (2,3)-oxydosqualene epoxydase catalyze the cyclization of squalene into squalene epoxide then following other steps to become sterol<sup>6, 10</sup>. To check the effect of terbinafine and DMSO as a solvent on inhibition of sterol synthesis as well as the production of artemisinin, the level of expression of sqs and ads were measured using relative quantification methods using actin as standard gene

Sqs is an enzyme which catalyze the condensation of two FDP molecule to form squalene, as a main precursor for sterol synthesis<sup>11</sup>. Sqs is important in the branch of isoprenoid pathway due to its role to control carbon flux in regulation of sterol synthesis toward other terpenoid compounds. Therefore inhibition of sterol biosynthetic pathway cause a shift of carbon flux into the formation of terpenoid non-sterol compound including artemisinin<sup>4</sup>. Statistical analysis for quantification of expression level of sqs after treatment with terbinafine 30  $\mu$ M during 24 and 50 hours was not significantly different to untreated control (Fig. 3). DMSO was not also significantly different to control. It showed that terbinafine and DMSO did not significantly influence the the expression sqs in mRNA level. This is same as result reported by Wentzinger<sup>6</sup>, where the treatment of terbinafin in tobacco enhanced the squalene production but did not influence expression level of sqs. This indicated that there was no negative regulation effect in gene transcription in squalene enhancement<sup>6</sup>. Regulation mechanism of sqs toward inhibitor antifungal was not much known, so the further analysis of squalene and stigmasterol production and expression level of squalene epoxydase is needed to know their relation in sterol biosynthesis.

In artemisinin biosynthetic pathway, ads is a key enzyme which catalyze the cyclization of FDP to form amorpho-4,11-diene, a precursor for alcohol artemisinate, dihydroartemisinate acid and aldehyde, furthermore into artemisinin. Statistical analysis of gene expression of ads showed that terbinafine was not significantly different to control, while showed the enhanceent of relative ads expression level 3.87 and 4.04 fold in incubation time 24 and 50 hours respectively (Fig. 3). The enhancement of ads expression level by DMSO was assumed by its effect on the enhancement of mRNA transcription level<sup>12</sup>. At low concentration, DMSO (<10%), could change RNA polymerase structure so enhance its activity in initiatin of transcription process. Low effect of terbinafine on ads expression level suggest that there was no combination effect between terbinafine and DMSO. The enhancement of ads expression level was shown only by DMSO treatment, but not with terbinafine dissolved in DMSO.

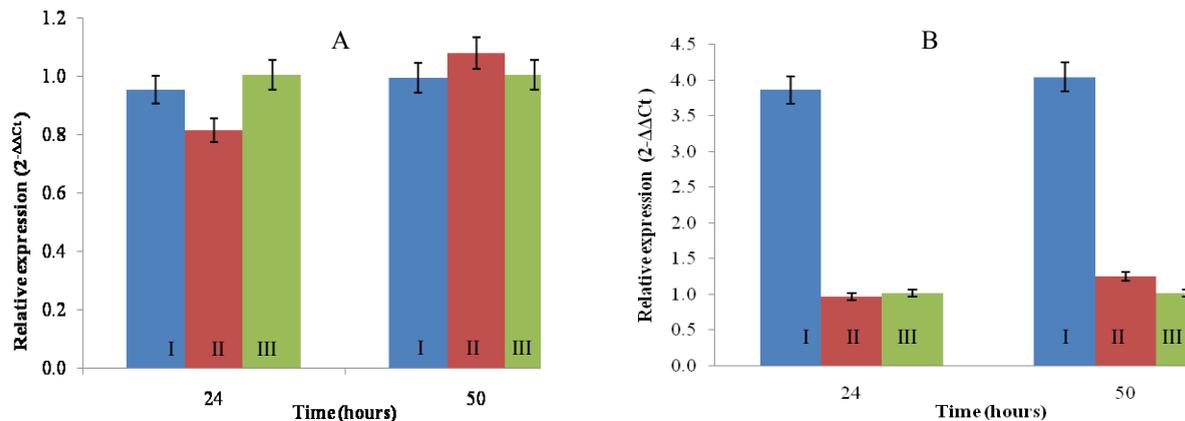


Figure 3. Relative expression level of A) squalene synthase (sqs), and B) amorpho 1,2-diene synthase (ads), I (treated with DMSO), II (treated with terbinafine), III (control)

Determination of artemisinin content has been performed by HPLC to see the relation between the effect of terbinafine on RNA level and artemisinin production. Before injected to the HPLC coloum, artemisinin was modified into Q260 to give a cromophor in artemisinin structure to be able monitored by UV detector<sup>12</sup>. This modification was done by adding NaOH 0,05 M to hydrolize artemisinin into Q292 compound and CH<sub>3</sub>COOH 0,2 N to change Q292 into Q260 compounds which is a stable compound and could be deteted in UV wavelength 260 nm (Fig. 4).

Artemisinin production could be enhanced by adding 30  $\mu\text{M}$  terbinafine with incubation time 50 hours on the *A. annua* cultures (Fig. 5) about 1.36 folds compared to the control cultures. Highest artemisinin content was found in the treatment with DMSO, incubation time 50 hours about 2.42 folds compared to the control cultures. This showed that DMSO has influence on the enhancement of artemisinin production higher than DMSO. This agree with the previous results on the hairy root cultuers of *A. annua* elicited on terbinafine which gave the lower enhancement of artemisinin production than DMSO<sup>12</sup>. DMSO was reported to have a role in enhancement of artemisinin content through stimulation of reactive oxygen compound and serving oxygen for photooxydative non-enzymatic reaction which is a final step on artemisinin synthesis<sup>13</sup>. DMSO has been also reported to enhance secondary metabolite from other plants cultures. In *Catharantus roseus*, DMSO enhanced the alkaloid production through improving cell permeability<sup>14</sup>, in *Phoenix dactylifera* L., DMSO enhanced the production of phenolic an peroxyde compound<sup>15</sup>, while in *Grindelia pulchella* DMSO together with  $\text{CuSO}_4$  enhanced the production of grindelic acid<sup>16</sup>

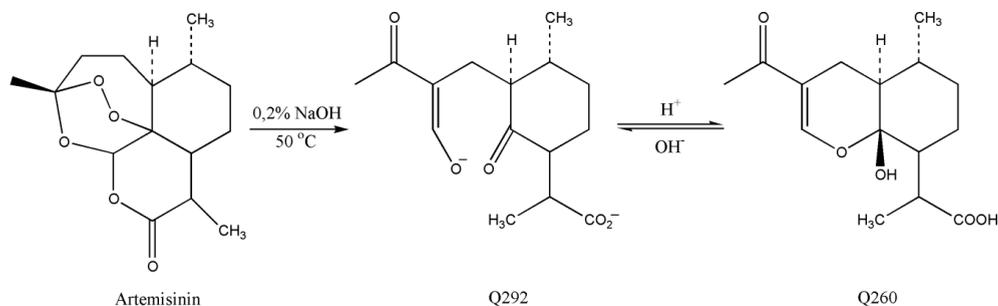


Figure 4. Modification of artemisinin into Q260 compound

Artemisinin production could be enhanced by adding 30  $\mu\text{M}$  terbinafine with incubation time.

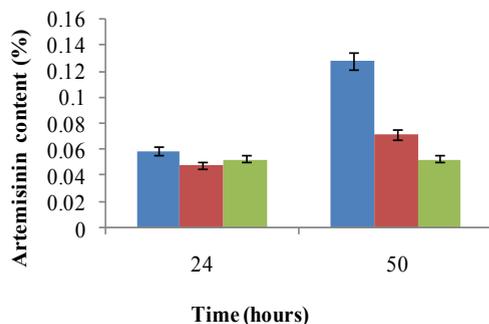


Figure 5. Artemisinin content of *A. annua* I (treated with DMSO), II (treated with terbinafine), III (control)

The enhancement of artemisinin production by terbinafine 30  $\mu\text{M}$  was not really high, but the effect of enhancement showed that the terbinafine could influence on the biosynthetic pathway of artemisinin. Caretto<sup>17</sup> reported another antifungal miconazol which is an inhibitor of sterol synthesis could enhance the production of artemisinin. Although the effect of terbinafine and miconazol is on different target, but both compounds have a role in inhibition of sterol synthesis, so like miconazol, terbinafine in higher concentration and longer incubation time probably will enhance artemisinin production in higher level

#### 4. Conclusion

At mRNA levels, terbinafin 30  $\mu$ M had no significant effect on SQS and ADS expression levels, but it increased the amount of artemisinin at 50 hours incubation up to 1.36 times higher than control. DMSO increased level expression of ADS up to 3-4 times and increased the content of artemisinin up to 2.42 times higher than control at 50 hours incubation time

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