Expression of artemisinin biosynthesis and trichome formation genes in five Artemisia species

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Abstract

Artemisinin, a sesquiterpene lactone produced by some Artemisia species, is an efficacious anti-malarial drug, effective against cancer, hepatitis, and schistosomiasis. A. annua is a main source of artemisinin while other Artemisia species produce less artemisinin content. The aim of the current study was to identify the limiting factor of artemisinin biosynthesis in studied Artemisia species, compared to A. annua. The specialized 10-celled biseriate glandular trichomes on the leaves, stems, and inflorescences of some Artemisia species are as a site of artemisinin synthesis. The leaves of five Artemisia species, having different artemisinin contents were assessed in terms of the glandular trichomes density, and area per leaf, and the expression of artemisinin biosynthesis genes and two genes (Aa-TTG1 and Aa-TFAR1) involved in trichome formation. This study identified one novel plant sources of artemisinin (A. deserti, 5.30 mg g(-1) DW) that statistically performed as well as A. annua of Iran (6.27 mg g(-1) DW), but inferior to A. annua cv. Anamed (14.50 mg g(-1) DW) at the flowering stage. A. deserti had the highest trichome area per leaf area accompanied with a high expression of Aa-ADS, Aa-A1DH1, Aa-CYP71AV1, Aa-TTG1, and Aa-TFAR1 genes. A. persica with low artemisinin content had a high density of glandular trichome, high expression of TTGI and TFAR1, but low expression of artemisinin biosynthetic genes. A. khorassanica with no artemisinin content had a very low density of glandular trichome and gene expression. The artemisinin content of A. deserti is significantly as same as A. annua of Iran and inferior to A. annua cv. Anamed despite having the highest glandular trichome area per leaf, and high relative expression of Act-ADS, Aa-ALDH1, Aa-CYP71AV1, Aa-TTG1, and Aa-TFARI. We suggest that it is related to the preferential oxidation of artemisinic aldehyde to artemisinic add than the reduction of the artemisinic aldehyde to dihydroartemisinic aldehyde, due to the very high expression of Aa-ALDHI and Aa-CYP71AV1, and the low expression of Aa-DBR2. It is possible to develop high artemisinin producer plant by overexpression of Aa-DBR2 in A. deserti. It is concluded that there is a relationship between the enhancement of artemisinin content and increased expression of some genes.

Keywords

Author Keywords: Artemisia species; Artemisinin biosynthesis genes; Glandular trichome; Relative gene expression; Scanning electron microscopy; Trichome formation genes

KeyWords Plus: ANNUA L; GLANDULAR TRICHOMES; MOLECULAR-CLONING; TERPENE METABOLISM; TISSUES; ASTERACEAE; REDUCTASE; CYP71AV1; PLANTS; LOCALIZATION

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Research paper

Expression of artemisinin biosynthesis and trichome formation genes in five *Artemisia* species

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ABSTRACT

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1. Introduction

Malaria is a global health problem which is the main reason of disease and death in humans for over a century (Xiao et al., 2016; Muangphrom et al., 2016). Artemisinin, a sesquiterpene lactone, an efficacious anti-malarial drug and effective against a number of cancers and viral diseases (Efferth et al., 2009), is produced by some *Artemisia* species (Duke et al., 1994; Willcox et al., 2004; Arab et al., 2006; Hsu, 2006; Zia et al., 2007; Mannan et al., 2010; Ranjbar et al., 2015). Tu was awarded her Nobel Prize in Physiology or Medicine in 2015 for the discovery of this effective antimalarial compound as a head of a scientific group in 1967–1969. *Artemisia* L. is a genus of small herbs and shrubs, belonging to an important family Asteraceae. It has over 500 species which are mainly found in Asia, Europe, and North America (Bora and Sharma, 2011) and Iran has 35 species of the genus (Abad et al., 2012). *Artemisia* species inhabit in all provinces of Iran, some of those are limited to the special area (Naghavi et al., 2014), and therefore the science of sesquiterpene biosynthesis in *Artemisia* species is substantial for natural products research in the near future. In Flora Iranica, Podlech, (1986) classified *Artemisia* genus into three subgenera,

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Artemisia, *Dracunculus*, and *Seriphidium*. Duke et al. (1994) reported that artemisinin is produced solely in glandular trichomes of *A. annua* L. They evaluated the normal biotype of *A. annua* with both filamentous and glandular trichomes, and a biotype with only filamentous trichomes, observed that just in the presence of glandular trichomes, artemisinin was detected. Then, it was reported that only three *Artemisia* species, including *A. annua*, *A. apiacea*, and *A. lanceolata* produced artemisinin (Willcox et al., 2004). Recent studies have reported that artemisinin is also produced in many other *Artemisia* species, *e.g. A. afftangutica*, *A. absinthium*, *A. bushriences*, *A. cina*, *A. dracunculus*, *A. dubia*, *A. indica*, *A. japonica/em*, *A. moorcroftiana*, *A. parviflora*, *A. roxburghiana*, *A. sieberi*, *A. vulgaris*, *A. campestris*, *A. diffusa*, *A. scoparia*, and *A. sieberi* (Arab et al., 2006; Hsu, 2006; Zia et al., 2007; Mannan et al., 2010; Ranjbar et al., 2015). All of those species produced less artemisinin contents than *A. annua.* Glandular secretory trichomes as a site of artemisinin synthesis are very important. The morphology of its structure can vary highly with tissue and species (Wagner, 1991). Nevertheless, biseriate and capitate glandular trichomes are common in certain genera of Asteraceae (Fahn, 1988), such as *Artemisia* (Kelsey and Shafizadeh, 1980; Slone and Kelsey, 1985; Cappelletti et al., 1986; Ascensão and Pais, 1987). Bi-seriate 10-celled glandular trichomes have been reported on both leaf surfaces of *A. nova* (Kelsey and Shafizadeh, 1980), in floral stalks of *A. tridentata* (Slone and Kelsey, 1985), on both leaf surfaces and on the ovary surfaces of *A. umbeliformis* (Cappelletti et al., 1986), on the adaxial leaf surface of *A. campestris* ssp. maritima (Ascensão and Pais, 1987), in the leaf (Duke and Paul, 1993) and flower (Ferreira and Janick, 1995) of *A. annua*, and in the different parts of *A. nitida* Bertol (Corsi and Nencioni, 1995). However, the exudate accumulation capacity may be related to gland size. Two putative transcription factors including transparent testa glabra1 (*Aa-TTG1*) and enhancer of glabra3 (*Aa-GL3*) (Liu et al., 2009), a cuticular wax biosynthesis gene, trichome-specific fatty acyl-CoA reductase (*Aa-TFAR1*) (Maes et al., 2011), a transcription factor of AP2/ERF superfamily, trichome and artemisinin regulator 1 (*Aa-TAR1*) (Tan et al., 2015), and *AaMYB1* (Matías-Hernández et al., 2017), involved in glandular secretory trichome development and artemisinin biosynthesis were identified. *AaMYB1* plays a role in trichome initiation and trichome branching. It may contribute to improve artemisinin production either by downregulating its competitive pathway, or by upregulating pathway such as the GA metabolism pathway that is indirectly useful (Matías-Hernández et al., 2017). Ranjbar et al. (2015) studied the artemisinin biosynthetic pathway (*Aa-ADS*, *Aa-CYP71AV1*, *Aa-ALDH1*, *Aa-DBR2,* and *Aa-RED1*; Fig. 1) in eight *Artemisia* species, where *A. absinthium* showed a higher expression level of both genes *Aa-ALDH1* and *Aa-CYP71AV1* compared to *A. annua* at all developmental stages. Komori et al. (2013) were unable to detect the expression of *Aa-ADS* in *A. afra* and *A. absinthium*, but they reported that *Aa-CYP71AV1* expressed in both species. Moreover, Muangphrom et al. (2014) detected the expression of *Aa-DBR2* in *A. absinthium*. The enzymes coding by these genes showed similar activities to those coded by *Aa-CYP71AV1* and *Aa-DBR2* in *A. annua* (Komori et al., 2013; Muangphrom et al., 2014). In any *Artemisia* species other than *A. annua*, there are no published studies on the genes involved in trichome formation. It is noteworthy that *A. annua* is still the main source of artemisinin and the productivity of artemisinin in the wide-type of *A. annua* is very low and inadequate to cover the demand of all patients (Xiao et al., 2016). A promising method for overcoming the natural barriers of production is genetic manipulation of an organism (Naghavi et al., 2014). Metabolic engineering is a potent approach to increase the range of bioactive compounds, but none of the metabolic engineering methods of *A. annua* for commercialization of artemisinin has been successful (Tang et al., 2014; Yuan et al., 2015). Lacking genetic evidence of biosynthesis pathway has hampered efforts of metabolic engineering for the high production of artemisinin (Xie et al., 2016). Glandular secretory trichomes of *A. annua* possess all urgent elements, such as pathway enzymes genes and transcription factors and oil environment that are essential in artemisinin biosynthesis (Xiao et al., 2016). The knowledge of factors affecting trichome density and morphology, whole biosynthesis pathway and regulatory mechanisms controlling the start and the flux of the pathway, can be resulted in a successful metabolic engineering. In the current study, we tried to detect the limiting factor of artemisinin biosynthesis in *Artemisia* species other than *A. annua*. It was aimed to determine the artemisinin contents of 17 *Artemisia* species and select species with high, medium, low, and no artemisinin contents and then evaluate them in the view of glandular trichomes, and the expression of artemisinin biosynthesis genes and two genes (*TTG1* and *TFAR1*), involved in trichome formation at the flowering stage.

2. Materials and methods

Seeds of 16 *Artemisia* species were collected from different parts of Iran (Table 1). Plants were propagated and their seeds were collected and cultured in Iranian Biological Resource Center. Furthermore, *A. annua* cv. Anamed as a high artemisinin cultivar, and *A. annua* of Iran were included as controls. For primary screening, the leaves of 16 *Artemisia* species and *A. annua* cv. Anamed and *A. annua* of Iran were sampled at the vegetative stage for determining artemisinin content. Then, *A. annua* cv. Anamed and *A. annua* of Iran as controls and five *Artemisia* species having high, low, and no artemisinin contents were selected and their leaves were assessed in the view of artemisinin content, density, and area of glandular trichomes, using fluorescent microscopy and scanning electron microscopy (SEM), respectively at the flowering stage. Half of each leaf was cut and mixed for RNA extraction and expression analyses, and another half was considered for artemisinin measurement. Thence based on artemisinin content, and the morphology of glandular secretory trichome in the previous step, five *Artemisia* species, including *A. annua* L. of Iran (S1) as a control species, *A. khorassanica* Podlech. (S2), and *A. persica* Boiss. (S3) as endemic species of Iran having no and low artemisinin contents, respectively, *A. deserti* Krasch. (S4) with high artemisinin content, and *A.*

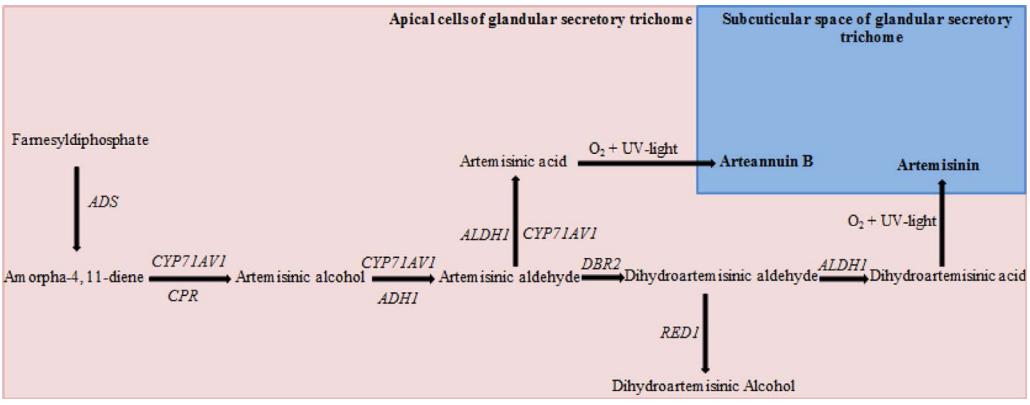

Fig. 1. Summary of artemisinin biosynthesis pathway. Aa-ADS: amorpha-4,11-diene synthase, Aa-CYP71AV1: amorphadiene-12-hydroxylase, Aa-CPR: cytochrome P450 reductase, Aa-ADH1: alcohol dehydrogenase 1, Aa-ALDH1: aldehyde dehydrogenase 1, Aa-DBR2: artemisinic aldehyde Δ11(13) reductase, Aa-RED1: dihydroartemisinic aldehyde reductase.

Table 1
Local information of studied *Artemisia* species.

Species	IBRC No	Local collection sites	Latitude Longitude	Altitude (m)	Collection date	Codes and properties of selected species
Subgenus *Artemisia*						
A. annua L.	IBRC P1003898	Emamzadeh Hashem,Gilan, Iran	37° 02′ N 49° 40′ E	76	December 16, 2010	S1: Control species
A. annua L cv. Anamed	–	Anamed, Germany (www.anamed.net)	–		–	
A. austriaca Jacq.	IBRC P1000187	Pir Ahmad Kandi, West Azerbaijan, Iran	39° 22′ N 44° 06′ E	1793	November 13, 2009	
A. vulgaris L.	IBRC P1000196	Khoy, West Azerbaijan, Iran	38° 36′ N 44° 49′ E	1252	November 13, 2009	
A. absinthium L.	IBRC P1000023	Minudasht, Golestan, Iran	37° 17′ N 55° 18′ E	65	October 25, 2009	
A. persica Boiss.	IBRC P1006530	Deh Bala, Yazd, Iran	31° 37′ N 54° 05′ E	3304	October 28, 2011	S3: Low artemisinin content, and medium density, and low area of glandular trichomes
A. incana (L.) Druce	IBRC P1000195	Khoy, West Azerbaijan, Iran	38° 40′ N 44° 41′ E	1320	November13, 2009	
Subgenus *Dracunculus*						
A. marschalliana Sprengel.	IBRC P1000141	Zaviyeh, East Azerbaijan, Iran	38° 50′ N 47° 00′ E	1806	November 11, 2009	S5: Medium artemisinin content, and high density, and low area of glandular trichomes
A. dracunculus L.	IBRC P1007121	Imported Seed	–	–	–	
Subgenus *Seriphidium*						
A. santolina Schrenk	IBRC P1006588	Kahnok, Sistan and Baluchestan, Iran	28° 43′ N 60° 46′ E	1599	November 1, 2011	
A. ciniformis Krasch. & Popov ex. Poljakov	IBRC P1000633	Baba Aman, North Khorasan, Iran	37° 29′ N 57° 25′ E	1096	January 13, 2010	
A. fragrans Will.	IBRC P1000596	Hemmatabad, Golestan, Iran	37° 54′ N 55° 41′ E	157	January 11, 2010	
A. khorassanica	IBRC P1000307	Kalateh Khij, Semnan, Iran	36° 36′ N 55° 23′ E	1268	November 25, 2009	S2: No artemisinin content, and very low density of glandular trichomes
A. aucheri Boiss.	IBRC P1000557	Shurek Chal, Mazandaran, Iran	35° 56′ N 53° 00′ E	2103	December 27, 2009	
A. deserti Krasch.	IBRC P1000261	Aftar, Semnan, Iran	35° 37′ N 53° 06′ E	1793	November 22, 2009	S4: High artemisinin content and high density, and area of glandular trichomes
A. oliveriana J. Gay ex DC.	IBRC P1000677	Chenaran, Razavi Khorasan, Iran	36° 47′ N 59° 00′ E	1392	January 15, 2010	
A. kopetdaghensis Krash., Popov & Lincz. ex Poljakov	IBRC P1000634	Baba Aman, North Khorasan, Iran	37° 29′ N 57° 25′ E	1096	January 13, 2010	
A. turcomanica Gand.	IBRC P1000636	Baba Aman, North Khorasan, Iran	37° 29′ N 57° 25′ E	1096	January 13, 2010	

Table 2
Primer nucleotide sequences used in qRT-PCR.

Genes	accession number (Gene Bank)	Forward and Reverse Primer Sequences	Fragment size (bp)
Aa-β-Actin	EU531837	F: 5′-CCCCTGCTATGTATGTTGCCA-3′ R: 5′-CGCTCGGTAAGGATCTTCATCA-3′	202
Aa-CPR	EF197890	F: 5′-CGGAACAGCCATCTTATTCTTCG-3′ R: 5′-GTTGCACGTACTCCTTAGTGG-3′	149
Aa-ADS	HQ315833	F: 5′-CCGAGCAAGAAAGAAAACATAG-3′ R: 5′-AACTTCAAGAAACTGGCACA-3′	203
Aa-ALDH1	FJ809784	F: 5′-GATGTGTGTGGCAGGGTCTC-3′ R: 5′-GAGTGGCGAGATCAAAAGGGT-3′	119
Aa-CYP71AV1	DQ453967	F: 5′-CCGAGACTTTAACTGGTGAGAT-3′ R: 5′-CACGAAGCGACTGAAATGAC-3′	147
Aa-DBR2	EU704257	F: 5′-GCGGTGGTTACACTAGAGAACTT-3′ R: 5′-CAAAACTAGAGGAGTGACCC-3′	223
Aa-RED1	GU167953	F: 5′-TGTCAACTGTGTCCATCCAGGT-3′ R: 5′-ACCATCATCGGGCAACAAAGC-3′	118
Aa-TTG1	–	F: 5′-AATCCCATTCGAGCCCACT-3′ R: 5′-GACTTTGCCTGTTGCGGAG-3′	142
Aa-TFAR1	GU733320	F: 5′-AGTCGCTCAATGGAACAAGTGG-3′ R: 5′-CATCTCCCCTAATGCCTTGGTA-3′	197

marschalliana Sprengel. (S5) with medium artemisinin content were finally selected for determining the expression of artemisinin biosynthesis genes and two genes (*TTG1* and *TFAR1*), involved in trichome formation.

2.1. Artemisinin extraction

For comparisons of the artemisinin concentration assessed by HPLC (high-performance liquid chromatography), sampled plants (upper branches) were put in a forced-air oven set at 50 °C for 48 h. Artemisinin was extracted by refluxing 0.5 g of sieved dry leaves with 50 ml of hexane at 75 °C for 1 h (> 95% artemisinin recovery). The hexane extracts were transferred to glass beakers and let to evaporate to dry in a fume hood. Within 24 h, samples were reconstituted in 10 ml of acetonitrile, filtered through pre-wetted 0.2 µm (25 mm) nylon Millex-GN filters (Millipore Corporation, Bedford, MA), connected to disposable 3-ml syringes (Peng et al., 2006). The HPLC analyses were performed on a C18 column (250 mm × 4.6 mm) and detection was conducted at 210 nm wavelength. The acetonitrile: water 65:35% (v/v); was used as a mobile phase with 1 ml/min of the flow rate (Lapkin et al., 2009). The retention times of artemisinin standard and the artemisinin of *Artemisia* species were 8.35 and 8.35–8.45 min, respectively. The calibration curve was constructed by plotting the peak area (y) against concentration (150, 300, 600, 1200, and 2400 ppm) of standard solutions (x). The determination coefficient (R^2) was 0.9975. The contents of artemisinin (mg/g DW) was determined, using calibration curves.

2.2. Fluorescence microscopy

Trichome density was determined for the abaxial leaf epidermis (upper branches). Leaf samples were analyzed under the Olympus IX-71 Inverted Fluorescence Microscope (Olympus, Tokyo, Japan). All tissue images have been taken by the same magnification (4 x objective, 10 x on ocular). Each replication was the average of three samples.

2.3. Scanning electron microscopy (SEM)

Trichome area was determined for the abaxial leaf epidermis. After dehydrating the leaf (upper branches) tissue in ethanol, the tissues were dried in an E3100 critical point drier (Quorum Technologies Ltd., Laughton, East Sussex, UK). Then, the dried specimens were placed on aluminium specimen stubs with silver conductive paint, coated with 100 A° of golden in a SBC-12 sputter coater (KYKY Technology Development Co., Ltd., Beijing, China), and photographed in a KYKY-EM3200 Digital Scanning Electron Microscope (KYKY Technology Development Co., Ltd., Beijing, China) at 26 kV. The major (A) and minor (B) axes of the ellipse were measured. The area of glandular trichome was calculated, according to the formula, area = $AB\pi/4$ (Maes et al., 2011). Each replication was the average of three samples.

2.4. Real-time RT-PCR

Total RNA was extracted, using RiboEx Total RNA reagent (GeneAll Biotechnology Co., Ltd., Songpa-gu, South Korea), according to the manufacturer's instructions. In addition, the remaining genomic DNA was removed, using Qiagen RNase-Free DNase (Qiagen, 79254, Qiagen Inc., Midland, ON, Canada). cDNA was synthesized with 1 µg total RNA, using Thermo Scientific Revert-Aid™ First-Strand cDNA Synthesis Kit (Fermentas, K1622, Thermo Fisher Scientific, Hudson, NH, USA), following the manufacturer's protocol, to finally obtain a 20 µl cDNA solution. The qRT-PCR primers were designed, using Oligo 7 primer analysis software and then check with oligo analyzer tool (eu.idtdna.com/calc/analyzer) and NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomewww.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The OPR3-like gene (12-oxophytodienoate reductase, Genbank accession number EU848577) and BOS (α-bisabolol synthase, Genbank accession number JQ717161) of *A. annua* show a very high similarity to *Aa-DBR2* and *Aa-ADS*, respectively. Designed primers were specific for *Aa-DBR2* and *Aa-ADS*. *Aa-β-Actin* and *Aa-CPR* were used as reference genes (Table 2). The qPCR was performed, using specific primers (Table 2) on a BioRad MiniOpticon real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) with the fluorescent dye SYBR®Green Master Mix 2X (Ampliqon, A323402, Denmark) in accordance with the manufacturer's instructions. One µL of the first strand cDNA was used as a template in 20 µl reactions, including 10 µl SYBR®Green PCR Master Mix and three pmol of each primer. The qRT-PCR was run at 95 °C (15 min), 40 cycles at 95 °C (20 s), 57 °C (30 s), 72 °C (30 s), followed by gradient: 60–95 °C (5 s). The dissociation stage was accomplished to determine the PCR product size and to detect possible primer dimers. Triplets of all samples were run, and the negative control of the Master Mix in addition of primers was performed in all qPCR runs. Relative expression levels were calculated, using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Sehringer et al., 2005).

2.5. Statistical analysis

The experiment was conducted, using a completely randomized design (CRD) with three replications. After initially testing the normal

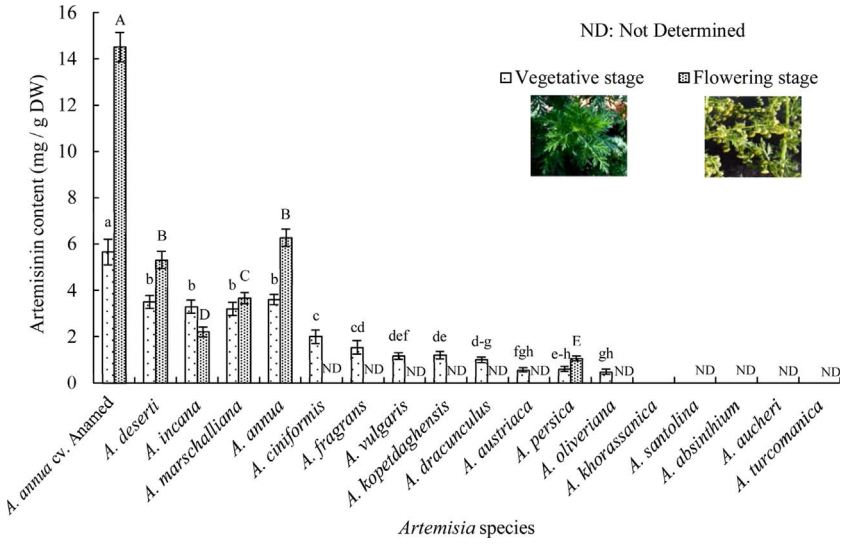

Fig. 2. Artemisinin content of 17 *Artemisia* species in the vegetative stage and six of the same species with high, low and no artemisinin content in the flowering stage.

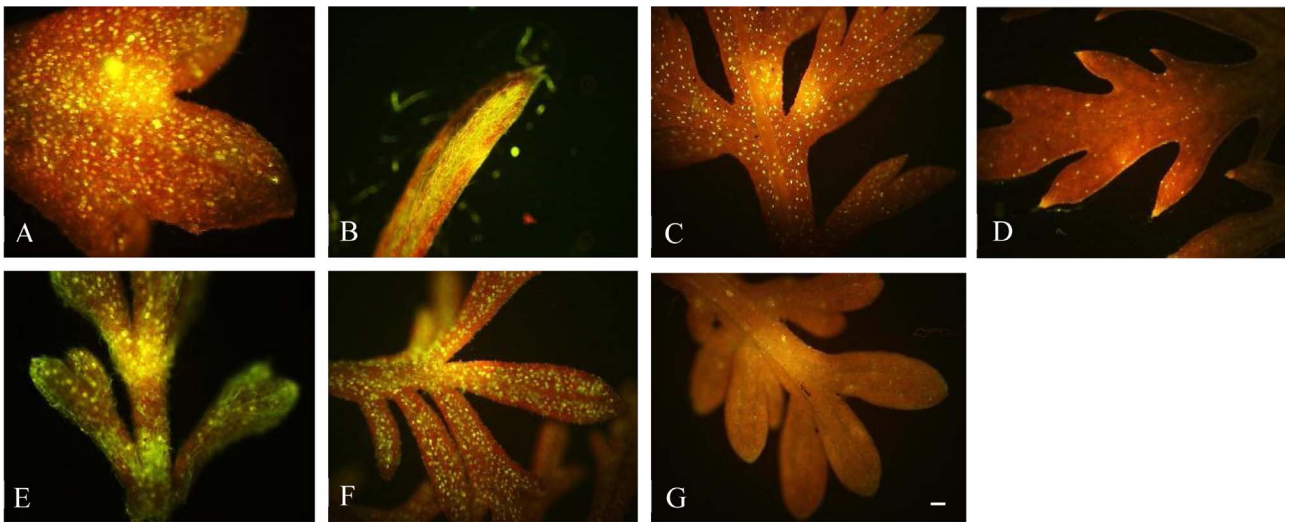

Fig. 3. Glandular trichomes of *A. deserti* (A), *A. marschalliana* (B), *A. annua* cv. Anamed (C), *A. annua* of Iran (D), *A. incana* (E), *A. persica* (F) and *A. khorassanica* (G) showing the content of autofluorescing aromatic oils. Scale bar 200 μm.

distribution of data, analyses of variances were carried out, using PROC GLM of SAS (SAS Institute, 2002). Mean comparisons were done by Fisher's least significant differences (LSDs) at 0.05 or 0.01 probability levels. Also, the standard error (SE) was calculated. Correlation and linear regression analyses were conducted to assess the phenotypic relationship between the traits.

3. Results and discussion

3.1. Artemisinin content

Artemisinin concentration of 16 *Artemisia* species of Iran and *A. annua* L. of Iran, and *A. annua* L. cv. Anamed were analyzed, using HPLC at the vegetative stage (Fig. 2). The highest artemisinin concentration was detected in *A. annua* L. cv. Anamed (5.65 mg g^{-1} DW), followed by *A. annua* L. of Iran (3.6 mg g^{-1} DW), *A. deserti* Krasch. (3.5 mg g^{-1} DW), *A. incana* (L.) Druce (3.3 mg g^{-1} DW), and *A. marschalliana* Sprengel. (3.2 mg g^{-1}DW) (Fig. 2). Three *Artemisia* species as well as *A. annua* L. of Iran as a high artemisinin producer plants, *A. persica* Boiss., and *A. khorassanica* Podlech. (endemic species of Iran) with low (0.61 mg g^{-1} DW), and no artemisinin content, respectively (Fig. 2) were selected for HPLC, fluorescence and SEM microscopic analysis at the flowering stage. In this stage, *A. annua* L. cv. Anamed possessed the highest amount of artemisinin (14.50 mg g^{-1} DW), followed by *A. annua* L. of Iran (6.27 mg g^{-1} DW), *A. deserti* Krasch. (5.30 mg g^{-1} DW), *A. marschalliana* Sprengel. (3.67 mg g^{-1} DW), *A. incana* (L.) Druce (2.2 mg g^{-1} DW), and *A. persica* Boiss. (1.06 mg g^{-1} DW). No artemisinin content was detected in *A. khorassanica* Podlech. (Fig. 2). The higher artemisinin content was obtained at flowering stage in *A. annua* L. cv. Anamed, *A. annua* L. of Iran, *A. deserti* Krasch., *A. marschalliana* Sprengel., and *A. persica* Boiss. while, *A. incana* (L.) Druce. was shown to have a higher artemisinin content at vegetative stage (Fig. 2). Ranjbar et al. (2015), studying on eight *Artemisia* species of Iran reported that *A. scoparia* Waldst. & Kit is the only species showing the highest artemisinin content as well as the highest trichome density prior to the reproductive stage. Generally, all previous studies reported that other *Artemisia* species produced less artemisinin content than *A. annua* L. (Arab et al., 2006; Hsu, 2006; Zia et al., 2007; Mannan et al., 2010; Ranjbar et al., 2015). The current study identified a novel plant source of artemisinin (*A. deserti* Krasch.) that performed as well as *A. annua* L. of Iran. Hence, no significant difference was detectable between the artemisinin content of *A. deserti* Krasch. and *A. annua* L. of Iran. It is noteworthy that *A. annua* L. of Iran is a low artemisinin producer plant (Yang et al., 2015).

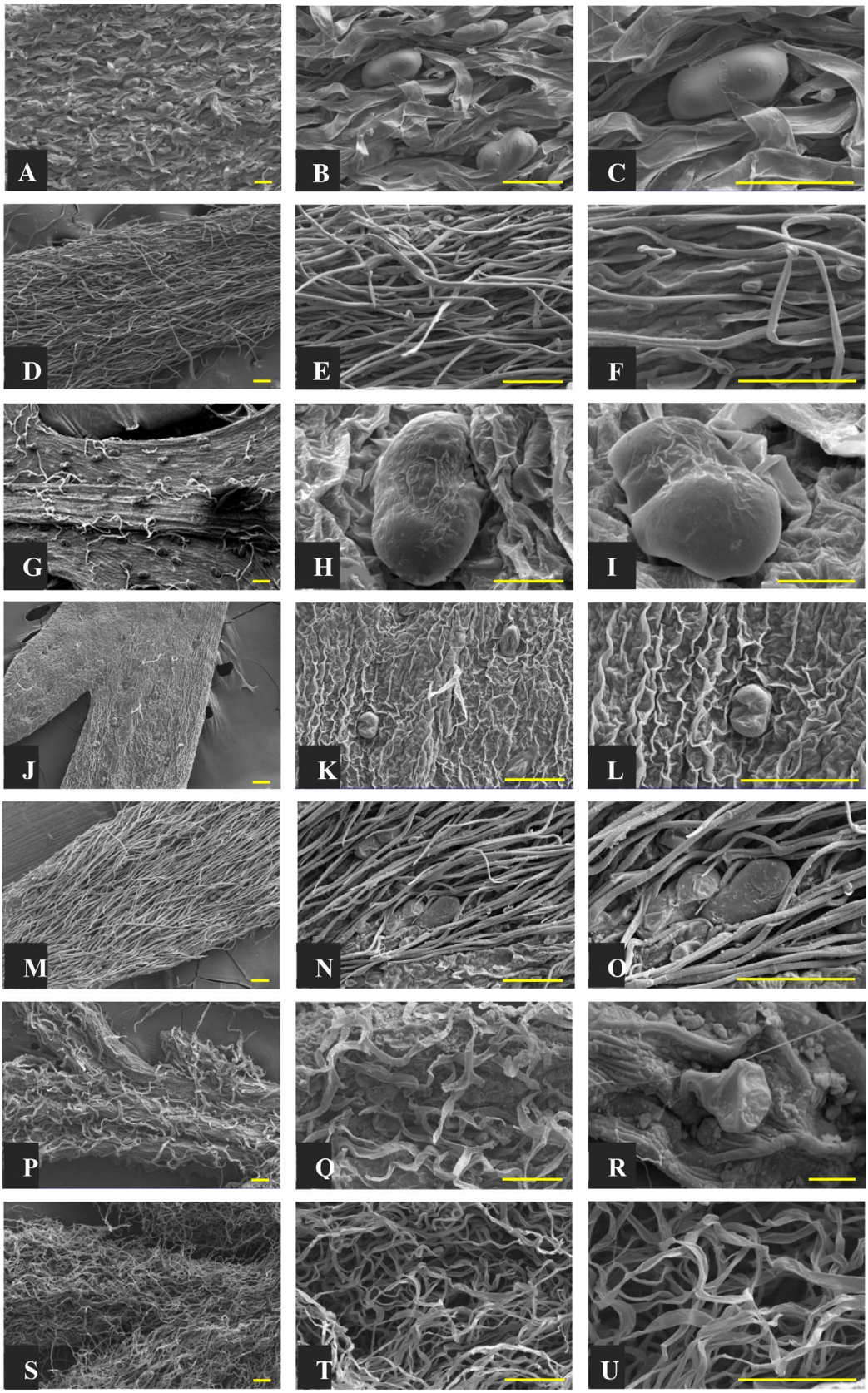

Fig. 4. Scanning electron micrograph of leaves of *A. deserti*. (A, B, C), *A. marschalliana* (D, E, F); *A. annua* cv. Anamed (G, H, I); *A. annua* of Iran (J, K, L); *A. incana*. (M, N, O); *A. persica* (P, Q, R); *A. khorassanica* (S, T, U). A, D, J, M, P, S (×75, Scale bar 100 μm); B, E, K, N, Q, T (×250, Scale bar 100 μm); C, F, L, O, U (×500, Scale bar 100 μm); G (×71, Scale bar 100 μm); H (×1462, Scale bar 20 μm); I (×1685, Scale bar 20 μm); R (×2000, Scale bar 10 μm).

3.2. Density and area of glandular trichomes

Leaves of five *Artemisia* species, *A. annua* L. cv. Anamed and *A. annua* L. of Iran were assessed by fluorescence microscopy and SEM. (Figs. 3 and 4). There was no significant relationship ($R^2 = 0.024^{ns}$) between glandular trichome density and artemisinin content. The highest trichome area per leaf was detected in *A. deserti* Krasch. (284.3 $mm^2\ mm^{-2}$), followed by *A. annua* L. cv. Anamed

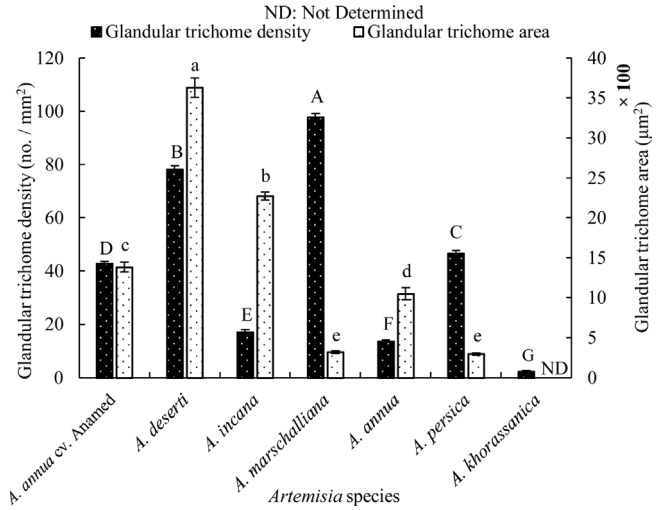

Fig. 5. Density and area of Glandular trichome of some *Artemisia* species in the flowering stage. Error bars are shown as SE (n = 3). Means within each trait followed by the same letter are not significantly different according to LSD at 0.01 probability level.

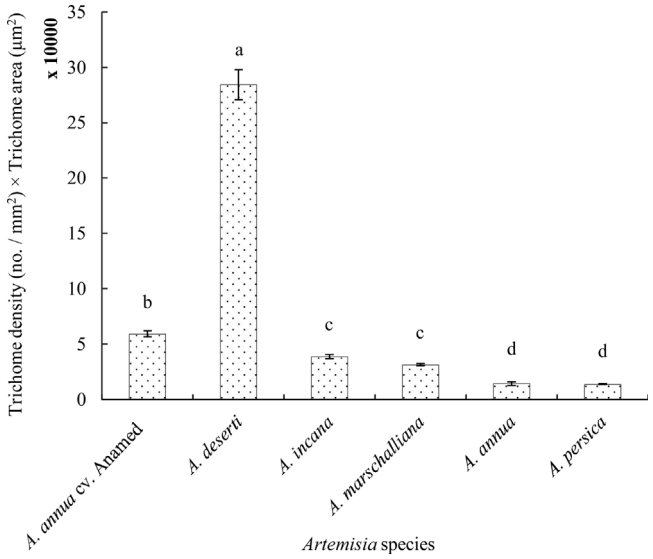

Fig. 6. Glandular trichome area per leaf area of 5 *Artemisia* species in the flowering stage. Error bars are shown as SE (n = 3). Means followed by the same letter are not significantly different according to the LSD at 0.01 probability level.

(59.1 mm^2 mm^{-2}), *A. incana* (L.) Druce. (38.7 mm^2 mm^{-2}), *A. marschalliana* Sprengel. (31.2 mm^2 mm^{-2}), *A. annua* L. (14.5 mm^2 mm^{-2}), and *A. persica* Boiss. (13.8 mm^2 mm^{-2}) (Fig. 6). In the current study, *A. annua* L. cv. Anamed (14.50 mg g^{-1}DW, and 43 no. mm^{-2}, respectively) had more artemisinin content and density of glandular trichome than *A. annua* L. of Iran (6.27 mg g^{-1} DW, and 14 no. mm^{-2}, respectively). *A. khorassanica* Podlech. had a very low density of glandular trichomes (2 no. mm^{-2}, Fig. 5), accompanied by having no artemisinin content Fig. 2. It should be expected that more trichomes would result in more artemisinin production, because glandular secretory trichomes are the sites of artemisinin synthesis. The SEM micrographs showed that the leaves of *Artemisia* species had not only glandular trichomes, in which artemisinin was produced but also non-glandular (filamentous) trichomes (Fig. 4). The leaves of *A. annua* L. possess high glandular trichomes density and very low density of non-glandular (filamentous) trichome, but other *Artemisia* species possess a high density of such filamentous trichomes (Fig. 4). In *A. nova* Nelson (black sagebrush) (Kelsey and Shafizadeh, 1980) and in *A. umbeliformis* (Cappelletti et al., 1986) were reported that the filamentous trichomes form a thick covering over the leaf surface, obstructing direct observation of the glandular trichomes. The highest area of glandular trichome was identified in *A. deserti* Krasch. (3630 μm^2), followed by *A. incana* (L.) Druce. (2272 μm^2; Fig. 5). In the current study, *A. annua* L. cv. Anamed had higher artemisinin content and larger (24.1%) glandular trichome than *A. annua* L. of Iran. In *A. annua* L., Maes et al. (2011) observed that trichomes of high artemisinin producer plants were 26.5% larger than those of low artemisinin producer plants. Despite the highest density of glandular trichome in *A. marschallina* Sprengel. (98 no. mm^{-2}) and the higher density of glandular trichome in *A. persica* Boiss. (47 no. mm^{-2}) than *A. annua* of Iran (14 no. mm^{-2}), these species had less artemisinin content than *A. annua* L. of Iran. It may be related to the different relative expression of artemisinin biosynthesis genes. The highest glandular trichome area per leaf area was observed in *A. deserti* Krasch. with high artemisinin content (Fig. 6). Based on these results, we selected *A. annua* L. of Iran (S1, subgenus *Artemisia*) as a control species, *A. khorassanica* Podlech. (S2, subgenus *Seriphidium*) with no artemisinin content and very low glandular trichome density, *A. persica* Boiss. (S3, subgenus *Artemisia*) with low artemisinin content and relatively high trichome density, *A. marschalliana* Sprengel. (S5, subgenus *Dracunculus*) with medium artemisinin content and high density of glandular trichome, and *A. deserti* Krasch. (S4, subgenus *Seriphidium*) with high artemisinin content, density, and area of glandular trichome for analyzing relative expression of five artemisinin biosynthesis genes and two genes (*Aa-TTG1*, *Aa-TFAR1*), involved in trichome formation.

3.3. Gene expression

One important regulatory mechanism of secondary metabolite pathways are transcriptional co-regulation of genes involved in such pathways. The qRT-PCR method was used to understand the relationship of artemisinin content with the expression pattern of artemisinin biosynthesis genes and two genes (*Aa-TTG1*, *Aa-TFAR1*), involved in trichome formation. The transcription levels of five artemisinin biosynthesis genes and above-mentioned two genes involved in trichome formation of four species, including *A. deserti* Krasch. (S4) with high artemisinin content, *A. marschalliana* Sprengel. (S5) with medium artemisinin amount, *A. persica* Boiss. (S3) with low artemisinin content, and *A. khorassanica* Podlech. (S2) with no artemisinin content were compared relative to *A. annua* L. of Iran (S1), which was chosen as the reference species.

3.3.1. Relative expression analysis of artemisinin biosynthetic genes

We evaluated the relative gene expression of five enzymes, including Aa-ADS (amorpha-4,11-diene synthase), Aa-CYP71AV1 (amorphadiene-12-hydroxylase), Aa-ALDH1 (aldehyde dehydrogenase 1), and Aa-DBR2 (Artemisinic aldehyde Δ11(13) reductase), involved in the conversion of farnesyldiphosphate (FDP) to dihydroartemisinic acid, which is a late precursor of artemisinin (Brown and Sy, 2004), and Aa-RED1 (dihydroartemisinic aldehyde reductase), the enzyme can potentially convert dihydroartemisinic aldehyde into dihydroartemisinic alcohol (dead end product) and withdraw dihydroartemisinic aldehyde from further conversion to artemisinin (Rydén et al., 2010) (Fig. 1). According to our results, the relative expression of *Aa-ADS*, *Aa-CYP71AV1*, and *Aa-ALDH1* were around 3.33, 150.50, 177.89 fold, respectively higher in S4 than in S1 while the level of *Aa-DBR2* gene expression in S4 (1.12 fold) was nearly equal to S1 (the expression of *Aa-DBR2* was low in all five species, Fig. 7). It is noteworthy that in the study of Liu et al. (2009), *Aa-DBR2* was not found in a subtractive library between blooming flowers and flower buds with different artemisinin contents. Furthermore, the study of the transformation of two *Artemisia* species (*A. annua* and *A. dubia*) with *rol ABC* genes demonstrated that the artemisinin content (~9 and ~25 fold in *A. annua* and *A. dubia*, respectively), and the expression levels of *Aa-ADS* (~11 and ~270 fold in *A. annua* and *A. dubia*, respectively), *Aa-*

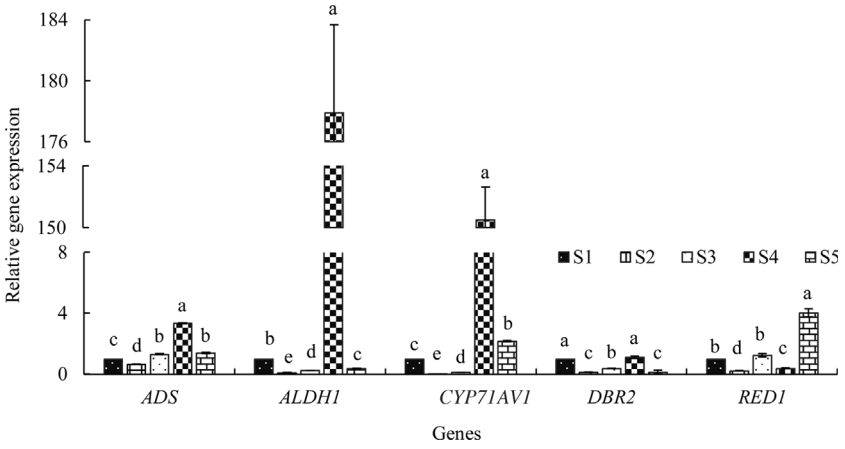

Fig. 7. Relative expression of artemisinin biosynthetic genes in five *Artemisia* species including S1 (*A. annua* of Iran), S2 (*A. khorassanica*), S3 (*A. persica*), S4 (*A. deserti*), and S5 (*A. marschalliana*). Error bars are shown as SE (n = 3). Means within each gene followed by the same letter are not significantly different according to the LSD at 0.01 probability level.

CYP71AV1 (~5 and ~120 fold in *A. annua* and *A. dubia*, respectively), and *Aa-ALDH1* (~7 and ~5 fold in *A. annua* and *A. dubia*, respectively) were significantly increased in transformed plants of both *Artemisia* species compared to untransformed plants (Kiani et al., 2016). *Aa-ALDH1* and *Aa-CYP71AV1* may work together to convert the artemisinic aldehyde into artemisinic acid, which is a late precursor of arteannuin B (Teoh et al., 2006). Yang et al. (2015) reported that *Aa-DBR2* plays an important role in high artemisinin producer chemotype of *A. annua* and low expression of *Aa-DBR2* may limit artemisinin biosynthesis in the low artemisinin producer varieties. By optimizing the production pathway in yeast, the synthesis of 25 g per L artemisinic acid was achieved (Paddon and Keasling, 2014) and for the final step, a novel chemistry process was developed for the conversion of the purified artemisinic acid to artemisinin (Paddon et al., 2013). The results of the current study suggest that S4 may be produced artemisinin acid/arteannuin B more than artemisinin. It was reported that the upregulation of *Aa-ADS* and *Aa-CYP71AV1* expression level under the condition of UV light, heat, and cold shocks resulted in the increase of artemisinin content (Yin et al., 2008). Some studies found that the *Aa-CYP71AV1* did not effect on the artemisinin content, while other studies showed that the Aa CYP71AV1 enzyme had an effect on artemisinin content (Yin et al., 2008; Jing et al., 2009; Pu et al., 2009; Liu et al., 2010; Lei et al., 2011). The higher relative expression of *Aa-ADS* (1.39 fold), *Aa-CYP71AV1* (2.16 fold), and *Aa-RED1* (4.02 fold) were observed in S5 than S1 (Fig. 7). Consequently, Aa-RED1 enzyme appears to influence the yield of the artemisinin precursor dihydroartemisinic acid. The least relative expression of examined artemisinin biosynthesis genes (Fig. 7) with very low density of glandular trichome and no artemisinin content in S2 may underscore glandular trichomes as a site of artemisinin production. Soetaert et al. (2013) detected the transcripts of artemisinin biosynthesis genes in filamentous trichomes. This is in agreement with the report of Wang et al. (2009) in which the expression of *ADS* in filamentous trichomes was identified at very low level, using RT-PCR. While in a study of promoter-GUS fusion with the *Aa-ADS* promoter, no stained filamentous trichomes were observed (Wang et al., 2011). In addition to this, no artemisinin has been detected in the biotype of *A. annua* with only filamentous trichomes (Duke et al., 1994). In the current study, S3 with low artemisinin content had the less relative expression of artemisinin biosynthetic pathway genes than S1 (Fig. 7). The low artemisinin content of *A. persica* Boiss. (S3) in spite of high density of glandular trichome may be related to the low expression of artemisinin biosynthetic genes.

The relative level of transcripts ($2^{-\Delta\Delta CT}$ method, Livak and Schmittgen, 2001) in combination with kinetic data can be used to estimate the relative turnover potential of the different enzymes of the artemisinin biosynthesis (Olofsson et al., 2011). For this estimation, it was assumed that the number of enzyme active sites was proportional to the transcription level and that the enzymes were working at substrate saturation with an optimal NADPH/$NADP^+$ ratio. In this situation, the k_{cat}-value is a good indicator of the conversion of substrate to product. Amongst the five studied *Artemisia* species, the highest relative turnover potential of Aa-ADS, Aa-ALDH1, and Aa-DBR2 was found in S4, followed by S1, which is reflected in the amount of artemisinin (Table 3). Aa-ADS is the rate-limiting enzyme due to the low k_{cat}-value of Aa-ADS, which is a general feature of sesquiterpene synthases (Cane, 1990). In S4, the relative turnover of Aa-DBR2 and Aa-ALDH1 were 2.11 and 236.38, respectively (Table 3). Aa-ALDH1 exhibits a 5-fold higher potential conversion of dihydroartemisinic aldehyde as compared to artemisinic aldehyde due to differences in the k_{cat}-value. Due to the higher potential conversion capacity of Aa-ALDH1 than Aa-DBR2, it may be supposed that as amorpha-4,11-diene is formed by Aa-ADS, it is efficiently converted more the way to artemisinic acid in S4. Yang et al. (2015) reported that artemisinin is a major product in the *A. annua* L. cv. Anamed, whereas in *A. annua* of Iran, the flux is promoted towards arteannuin B by biosynthetic machinery. Thus, we suggest that the main factor caused low artemisinin content of S4 in spite of very high relative expression of *Aa-ALDH1* and *Aa-CYP71AV1* and the higher expression of *Aa-ADS* than S1 is preferential oxidation of the artemisinic aldehyde to artemisinic acid than the reduction of the artemisinic aldehyde to dihydroartemisinic aldehyde due to the high expressions of *Aa-ALDH1* and *Aa-CYP71AV1*, and the low expression of *Aa-DBR2*. Ting et al. (2013) reported that the expression of *Aa-CYP71AV1* in high and low artemisinin producer cultivars of *A. annua* L. were similar, but Aa-CYP71AV1 in high artemisinin producer cultivar showed less enzyme activity and changed the metabolic flux to artemisinin production. In addition, the high turnover potential of Aa-ALDH1 may indicate that this enzyme is involved in the oxidation/reduction of other compounds in S4.

3.3.2. Relative expression analysis of two genes involved in trichome formation

Aa-TTG1 (transparenta testa glabra 1) related to trichome initiation and *Aa-TFAR1* (trichome-specific fatty acyl-CoA reductase 1) involved in cuticular wax formation during glandular trichome development in the leaves and flowers of *A. annua* L. plants were reported by Liu et al. (2009) and Maes et al. (2011), respectively. The level of genes expression of *Aa-TTG1* (6.18 fold) and *Aa-TFAR1* (3.26 fold) were higher in S4 (with a high density of glandular trichome) than S1 (Fig. 8). Moreover, S5 with the highest density of glandular trichome had a high relative expression of *Aa-TTG1* (2.68 fold) and *Aa-TFAR1* (8.82 fold) than S1. S3 with low artemisinin content and relatively high glandular trichome density had higher expression of *Aa-TTG1* (4.78 fold) and *Aa-TFAR1* (5.39 fold) than S1 (Fig. 8). S2 with no artemisinin content and very low density of glandular trichome had a less relative expression of *Aa-TFAR1* (0.38 fold) and higher expression of *Aa-TTG1* (1.76 fold) than S1. It is interesting to note that the leaves of *A. annua* possess high

Table 3
Estimation of relative turnover potential for four enzymes of artemisinin biosynthesis using the $2^{-\Delta\Delta CT}$ method.

Enzyme	Substrate	K_m (μM)	K_{cat} (S^{-1})	ΔΔCT					Normalized transcript amount relative to RED1 $2^{-\Delta\Delta CT}$					Relative turnover					Reference for kinetic constants
				S1	S2	S3	S4	S5	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5	
ADS	FDP	2	0.004	−2.31	−3.88	−2.41	−5.46	−0.78	4.96	14.76	5.300	44.12	1.720	0.02	0.06	0.02	0.18	0.01	Picaud et al. (2005)
DBR2	AA	19	2.600	2.17	2.90	3.94	0.30	7.12	0.22	0.13	0.065	0.81	0.007	0.58	0.35	0.17	2.11	0.02	Zhang et al. (2008)
ALDH1	DHAA	8.8	7.700	1.59	2.89	3.97	−7.30	5.07	0.33	0.13	0.064	157.59	0.030	2.56	1.04	0.49	1213.41	0.23	Teoh et al. (2009)
	AA	2.6	1.500	1.59	2.89	3.97	−7.30	5.07	0.33	0.13	0.064	157.59	0.030	0.50	0.20	0.09	236.38	0.04	
RED1	DHAA	67	0.280	0	0	0	0	0	1	1	1	1	1	0.28	0.28	0.28	0.28	0.28	Rydén et al. (2010)

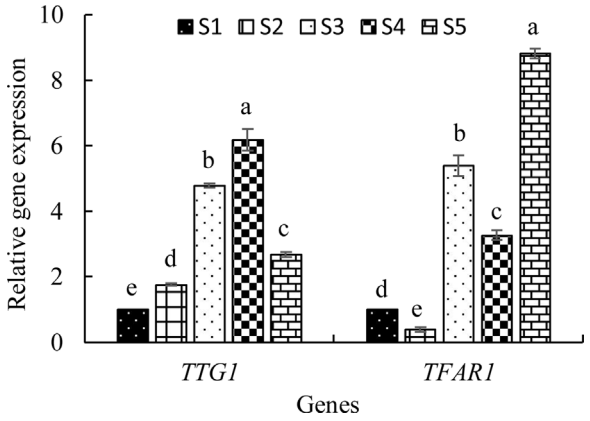

Fig. 8. Relative expression of two genes involved in trichome formation in five *Artemisia* species including S1 (*A. annua* of Iran), S2 (*A. khorassanica*), S3 (*A. persica*), S4 (*A. deserti*), and S5 (*A. marschalliana*). Error bars are shown as SE (n = 3). Means within each gene followed by the same letter are not significantly different according to the LSD at 0.01 probability level.

glandular trichomes density and very low non-glandular (filamentous) trichome density, but other *Artemisia* species possess high filamentous trichomes density. It may be concluded that Aa-TTG1 is a transcription factor promoting the initiation of both filamentous and glandular trichomes. Kiani et al. (2016) detected a significant increase in expression of *Aa-TFAR1* in the transformed plant of *A. annua* (~10) and *A. dubia* (~300) by transformation with *rol ABC* genes. Furthermore, transformed leaves of both *Artemisia* species produced more glandular trichomes (~222 trichomes/5 mm^2 and 173 trichomes/5 mm^2 in transformed *A. annua* and *A. dubia*, respectively) compared to the controls (~120 trichomes/5 mm^2 and 73 trichomes/5 mm^2 in *A. annua* and *A. dubia*, respectively).

4. Conclusion

The aim of our study was to detect the limiting factor of artemisinin biosynthesis in *Artemisia* species other than *A. annua.* It was thought that the morphology (including the cell number, area, and density) of glandular secretory trichomes as a site of artemisinin production is effective in artemisinin production, but the previous studies were shown that the biseriate, capitate glandular trichomes are common in the genus of *Artemisia*. Furthermore, our results showed that the density of glandular trichome ($R^2 = 0.024^{ns}$) and gland size ($R^2 = 0.010^{ns}$) had no significant relationship with artemisinin content. The current study identified a novel plant source of artemisinin (*A. deserti* Krasch.; S4) that statistically performed as well as *A. annua* L. of Iran (S1), but inferior to *A. annua* L. cv. Anamed at flowering stage. The high expression levels of genes involved in artemisinin production (*Aa-ADS*, *Aa-ALDH1,* and *Aa-CYP71AV1*) in S4 indicate a much higher capacity to produce artemisinin precursors which are partly due to the considerably higher density of glandular trichomes in S4 than S1, notifying that S4 had the highest trichome area per leaf area (Fig. 6). However, S5 with the highest density of glandular trichomes had less artemisinin content than S1, because of the expression of *ALDH1* was less and the expression of *RED1* was higher than S1. Consequently, Aa-RED1 enzyme appears to influence the yield of the artemisinin precursor dihydroartemisinic acid. The relative turnover potential of Aa-DBR2 and Aa-ALDH1 were 2.11 fold and 236.38 fold, respectively in S4, but the relative turnover potential of Aa-DBR2 (0.58 fold) and Aa-ALDH1 (0.50 fold) were equal in S1. Since Aa-DBR2 and Aa-ALDH1 are acting on the same pool of intermediates and relative turnover of Aa-ALDH1 is very higher than Aa-DBR2 in S4. Hence, it may be concluded that S4 produces artemisinic acid/arteannuin B more than artemisinin. The flux of intermediate through the two branches of the pathway may be the main factor of low artemisinin content in S4. It is possible to develop high

artemisinin producer plant By the overexpression of *Aa-DBR2* in S4.

Conflict of interest

None.

Author contribution

M. Salehi carried out the experiments and the preparation of manuscript under the joint supervision of Assoc. prof. G. Karimzadeh and Prof. M. R. Naghavi, and the advisory of Assoc. Prof. H. Naghdi Badi, and Assist. Prof. S. Rashidi Monfared. All authors read and approved the final manuscript.

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