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## CORRELATION OF THE EXPRESSION LEVEL OF AMORPHA DIENE SYNTHASE GENE (ADS) WITH THE ARTEMISININ LEVEL IN *Artemisia annua* L. PLANT

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Artemisinin is an important therapeutic drug that, along with its derivatives, is a proven treatment for a number of diseases besides malaria (Dhingra *et al*., 2000), other parasites like schistosomiasis (Utzinger *et al*., 2001; Borrmann *et al*., 2001), and more recently cancer and hepatitis B (Romero *et al*., 2005). Artemisinin has also been shown to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon, and small-cell lung carcinomas (Efferth *et al*., 2001; Singh and Lai, 2001). Furthermore, artemisinin may be especially effective in treating drug resistant cancers (Sadava *et al*., 2002; Efferth *et al*., 2002). However, the drug is in short supply as its complex structure still requires extraction from plants. Although other researchers are working on a synthetic trioxolane (Vennerstrom *et al*., 2004) and bacterial produced artemisinin precursors (Martin *et al*., 2003) that may replace artemisinin as an inexpensive therapeutic. *A. annua* plants are still the only current source of the drug.

In spite of the therapeutic importance of artemisinin, its biosynthetic pathway post-FDP production is not yet completely elucidated. It is clear that the first dedicated step in the biosynthesis of artemisinin is the cyclization of farnesyl diphosphate (FDP) by amorphadiene synthase (ADS) to amorpha-4,11-diene (Fig. 1; Bouwmeester *et al*., 1999). Several authors have demonstrated that artemisinic acid and/or dihydroartemisinic acid are further intermediates in the formation of artemisinin (Fig. 1; Wallaart *et al*., 1999a; Abdin *et al*., 2003). Little is known about the enzymes involved in the conversion of amorpha-4,11-diene to dihydroartemisinc acid, and until recently (Bertea *et al*., 2005), none of the putative intermediate products had been identified. Modification of the amorpha-4,11-diene carbon skeleton to produce artemisinic acid was thought to involve cytochrome P450 enzyme leading to the production of artemisinic alcohol which could then be oxidized twice by either cytochrome P450 enzymes or dehydrogenases to yield artemisinic acid (Fig. 1; Bouwmeester *et al*., 1999). The C11– C13 double bond of artemisinic acid is thought to be reduced to yield dihydroartemisinic acid, which was shown by others to be converted non-enzymatically to artemisinin (Wallaart *et al*., 1999a,b; Abdin *et al*., 2003).

The first committed step of artemisinin biosynthesis is the cyclization of farnesyl diphosphate (FDP) by a sesquiterpene synthase (cyclase) to produce the characteristic 15 carbon ring system, Amorpha-4,11-diene. Although the complete biosynthetic pathway for artemisinin has not yet been established, artemisinic acid is now considered to be the biogenic precursor of artemisinin (Wallaart *et al*., 1999a,b; Brown *et al*., 2004). Artemisinic acid and amorpha-4,11-diene are structurally closely related, which made the latter the more likely candidate for the cyclization product. Detection and partial purification of amorpha-4,11-diene synthase (ADS) from the plant was first reported by Bouwmeester *et al*. (1999). The low level of the volatile amorpha-4,11-diene in the plant and the high amorphadiene synthase activity were considered to be strong evidence that amorpha-4,11-diene is an intermediate in the biosynthesis of artemisinin.

Several analytical methods to assay artemisinin have been described in the literatures, including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography coupled with mass spectrometry (GC-MS), and immunoassay. In addition, analytical procedures for biosynthetic precursors of artemisinin as well as metabolites in biological fluids have been developed. In this study we used Q260 HPLC method for estimation of artemisinin. Artemisinin has no chromophoric group and only absorbs at the low end of the UV spectrum, below 220 nm. In order to obtain a product with more specific and sensitive spectrometric charcteristics, Zhao and Zeng (1985) converted artemisinin into a product named Q260. First, artemisinin is treated with alkalie and converted into Q292, possessing a UV absorbtion at

292. Upon acidification, Q292 yields Q260, which has a strong UV absorption at 260 nm and is more stable than Q292. The reaction scheme is shown in Fig. (2). Q260 can be separated by means of HPLC on a reversed phase C18 column, with phosphate buffer-methanol as the mobile phase.

In the whole plant, artemisinin has been reported to accumulate in leaves, small green stems, buds, flowers and seeds (Liersch *et al*., 1986; Ferreira *et al*., 1995) with the highest levels in leaves and flowers. Neither artemisinin nor its precursors, however, were detected in roots (Charles *et al*., 1990). Artemisinin content in full bloom flowers was 4-5 times higher than in leaves (Ferreira *et al*., 1995) and both leaves and flowers of *A. annua* have trichomes. Duke *et al*. (1994) showed that artemisinin is sequestered in the glandular trichomes of *A. annua*, and that glandless types produce no artemisinin (Ferreira *et al*., 1995). Some investigators reported that artemisinin content is highest just prior to flowering (Liersch *et al*., 1986; Woerdenbag *et al*., 1994) while others found an artemisinin peak at full flowering stage (Morales *et al*., 1993; Pras *et al*., 1991; Singh *et al*., 1988).

Reports differ regarding the stage at which the highest content of artemisinin occurs during plant development: just before plant flowering (Liersch *et al*., 1986; Woerdenbag *et al*., 1994; Chan *et al*., 1995), or at the point of full flowering (Singh *et al*., 1988; Ferreira *et al*., 1995). Both views suggested a possible linkage between flowering and artemisinin biosynthesis. In this study we correlated the expression level of ADS and the artemisisnin level detected by HPLC produced from different parts of the whole *A. annua* plant.

## MATERIALS AND METHODS

### *Plant materials*

*Artemisia annua* L. plants were grown in an experimental greenhouse in Worcester Polytechnic Institute, Worcester, Massachusetts, USA, using Hg- and Na vapor lamps 16 h/day and at a temperature of 22°C and a relative humidity of 40%.

### *Isolation of DNA*

Genomic DNA was isolated from plant leaves, stems, flowers and roots according to the extraction procedure of Stacey and Isaac (1994). Plasmid DNA samples were prepared by an alkaline lysis method with the Wizard DNA Purification System (Promega) according to the manufacturer's instructions.

### *Oligonucleotide primer design*

A sesquiterpene cyclase (SQC) that was previously identified as key gene from a sesquiterpenoid path in *A. annua* L. plant, is amorpha-4, ll-diene synthase (ADS) catalyzes the first committed step in the biosynthesis of artemisinin (Van Geldre *et al*., 2000). Forward and reverse primers were designed to amplify ADS fragment from the untranslated region

(3'UTR) that shows no homology with the gene. Forward primer of ADS (5' ATG AGG AGT ATG CCC AAA CC 3') and its reverse primer (5' TGC GTC TGA TTT ATT ATT GCC 3') were thus used with annealing temperatures of 55°C calculated according to the equation [2°*(A+T)+4°*(C+G)-2].

***RNA isolation and quantification***

RNA was extracted from different plant parts using phenol-chloroform method. Plastic wares and pipette tips were treated overnight with 0.1% DEPC-treated distilled water and autoclaved. All solutions were prepared in DEPC-treated water and autoclaved thereafter. The leaves, stems, flowers and roots were grinded in liquid nitrogen using a mortar and pestle. Then 100 mg of the fine powder were transferred to a 2-mL microcentrifuge tube. 500 µl of extraction buffer (50 mM Tris-HCl (pH 9), 150 mM NaCl, 1% sarcosyl, 20 mM EDTA, and 5 mM DTT) was added then vortexed vigorously and centrifuged at 21,910 *g* at 4°C for 5 min. The upper aqueous phase was carefully removed and 650 µL of guanidine buffer (8 M guanidine hydrochloride, 20 mM EDTA, 20 mM MES (pH 7), and BME (200 mM final concentration) was added then vortexed and Centrifuged at 21,910 *g* at 4°C for 5 min. Upper aqueous phase was removed and 500 µL of Chloroform was added. The upper aqueous phase was transferred into 2 microcentrifuge tubes (approximately 450 µL in each). To each tube, 45 µl of 3M sodium acetate (pH 5.2) and 900 µL of chilled absolute ethanol were added then incubated at -80°C for 90 min. The pellet was spun at 21,910 *g* at 4°C for 20 min then washed with 70% chilled ethanol (v/v) then Spun at 10,130 *g* at 4°C for 3 min. The supernatant was discarded and the pellet was dried at room temperature. The RNA pellet was dissolved in minimum amount of autoclaved DEPC water and stored at-80°C. The total RNA was quantified by monitoring the absorbance at 260 nm then the A260/A280 ratio was calculated. To check the integrity of the isolated RNA, 3-5 µg were run on a 1.2% denaturing agarose gel containing formaldehyde (Sambrook *et al*., 1989).

***RNA analysis with RT-PCR***

In order to isolate the corresponding cDNA sequences, DNase-treated RNA isolated from different plant organs (leaves, stems flowers and roots) was denatured for 10 min at 70°C and directly chilled on ice then subsequently transcribed to cDNA with AMV reverse transcriptase and oligo dT primer during 60 min at 42°C. The synthesized cDNA could be used immediately for PCR with 20 pmol of both specific primers and 2.5 U *Taq* in 10 mM Tris–HCl (pH 8.3), 50 mM KCl and 200 mM dNTP. The reaction conditions were 94°C during 1 min, 55°C during 1 min and 72°C during 2 min for a total of 40 cycles. The experiment was repeated using an indigenous constitutively expressed control (β actin) to ensure using the same amount of RNA in all samples.

***Analysis of artemisinin by Q260 HPLC method***

Powdered accurately weighed (0.1 g DW of L, F, S and R) plant materials were extracted with 10 ml of toluene in an ultrasonic bath for 30 min. After filtration, 500 µl of the toluene extract was evaporated to dryness and the residue redissolved in 200 µl methanol, then 800 µl of a 0.2% (w/v) sodium hydroxide solution was added. The mixture was vortexed and heated for 30 min in a water bath at 50°C. After cooling, 200 µl methanl and 800 µl 0.05 M acetic acid were added. The hydrolysis product of artemisinin, Q260, was assayed on a reversed phase Chromsep Microspher C18 column, 100 x 3mm i.d., equipped with a guard column (Chrompack, Middelburg, The Netherlands). The mobile phase used was 0.01 M phosphate buffer-methanol 55:45, pH 7, at flow rate of 1 ml/min. The artemisinin derivative was eluted from the column after about 6 min and was detected at 260 nm.

## RESULTS AND DISCUSSION

***RNA analysis and detection of sesquiterpene cyclase transcripts***

The amorpha-4, ll-diene synthase (ADS) catalyzes the first committed step in the biosynthesis of artemisinin (Van Geldre *et al*., 2000). In this study we correlated the expression level of ADS with the artemisinin level in different plant organs of *A. anna* L. plant. Total RNA was isolated from different plant organs (leaves, stems, flowers and roots) and detected on agarose gel as 28S and 18S as shown in Fig. (3). Distinct bands of 28S and 18S rRNA showed that RNA is intact. Reverse Transcription polymerase chain reaction (RT-PCR) was done to detect the expression of ADS gene in different plant organs of *A. annua*. It was detected that ADS was expressed in all plant organs (leaves, stems, flowers and roots) but is highly expressed and more abundant in flowers and the lowest expression level was detected in roots (Fig. 4A). The experiment was repeated using an indigenous constitutively expressed control (β actin) (Fig 4B). All RT-PCR experiments were combined with β actin as indigenous control to ensure using the same quantity of RNA in all samples. These data agreed with that obtained by Ferreira *et al*. (1995) as they showed that artemisinin is mainly present in leaves and flowers of *A. annua* L. plants, with the highest content around flowering time. Reports were different regarding the stage at which the highest content of artemisinin occurs during plant development either just before plant flowering (Liersch *et al*., 1986; Woerdenbag *et al*., 1994; Chan *et al*., 1995), or at the point of full flowering (Singh *et al*., 1988; Ferreira *et al*., 1995). Both views suggested a possible linkage between flowering and artemisinin biosynthesis. Wang *et al*. (2004) investigated whether such a linkage does indeed exist by altering the plant flowering time through transgenics. They transferred the flowering promoter factor (fpf1) from *A. thaliana* (Kania *et al.,* 1997) into *A. annua* via *A. tumefaciens*. The results induced shoots showed

that the fpf1 gene was transcriptionally expressed and the flowering time of the transgenic plants was about 20 d earlier than the non-transformed plants when grown under short-day conditions. No significant differences, however, were detected in artemisinin level between the flowering transgenic plants and the non-flowering non-transgenic plants. This work suggested that flowering does not appear to be a necessary factor for increasing the artemisinin content of *A. annua* plants, and that there may be no direct linkage between flowering and artemisinin biosynthesis. Charles *et al*. (1990) reported that neither artemisinin nor its precursors, however, were detected in roots) that explains why we detected very low expression of ADS in roots of *A. annua* L. plant as shown in Fig. (4A).

***High performance liquid chromatography (HPLC) for artemisinin assay***

Unfortunately, the relatively low yield of artemisinin in *A. annua* and non-availability of an economically viable synthetic protocol are the major obstacles for its commercial production and clinical use. Artemisinin has no chromophoric group and only absorbs at the low end of the UV spectrum, below 220 nm. In order to obtain a product with more specific and sensitive spectrometric charcteristics, Zhao and Zeng (1985) converted artemisinin into a product named Q260 by hydroxylation then acidification. In this study, we used this method to estimate the level of artemisinin in different plant organs as leaves, stems, flowers and roots to correlate the quantity of artemisinin with the expression levels of amorpha diene synthase gene (ADS). Figure (5) showed that the highest artemisinin level was detected in flowers (387.24) followed by leaves (277.82) and barely detected in stems (10.59 µg/g DW) and almost nothing is detected in roots (0.11 µg/g DW). This data agreed with the data shown in Fig. (4A) that the highest expression level of ADS was detected in flowers followed by leaves, stems then roots, therefore, there is a direct relationship between the expression of ADS and the production of artemisinin. Although the ADS mRNA exists in all tissues examined (root, stems, leaves and flowers), it is most likely to be expressed in only leaves and flowers i.e., the enzyme may not be active in all tissues or the amorphadiene produced in roots and stems is used to produce something other than artemisinin.

The low level of the volatile amorpha-4,11-diene in the plant and the high amorphadiene synthase activity were considered to be strong evidence that amorpha-4,11-diene is an intermediate in the biosynthesis of artemisinin. Abdin *et al*. (2003) reported that artemisinin concentration in *A. annua* is low, in the range of 0.01-0.8%; however, concentrations in some strains may be as high as 1.5% (J. Simon, personal communication). Although chemical synthesis is possible, it is complicated and economically not viable due to low yields (Abdin *et al*., 2003). Thus, the plant remains the only commercial source of the drug, and its relatively low yield in *A. annua* is a serious limita-

tion to its commercialization (Abdin *et al*., 2003; Bertea *et al*., 2005). The enhanced production of artemisinin either in tissue cultures or in whole plants of *A. annua* is, thus, highly desirable and should be achievable with a better understanding of the biosynthetic pathway and its regulation by both exogenous and endogenous factors. Furthermore, once the pathway genes and their regulatory controls have been elucidated, metabolic engineering can be employed.

## SUMMARY

Artemisinin, a sesquiterpene lactone, has been isolated in pure form in 1972, and its structure was determined in 1979 from the aerial parts of *Artemisia annua* L. plants. Artemisinin is effective against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. The concentration of aremisinin in *A. annua* is very low and its chemical synthesis is complicated and economically unviable due to the poor yields. However, this plant remains the only commercial source of the drug, but the relatively low yield of artemisinin in seriously limits the commercialization of the drug. Therefore, the enhanced production of artemisinin either in cell/tissue culture or in the whole plant of *A. annua* is highly desirable. It can be achieved by a better understanding of the biochemical pathway leading to the synthesis of artemisinin. Our efforts are focused on the overproduction of this valuable medicine by genetically engineered *A. annua* plants. Amorpha-4, 11-diene synthase (ADS) is a sesquiterpene cyclase gene that catalyzes the first committed step in the biosynthesis of artemisinin (AN). Reverse transcription polymerase chain reaction (RT-PCR) was done on different parts of *A. annua* L. plant to compare the expression level of ADS using oligo dT as an initial primer in the RT-PCR. In this study we found direct correlation between the expression level of ADS and the artemisisnin level detected by HPLC produced from different parts of the whole *A. annua* plant. Additionally we observed that the flowers of *A. annua* plant are the most productive part of artemisinin and it is mainly accumulated in leaves and flowering tops of the plant. However, analysis of RNA from root, stem, leaf, and flower tissue revealed the presence of ADS mRNA in all tissues examined. Thus, the presence of artemisinin in leaf and flower tissues only is not due to the restriction of ADS expression to these tissues. There are a number of possible explanations that could reconcile the seemingly ubiquitous presence of the key terpene cyclase with the highly restricted location of artemisinin in whole plants that are recommended to be investigated in the future.

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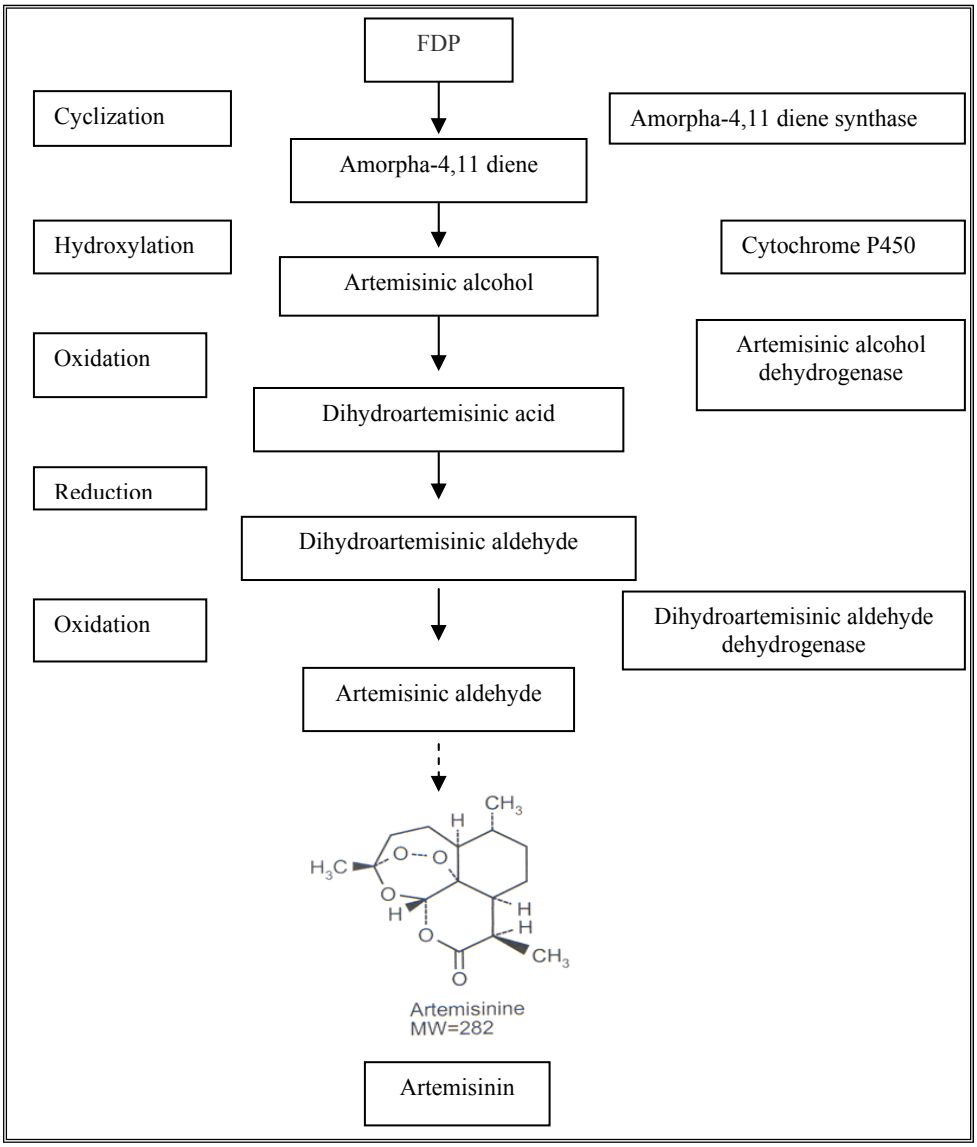


Fig. (1): Proposed biosynthetic pathway for biosynthesis of artemisinin from farnesyl diphosphate. On the left is the reaction action; on the right is the enzyme for each known enzymatic action. Adapted from Bertea *et al.* (2005).

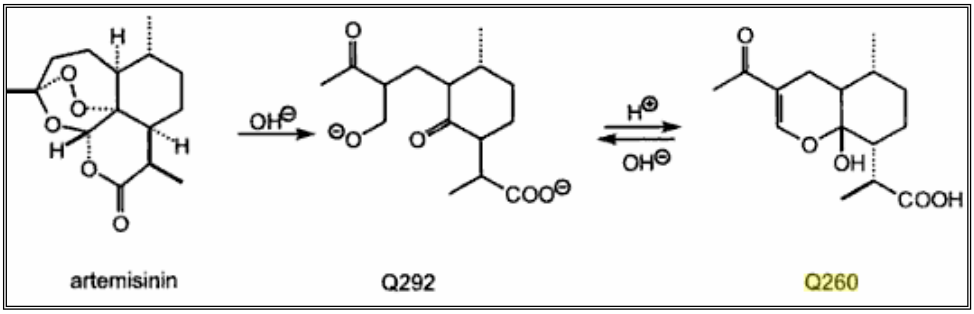


Fig. (2): Derivatization of artemisinin.

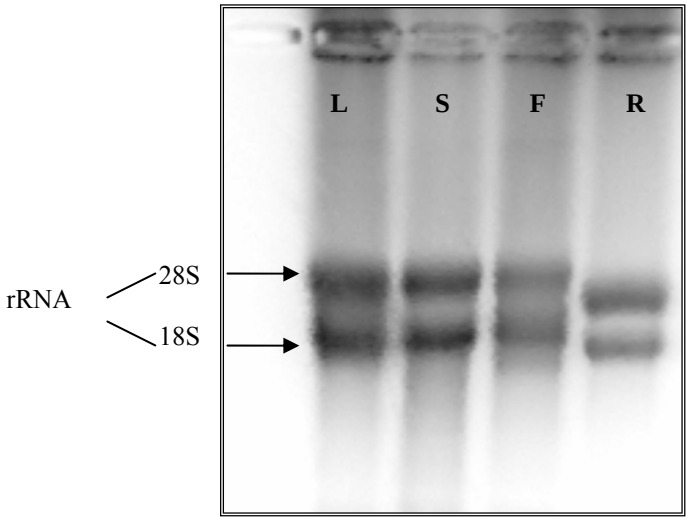


Fig. (3): RNA extracted from leaves (L), stems (S), flowers (F) and roots (R) were run on a 1.2% denaturing agarose gel containing formaldehyde to check the integrity. 28S and 18S rRNA were detected.

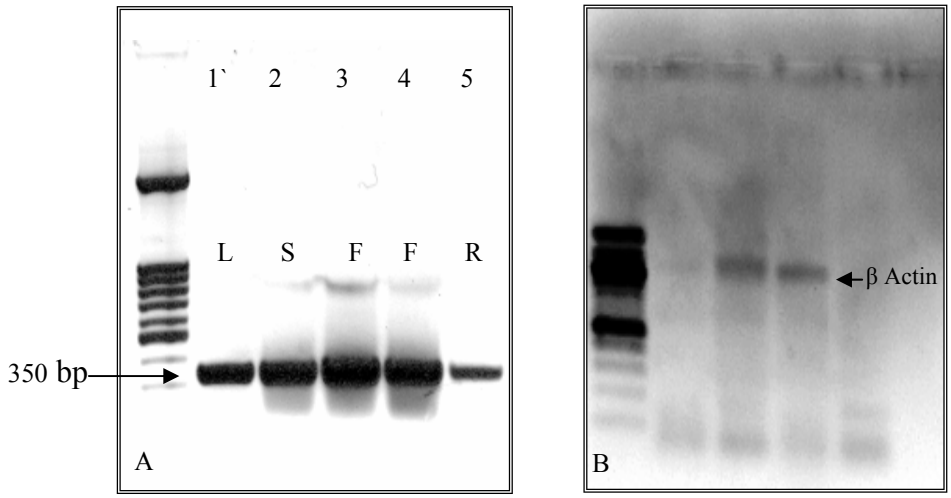


Fig. (4): Reverse transcription ploymerase chain reaction (RT-PCR) was done to test the expression of ADS gene in different plant organs (A) and (B) is an Endogenous control with β actin. Single stranded cDNA was generated from RNA extracted from Leaves (L), stems (S), flowers (F), roots (R) and the corresponding reverse primer and the reverse transcriptase. Double stranded cDNAs were obtained by PCR. The resulting RT-PCR products were analyzed on an agarose electrophoresis gel (1.5%).

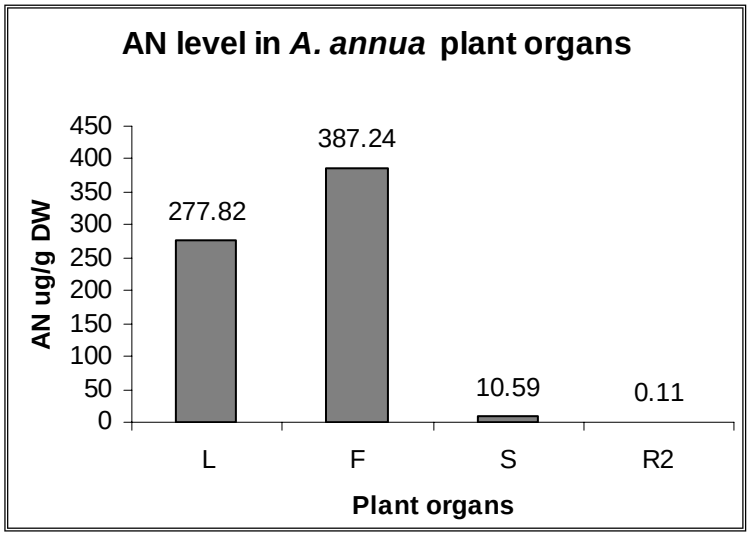


Fig. (5): Estimation of artemisinin level in different plant organs of *A. annua* L. plant using Q260 HPLC method. The level of artemisinin is measured as μg/g dry weight (DW).