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Research article

# Comparison of phytochemical properties and expressional profiling of artemisinin synthesis-related genes in various *Artemisia* species

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ABSTRACT

The *Artemisia* genus belongs to the Asteraceae family and is used in the treatment of many different diseases such as hepatitis and cancer. So far, around 500 species of *Artemisia* have been found in different regions of the world. Artemisinin is one of the medicinal compounds found in Artemisia species. Hence, this medical feature encourages researchers to pay attention to various species of this genus to discover more genetic and phytochemical information. In the present study, five species of Artemisia including *A. fragrans*, *A. annua*, *A. biennis*, *A. scoparia*, and *A. absinthium* were compared to each other in terms of the artemisinin content and other phytochemical components. Moreover, the relative expression profiles of eight genes related to the accumulation and synthesis of artemisinin [including *4FPSF*, *DBR2*, *HMGR1*, *HMGR2*, *WIRKY*, *ADS*, *DXS*, and *SQS*] were determined in investigated species. The result of high-performance liquid chromatography (HPLC) analysis showed that the content of artemisinin in various species was in the order of *A. fragrans* > *A. annua* > *A. biennis* > *A. scoparia* > *A. absinthium*. Based on the gas chromatography–mass spectrometry (GC-MS) analysis, 34, 26, 26, 24, and 20 phytochemical compounds were identified for *A. scoparia*, *A. biennis*, *A. fragrans*, *A. absinthum*, and *A. annua* species, respectively. Moreover, camphor (38.86%), β-thujone (68.42%), spathulenol (48.33%), β-farnesene (48.16%), and camphor (29.04%) were identified as the considerable compounds *A. fragrans*, *A. absinthium*, *A. scoparia*, *A. biennis*, and *A. annua* species, respectively. Considering the relative expression of the targeted genes, A. scoparia revealed higher expression for the *4FPSF* gene. The highest relative expression of the *DBR2*, *WIRKY*, and *SQS* genes was found in *A. absinthium* species. Moreover, *A. annua* showed the highest expression of the *ADS* and *DXS* genes than the other species. In conclusion, our findings revealed that various species of *Artemisia* have interesting breeding potential for further investigation of different aspects such as medicinal properties and molecular studies.

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## 1. Introduction

The *Artemisia* L. genus is one of the important medicinal plants belonging to the Asteraceae family. This genus consists of 200–500 species at the specific or sub-specific level [1], which are mainly distributed in the temperate regions of Australia, North America, Asia, North Africa, and Europe [2]. Among them, Asia has the greatest number of *Artemisia* species [3]. The secondary metabolites extracted from the *Artemisia* species have critical roles in the treatment of several diseases such as cancer, malaria, inflammation, hepatitis, and infections by viruses, fungi, and bacteria [4]. It has been reported that *Artemisia* species have bitter tastes and characteristic strong aromas, which are caused by the presence of terpenes and sesquiterpene lactones [5]. Nevertheless, several phenolic compounds such as phenolic acids, flavonoids, and coumarins have been identified in various species [5]. Moreover, various species of this genus have been served to synthesize essential oil used in food commodities, medicine, and cosmetics, due to their remedial and therapeutic medicinal virtues [6].

Artemisinin is one of the most abundant secondary metabolites in the genus *Artemisia* [7]. This metabolite and its derivatives are all sesquiterpene lactones containing an unusual peroxide bridge. It has been reported that this endoperoxide 1,2,4-trioxane ring is responsible for its antimalarial properties [8]. Moreover, several studies revealed the anticancer effect of artemisinin on lung, leukemia, breast, prostate, and ovarian cancers [9]. Hence, the medicinal properties of *Artemisia* species are attributed to as this secondary metabolite. It has been proved that artemisinin is synthesized through two distinct pathways: the non-mevalonate (MEP) in the plastid and the mevalonate (MVA) in the cytosol [10]. The MVA pathway has a critical role in artemisinin biosynthesis. In this pathway, the first specific precursor of artemisinin is formed via the conversion of farnesyl diphosphate (FDP) into amorpha-4,11-diene by using an amorpha-4, 11-diene synthase (ADS) catalyzer [11]. In this conversion pathway, sterol and sesquiterpene production are regulated, simultaneously. The production of sesquiterpene is dependent on sesquiterpene cyclase (SQC), while in the other pathway (sterol production) squalene synthase (SQS) plays a key role. In other words, by decreasing sterol production artemisinin production will increase, and vice versa. The regulation of *SQS* and *SQC* genes has been demonstrated by using miconazole to limit SQS in *A. annua* [12]. All artemisinin pathways are not fully known, however, two commonly known pathways along with the main involved genes in each of them are shown in Fig. 1.

It is clear that various factors such as plant and cell organs, phytohormones, signaling molecules and pathways, and environmental conditions affect the production of artemisinin [13]. Moreover, up-or down-regulation of different involved genes in artemisinin production pathways also affected artemisinin production. For instance, Olofsson et al. [14] and Wang et al. [15] reported that various sesquiterpene synthases genes may have a negative effect on the amount of produced artemisinin as a result of competition for the substrate FDP. Hence, an investigation of the expression of genes related to artemisinin biosynthesis can complete our knowledge of increasing artemisinin content.

In general, several studies demonstrated that artemisinin biosynthesis is unique to *A. annua* species [12,15,16], whereas recent studies [17–20] have revealed that other *Artemisia* species such as *A. indica*, *A. afftangutica*, *A. bushriences*, *A. absinthium*, *A. parviflora*,

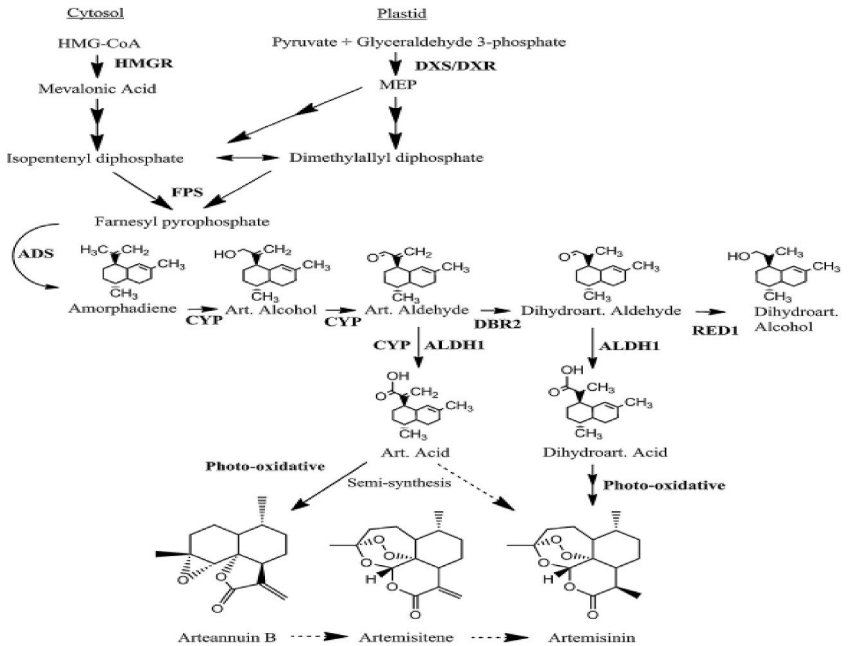


**Fig. 1.** Artemisinin biosynthetic pathway. *Art acid* artemisinic acid, *ADS* amorpha-4, 11-diene synthase, *CYP* cytochrome P 450 CYP71AV1, *DBR2* double bond reductase 2, *Aldh1* aldehyde dehydrogenase 1, *DXR* 1-deoxyxylulouse 5-phosphate reductoisomerase, *RED1* dihydroartemisinic aldehyde reductase 1, *DXS* 1-deoxyxylulose 5- phosphate synthase, *FPS* farnesyl diphosphate synthase, *MEP* nonmevalonate pathway, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, and *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA [13].

*A. vulgaris*, *A. sieberi*, *A. cina*, *A. dracunculus*, *A. dubia*, *A. moorcroftiana*, *A. roxburghiana*, and *A. japonica* can be used to the extraction of this important secondary metabolite. To our knowledge, there is no enough information on the artemisinin biosynthetic pathways in various *Artemisia* species. In the present study, we aimed to compare phytochemical profiles and the expression of eight genes involved in artemisinin biosynthesis among five different *Artemisia* species. We believe that the findings obtained in this study are important for natural products research in the near future.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The seeds of five *Artemisia* species, including *A. fragrans* Will. (Accession No. IBRC P1000099), *A. scoparia* Waldst & Kit (Accession No. IBRC P1006514), *A. biennis* Rchb. (Accession No. IBRC P1006581), *A. absinthium* L. (Accession No. IBRC P1000003), and *A. annua* L. (Accession No. IBRC P1000008), were collected from the Iranian Biological Resource Center (IBRC). All seeds of five species were sterilized with 20% (v/v) sodium hypochlorite for 20 min and then washed three times using tap water as proposed by Liu et al. [21]. Next, sterilized seeds were planted in plastic experimental pots filled with perlite and peat moss. The experimental trays were transferred into a growth chamber with controlled conditions (photoperiod of 16 h and light intensity of 5000 1X LUX). After 45 days, seedlings were transferred into plastic pots filled with soil and sand (1:1 ratio) and until the flowering stage kept under the same controlled conditions with minor modification (photoperiod of 12 h and light intensity of 7000 1X LUX).

### 2.2. High-performance liquid chromatography (HPLC)

To prepare of plant extracts, 2 g of dried leaf samples were powdered and added to 50 mL of 60% acetonitrile, then kept in an ultrasonic water bath for 10 min (Elmasonic E30H, 60Hz, Germany). In the next step, extracts were filtered using Whatman filter paper 0.45 μm. The HPLC analysis was performed using a Knauer HPLC-DAD system (DAD detector, Azura, Germany) with an Eclipse-XBD-C18 column (4.6 mm ID × 250 mm (5 μm 80A, USA) at room temperature. The mobile phase was acetonitrile:water (60–40) at a flow rate of 1 mL $min^{-1}$. The injected volume was 20 μL. The detection wavelength for artemisinin (Sigma-Aldrich, USA) was set at 260 nm [22]. The injection volume of the sample was 25 μL. The artemisinin accumulation was estimated from the standard curve of concentration versus the peak area.

### 2.3. Extract preparation and GC-MS analysis

0.5 g of dried and powdered leaves were extracted in 20 mL of methanol and maintained in an incubator shaker at 25 °C for 2 days. The extract was filtered using Whatman filter papers No. 1 and stored at 4 °C for further use. The phytochemical compounds of *Artemisia* species were identified using GC-MS (TRACE MS., TermoQuest-Finnigan) coupled to a 5973 MSD operated in electron impact mode at 70 eV ion source energy. The gas chromatograph was fitted with a DB-5 GC column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness). The oven temperature was programmed initially at 60 °C and raised to 250 °C at a rate of 5 °C $min^{-1}$. The injector and detector temperatures were set to 250 °C. The total run time of the sample was 40 min. Helium gas was used as the carrier with a flow rate of 1.1 mL $min^{-1}$. The detection of phytochemical compounds in the extract was achieved using the matches percentages and commercial libraries of the National Institute of Standards and Technology (WILEY 9th edition, NIST-08 MS library, Gaithersburg, MD, USA).

**Table 1**
The sequences of the selected genes related to *Artemisinin* accumulation.

| Gene | Forward/Reverse | Sequence | Reference |
|---|---|---|---|
| *ADS* | F | GTCGAATGGGCTGTCTCTGC | [24] |
| | R | CCATCAATAACGGCCTTGGA | |
| *DBR2* | F | CATCAACAAGCAAGCCCATTTC | [24] |
| | R | GCGATAGTCTTCAACCACCTC | |
| *HMGR-1* | F | GGTCAGGATCCGGCCCAAAACATT | [25] |
| | R | CCAGCCAACACCGAACCAGCAACT | |
| *HMGR-2* | F | TGCTGGTTCTCTTGGTGGAT | [22] |
| | R | CTCCAACTGTGCCAACCTCT | |
| *WIRKY* | F | CAAGAACTACCAAGACCGAATCC | [26] |
| | R | GGAGATAACAGGTGGCGAATAGAC | |
| *DXS* | F | ATGGGTTGGCGGGATTCAC | [25] |
| | R | CCGTCAAGATTGGCAGTAGGTAAA | |
| *FPS* | F | GTATGATTGCTGCGAACGATGGA | [25] |
| | R | CGGCGGTGAATAGACAATGAATAC | |
| *SQS* | F | TTTGAAAGCAGTATTGAAACAC | [27] |
| | R | CAGACAGCATCACGAAGC | |
| *Actin* | F | AGTGCTCCTGGTTAGTTGTC | [27] |
| | R | CTTGTTGCCTCGTAATCTTCG | |

### 2.4. RNA extraction, cDNA synthesis and real-time PCR

To investigate eight genes related to artemisinin accumulation (*4FPSF*, *DBR2*, *HMGR1*, *HMGR2*, *WIRKY*, *ADS*, *DXS*, and *SQS*) (Table 1), total RNA was extracted from young leaves of the investigated *Artemisia* species using DENAZIST ASIA kit (Tehran, Iran) according to the manufacturer's instructions. The concentration of the isolated RNA was determined using a Nano-Drop Spectrophotometers device (Thermo Scientific-2000C, USA). Then, cDNAs were synthesized using EasyTM cDNA Synthesis Kit (Parstos, Tehran, Iran) according to the manufacturer's instructions. Afterward, RT-qPCR analysis was carried out in a 12 μL volume containing 3.4 μL of RNAse-free water, 2 μL of cDNA (50 ng $\mu L^{-1}$), 6 μL of 2 × RealQ Plus 2 × Master Mix Green (Ampliqon), and 0.3 μL of (0.3 l M) forward and reverse primers. Amplifications were run in a MiniOpticon™ Real-Time PCR device (Bio-Rad, USA) under the following steps: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 53–60 °C for 20 s, 72 °C for 30 s, and finally the temperature was increased from 65 °C to 95 °C by one degree per second. Normalization of the relative expression of examined genes was done using the *Actin* gene. Based on the CT values for each reaction, the relative expression for each investigated gene was calculated as suggested by Pfaffl [23].

### 2.5. Statistical analysis

Based on the artemisinin content detected by HPLC, the differences among the studied species were investigated. An analysis of variance (ANOVA) was performed for the relative expression data based on a completely randomized design with three replications. Mean values were compared by using Duncan's multiple range test at the significant probability level for each experimental treatment. Data were analyzed by IBM SPSS Statistics software ver. 26 (Armonk, NY, USA). To investigate the association between the relative expression of studied genes and artemisinin content in each *Artemisia* species, Pearson's correlation coefficients were determined using IBM SPSS software ver. 26.

## 3. Results

### 3.1. Artemisinin content

In the present study, artemisinin content was estimated using the HPLC with a specific standard (Fig. 2A). As shown in Fig. 2B, there was significant difference among investigated species in term of artemisinin content ($P \leq 0.01$). The results demonstrated that *A. fragrans* has a significant amount of this compound (0.41 mg/g DW), followed by *A. annua* (0.27 mg/g DW), *A. biennis* (0.20 mg/g DW), *A. scoparia* (0.16 mg/g DW), and *A. absinthium* (0.12 mg/g DW). Indeed, the amount of artemisinin in *A. fragrans* was 1.52, 2.05, 2.56, and 3.42 times higher than in *A. annua*, *A. biennis*, *A. scoparia*, and *A. absinthium*, respectively (Fig. 3).

### 3.2. GC-MS analysis

A GC-MS analysis was performed to identify different phytochemical compounds in five investigated *Artemisia* species. Fig. 4 shows the rendered chromatograms for each species. Moreover, details of identified phytochemical compounds in each species are presented in Table 2. According to results, there was found many different phytochemical compounds in the investigated species. As a result, 34, 26, 26, 24, and 20 phytochemical compounds were identified for the species *A. scoparia*, *A. biennis*, *A. fragrans*, *A. absinthum*, and

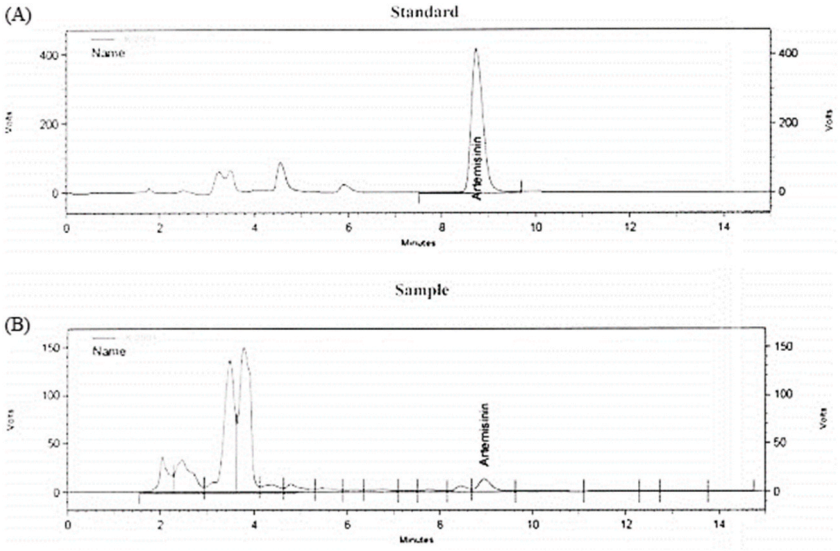


**Fig. 2.** The HPLC chromatogram for the standard (A) and extracted samples for *Artemisia* species (B).

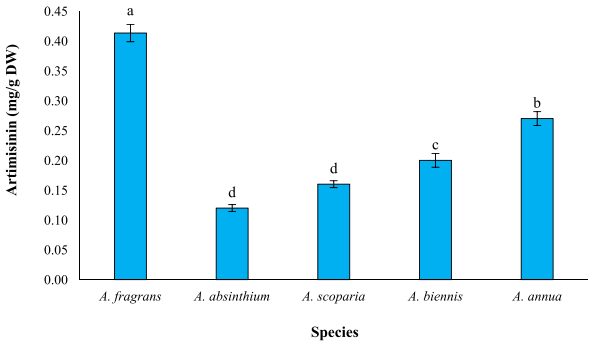


**Fig. 3.** The artemisinin content in different *Artemisia* species. Different letters show significant differences among species at 0.01 the probability level.

*A. annua*, respectively. In *A. fragrans*, the most compounds were related to camphor (38.86%), followed by borneol (14.28%) and β-terpineol (8.17%). The most identified compounds in *A. absinthium* were β-thujone (68.42%), followed by β-pinene (8.61%), and sabinene (3.83%). In *A. scoparia* species, spathulenol (48.33%), palmitic acid (4.47%), and oleic acid (4.31%) were identified as the most phytochemical compounds; whereas, the most compounds in *A. biennis* were β-farnesene (48.16%), spiroether (28.53%), and Octadecenoic acid (3.63%). In *A. annua* species, camphor (29.04%), β-selenin (27.87%), and *trans*-pinocarveol (7.54%) were highlighted as the dominant compounds. Among the identified compounds, p-Cymene and 1, 8-Cineol were common compounds in all investigated species. Species *A. fragrans*, *A. absinthium*, and *A. scoparia* were common in terms of 4-Terpineol and Spathulenol compounds. Moreover, the common compounds in *A. scoparia*, *A. biennis*, and *A. annua* were Hexahydrofarnesyl acetone and Palmitic acid. The Limonene and (E)-β-Farnesense were common compounds in *A. fragrans*, *A. absinthium*, and *A. scoparia* species. Other common compounds in various species are presented in Table 2.

### 3.3. Expression of the genes involved in the metabolite biosynthesis pathway

The relative expression of eight genes including *4FPSF*, *DBR2* (artemisinic aldehyde Delta (11(13)) reductase), *HMGR1* (3-hydroxy-3-methyl-glutaryl-CoA reductase 1), *HMGR2* (3-hydroxy-3-methyl-glutaryl-CoA reductase 2), *WIRKY*, *ADS* (amorpha-4, 11-diene synthase), *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), and *SQS* (squalene synthase) were investigated using qPCR technique. The result of ANOVA showed significant differences among investigated species in terms of the relative expression of *4FPSF*, *DBR2*, *WIRKY*, *ADS*, and *SQS* genes (Table 3). *A. scoparia* had a higher relative expression of *4FPSF* (11.75%), which was 1.89, 1.66, 2.06, and 1.83 times higher than *A. fragrans* (6.20%), *A. absinthium* (7.08%), *A. biennis* (5.71%), and *A. annua* (6.42%), respectively (Fig. 5A). The expression of *DBR2*, *HMGR1*, *HMGR2*, *WIRKY*, and *SQS* in *A. absinthium* was higher than other species. In *A. absinthium*, the expression of *DBR2* (4.56%) was 1.84-, 4.80-, 1.50-, and 2.23-fold higher than *A. fragrans*, (2.50%), *A. scoparia* (0.95%), *A. biennis* (3.04%), and *A. annua* (2.04%), respectively (Fig. 5B). Moreover, the expression of *WIRKY* (6.58%) was 1.48-, 3.41-, 1.30-, and 2.16-fold higher than *A. fragrans*, (4.44%), *A. scoparia* (1.93%), *A. biennis* (5.06%), and *A. annua* (3.05%), respectively (Fig. 5C); and the expression of *SQS* (10.11%) was 1.18-, 1.61-, and 1.06-fold higher than *A. fragrans*, (8.54%), *A. scoparia* (6.26%), and *A. biennis* (9.49%), respectively (Fig. 5F). The maximum number of transcripts for ADS and DXS genes were found in *A. annua*. Accordingly, the expression of *ADS* (10.71%) was 1.66-, 1.09-, 1.81-, and 1.65-fold higher than *A. fragrans*, (2.50%), *A. absinthium* (7.08%), *A. scoparia* (1.93%), and *A. biennis* (5.06%), respectively (Fig. 5D). Moreover, the expression of *DXS* (11.78%) was 1.36-, 1.10-, 3.25-, and 2.96-fold higher than *A. fragrans*, (8.63%), *A. absinthium* (10.74%), *A. scoparia* (3.62%), and *A. biennis* (3.98%), respectively (Fig. 5E).

### 3.4. Correlation analysis

To investigate relationships between artemisinin content and the relative expression of studied genes, Pearson's correlation analysis was performed based on the obtained data for each species, and the results are presented in Table 4. In *A. fragrans* and *A. absinthium* species, there was no significant correlation between artemisinin content and expression of studied genes. However, the relative expression of *HMGR1* with *DRB2* and *SQS* with *ADS* showed a positive and significant correlation. In *A. scoparia* specie, artemisinin content positively and significantly correlated with the relative expression of *DBR2* and *HMGR1* genes. Moreover, a significant and positive correlation was found between expression of *HNGR1* with *DBR2*, and *SQS* with *ADS* genes. Similar to *A. fragrans* and *A. absinthium* species, there was found a positive and significant correlation between the relative expression of *HMGR1* with *DRB2* and *SQS* with *ADS* genes. In *A. annua* species, a positive and significant correlation was only found between expression of *HMGR1* and *DBR2* genes.

## 4. Discussion

There are more than 300 species of *Artemisia* in the world [28]. *Artemisia* species differ from each other in terms of chemical composition, but some compounds are present in all of them. The common characteristic of these species is sesquiterpen lactones.

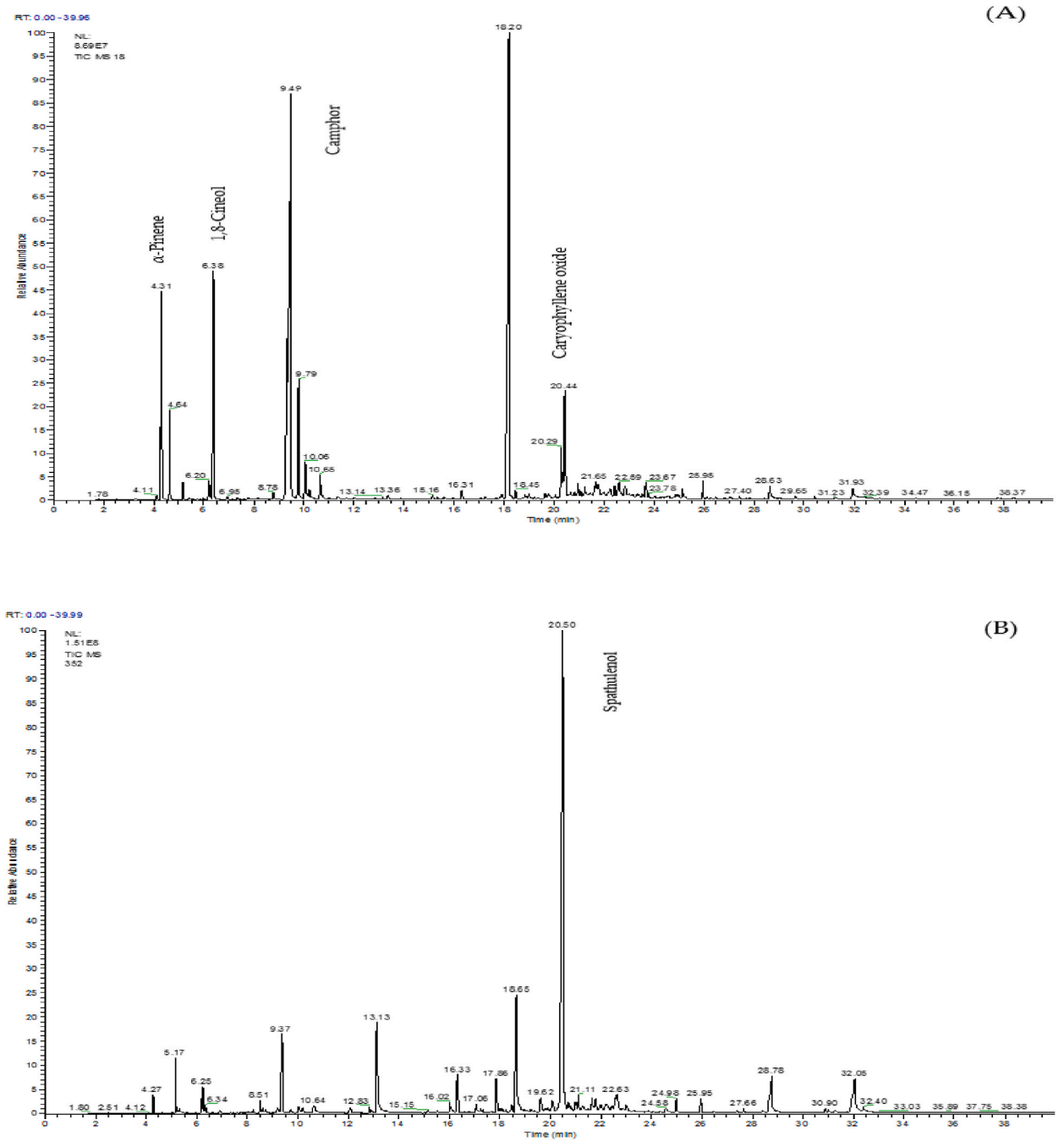


**Fig. 4.** Chromatograms of five different *Artemisia* species. (A) *A. annua*, (B) *A. scoparia*, (C) *A. absinthium*, (D) *A. fragrans*, and (E) *A. biennis*.

Artemisinin is a known sesquiterpen lactone that exists in *A. annua*, *A. abrotanum*, and *A. vulgaris* [29]. Similar to sesquiterpen lactones, the flavonoid composition is different in different species. The most common flavonoids of the *Artemisia* are artemetin and casticin, which are identified in the extracts of *A. abrotanum*, *A. absinthium*, and *A. annua* [30]. In addition, another common group of metabolites in *Artemisia* is coumarins [31]. In the present study, artemisinin content varied between 0.12 and 0.41 mg/g DW. The species *A. fragrans* revealed the highest amount of artemisinin, followed by *A. annua*, *A. biennis*, *A. scoparia*, and *A. absinthium*. Similarly, Salehi et al. [32] reported a wide range of variability in artemisinin content across different species of *Artemisia*. Various environmental and genetic factors affect the amount of artemisinin in *Artemisia* species [20]. Many genes are involved in artemisinin synthesis, and changes in their expression increase or decrease the amount of artemisinin. However, the extent of this change in gene expression and

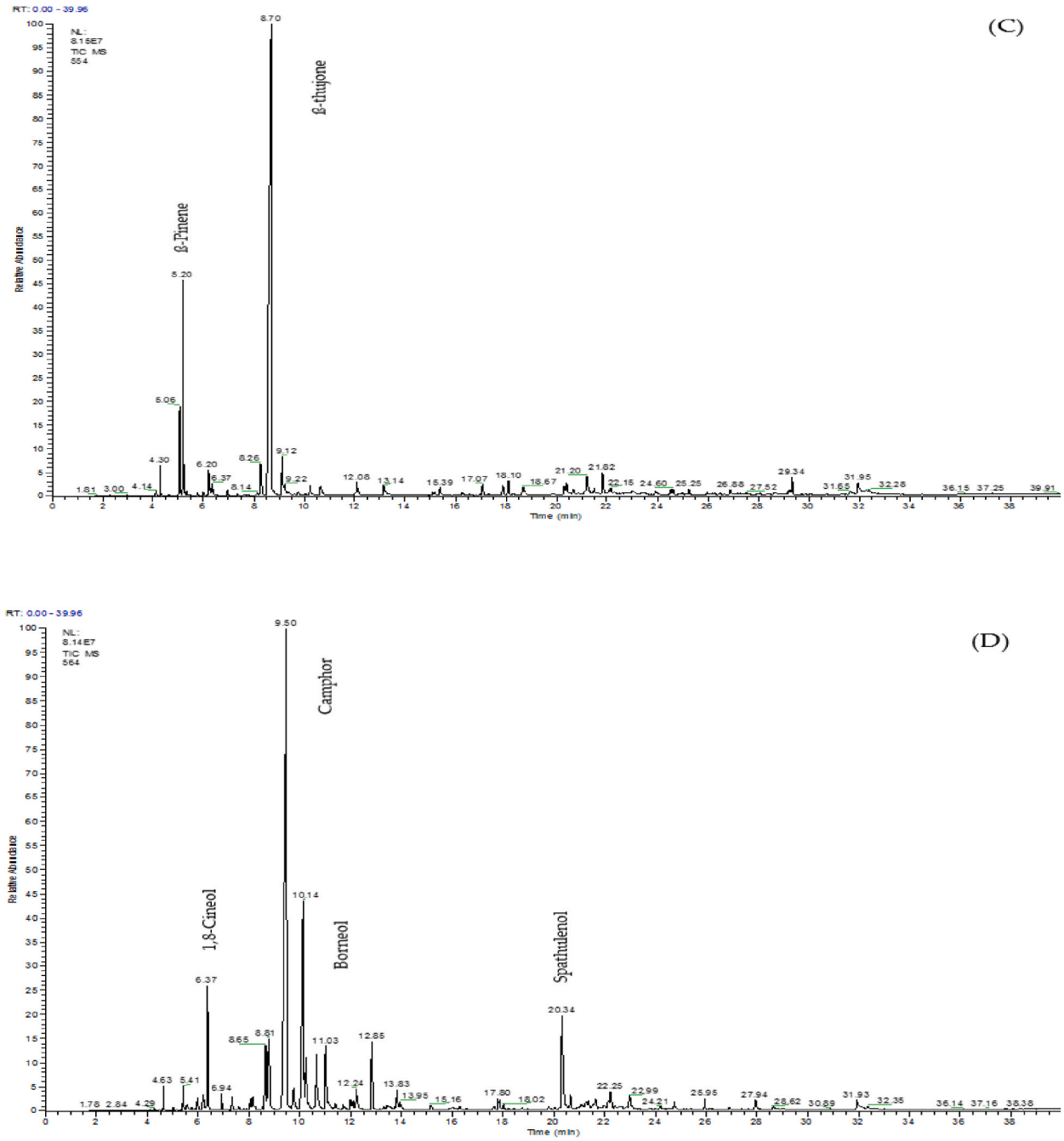


**Fig. 4.** (*continued*).

artemisinin biosynthesis also varies depending on the species and growth stages [33]. Artemisinin has been found in about 40 different species of *Artemisia*, varying from 0.0005% to 38.1% depending on the growth stage and plant organ [34]. In different studies, the highest amount of artemisinin was obtained in the *A. annua* species followed by the *A. deserti*, *A. marschalliana*, and *A. absinthium* [35–37]. Nomonov et al. [33] studied seven species of *Artemisia* in Tajikistan and found that the amount of artemisinin is between 0.07% and 0.45% of the dry mass. Salehi et al. [22] reported that *A. deserti* had 5.13 mg/g DW artemisinin. Salih et al. [32] showed that the leaf extract of *A. sieberi*, *A. Judaica*, and *A. monosperma* had about 3.01, 2.5, and 1.9 mg/g DW of artemisinin, respectively.

Plants use secondary metabolites to deal with both biotic and abiotic stresses. These compounds have medicinal properties and are used in the pharmaceutical industry [38]. So far, many of these medicinal compounds in nature have not been identified and many studies should be done for this purpose [39]. Different species of *Artemisia* are among the most important plants that have medicinal compounds and play an important role in traditional medicine [28]. Based on the result of GC-MS analysis, many bioactive compounds

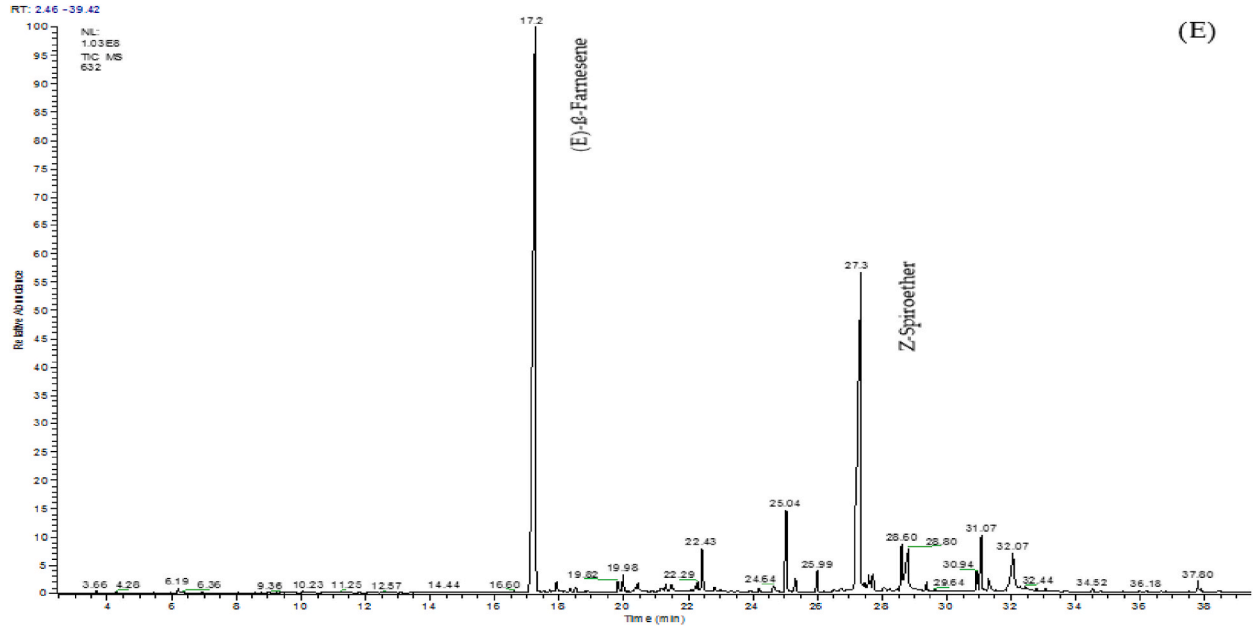


**Fig. 4.** (*continued*).

were found in different investigated species. Indeed, the high rate of variability in identified phytochemical compounds may be caused by the nature and physicochemical response of different species to environmental conditions. In addition, genetic, environmental, and seasonal factors can also be effective [40,41]. In the present study, the investigated species also differed in terms of their compositions. For example, monoterpenoids are abundant in the essential oils of *A. abrotanum*, *A. absinthium*, *A. annua*, and *A. vulgaris*, while phenylpropanoids are predominant in the essential oil of *A. dracunculus* [42]. However, our results indicated that alcohol esters of thujyl, α-thujone, β-thujone, camphene, (Z)-epoxycymene, *trans*-sabinyl acetate, and chrysanthyl acetate are common components in *A. absinthium*, which is in accordance with the findings reported by Kazemi et al. [43]. The most common monoterpenoids found in *A. abrotanum* are 1-terpineol, *trans*-piperitol, 1,8-cineole, and camphor [42]. While, camphene, camphor, β-pinene, borneol, and cuminal are commonly in *A. annua* [44]. Sabinene, terpinen-4-ol, β-osimene, *cis*-osimene, α-trans-osimene, limonene, α-flandrin, β-flandrin, (Z)-artemidin, and capylene have been identified in *A. dracunculus* [45]; and 1,8-cineole, sabinene, camphor, camphene, caryophyllene oxide, α-thugone, and β-thugone were founded in *A. vulgaris* [28]. From the geographical viewpoint, there are significant difference in phytochemical compositions. For instance, *A. vulgaris* sampled from the Republic of Bashkortostan have large amounts of Cpinene, trans chrysanthenol, 5-pinene, C-myrcene, and [46]. Mucciarelli et al. [47] emphasized that camphene and camphor are two main compounds in Italian *A. vulgaris*, and Pino et al. [48] reported caryophyllene oxide as the main compound of this plant from Cuba.

Genes play an important role in the synthesis of secondary metabolites, and increasing or decreasing their expression has a great impact on the production of secondary metabolites. For example, in a study, *SQS* gene silencing increased artemisinin synthesis in *A. annua* [26]. In addition, it has been reported that blocking the synthesis pathways of other secondary metabolites increases the synthesis of artemisinin [49]. In our study, the highest expression of the *SQS* gene was observed in the *A. absinthium* species, while *A. fragrans,* which had the highest content of artemisinin, showed the lowest relative expression of the studied genes. These results further confirmed the HPCL analysis (Figs. 3 and 5F). Therefore, this gene has a negative effect on artemisinin biosynthesis. *ADS* and *DBR2* genes play an important role in the production of artemisinin, and reducing their expression levels will reduce its production. The possible reason for the decrease in the expression of some studied genes in the mentioned species can be due to the decrease in the precursor material required for the activity of enzymes and the biosynthesis of artemisinin. Indeed, if the precursor material for artemisinin biosynthesis is consumed and becomes the final product, the expression of genes involved in this pathway will decrease. These results are further supported by the correlation analysis, where there was no significant association between artemisinin content and the relative expression of *ADS* and *DBR2* genes (Table 4). In previous studies, it has been reported that the *A. annua* species has a lower amount of artemisinin than other species, which is due to the low expression of these two genes [22,26]. Yuan et al. [50] reported that overexpression of *DBR2* gene in transgenic *A. annua* resulted in increasing the artemisinin concentration. Indeed, this finding was not in accordance with our results. As shown in Figs. 3 and 5B, there was no association between artemisinin content and the relative expression of *DBR2* gene, especially in *A. annua* species. Transcription factors regulate the synthesis of secondary metabolites by binding to *cis*-acting regulatory elements in promoters. The *WIRKY* transcription factor is one of the important transcription factors in the production of secondary metabolites, which affects the synthesis of artemisinin through binding to the W-box in the ADS promoter and activating its expression [51]. In another study, the overexpression of the *WIRKY* gene caused an increase in the expression of the *CYP71*AV gene, but it did not have a significant effect on the transcription of the *ADS* and *DBR2* genes [52]. However,

**Table 2**
Phytochemical compounds in the five Iranian *Artemisia* species detected using GC-MS analysis.

| No. | RT (min) | Compounds name | Area (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | A. fragrans | A. absinthium | A. scoparia | A. biennis | A. annua |
| 1 | 4.14 | α-thujene | – | 0.15 | – | – | – |
| 2 | 4.31 | α-Pinene | – | 1.02 | 0.56 | 0.07 | 5.26 |
| 3 | 4.63 | Camphene | 0.67 | – | – | – | 2.28 |
| 4 | 4.69 | Thuja-2,4(10)-diene | – | – | – | – | 0.12 |
| 5 | 5.06 | Sabinene | – | 3.83 | – | – | – |
| 6 | 5.17 | β-Pinene | – | 8.61 | 2.12 | – | 0.44 |
| 7 | 5.3 | β-Myrcene | – | – | 0.1 | – | – |
| 8 | 5.41 | dehydro-1,8-Cineole | 0.71 | – | – | – | – |
| 9 | 5.98 | α-Terpinene | 0.33 | – | – | – | – |
| 10 | 6.2 | p-Cymene | 0.57 | 1.12 | 0.62 | 0.15 | 0.66 |
| 11 | 6.25 | Limonene | – | 0.33 | 1.18 | 0.04 | – |
| 12 | 6.38 | 1,8-Cineol | 4.05 | 0.5 | 0.31 | 0.04 | 6.66 |
| 13 | 6.95 | γ-Terpinene | 0.56 | 0.18 | – | 0.05 | – |
| 14 | 7.34 | Cis-Sabinene hydrate | 0.54 | – | – | – | – |
| 15 | 8.05 | Filifolone | 0.4 | – | – | – | – |
| 16 | 8.16 | Trans-Sabinene hydrate | 0.8 | – | – | – | – |
| 17 | 8.22 | cis-Thujone | – | – | 0.11 | – | – |
| 18 | 8.26 | α-thujone | – | 2.21 | – | – | – |
| 19 | 8.51 | trans-Thujone | – | – | 0.53 | – | – |
| 20 | 8.63 | Chrysanthenone | 2.83 | – | 0.19 | – | – |
| 21 | 8.7 | β-thujone | – | 68.42 | – | – | – |
| 22 | 8.72 | 1-Terpineol | 4.59 | – | 0.14 | – | – |
| 23 | 8.78 | α-Campholenal | – | – | – | – | 0.24 |
| 24 | 9.12 | Thujanol <neo-3-> | – | 2.24 | – | – | – |
| 25 | 9.35 | trans-Pinocarveol | – | 0.87 | 0.32 | – | 7.54 |
| 26 | 9.39 | β-Terpineol | 8.17 | – | – | – | – |
| 27 | 9.49 | Camphor | 38.86 | – | 4.25 | – | 29.04 |
| 28 | 9.79 | Pinocarvone | 1.1 | – | 0.12 | – | 4.81 |
| 29 | 10.03 | Borneol | 14.28 | – | 0.31 | – | – |
| 30 | 10.06 | 4-Terpineol | 1.73 | 0.59 | 0.18 | – | 1.54 |
| 31 | 10.64 | Myrtenol | – | 0.52 | 0.59 | – | – |
| 32 | 10.65 | Myrtenal | – | – | – | – | 1.21 |
| 33 | 10.67 | α-Terpineol | 2.91 | – | – | – | – |
| 34 | 11.03 | Trans-Piperitol | 3.12 | – | – | – | – |
| 35 | 12.08 | Cis-Chrysanthenyl acetate | – | – | 0.19 | – | – |
| 36 | 12.08 | Carvotanacetone | – | 0.83 | – | – | – |
| 37 | 12.24 | Piperitone | 1.07 | – | – | – | – |
| 38 | 12.83 | Bornyl acetate | 2.67 | – | 0.28 | – | – |
| 39 | 13.14 | E-Anethole | – | 1.06 | – | – | – |
| 40 | 15.39 | β-Bourbonene | – | 0.3 | – | – | – |
| 41 | 16.02 | Methyl eugenol | – | – | 0.73 | – | – |
| 42 | 16.33 | *trans*- Caryophyllene | – | – | 1.95 | – | – |
| 43 | 17.06 | (E)-β-Farnesene | – | 0.45 | 0.5 | 48.16 | – |
| 44 | 17.86 | Ar-Curcumene | – | – | 2.04 | 0.5 | – |
| 45 | 17.88 | Germacrene D | – | 0.48 | – | – | – |
| 46 | 18.2 | β-Selinene | – | 0.73 | – | – | 27.87 |
| 47 | 18.34 | Ledene | – | – | – | 0.13 | – |
| 48 | 18.48 | β-Bisabolene | – | – | 0.41 | 0.16 | – |
| 49 | 18.75 | δ-Cadinene | – | – | 0.74 | – | – |
| 50 | 19.82 | (E)-Nerolidol | – | – | – | 0.48 | – |
| 51 | 20.08 | Citronellyl butanoate | – | – | 0.97 | – | – |
| 52 | 20.44 | Caryophyllene oxide | 0.61 | 0.71 | – | 0.78 | 4.99 |
| 53 | 20.46 | Globulol | – | – | – | 0.42 | – |
| 54 | 20.5 | Spathulenol | 4.74 | 0.4 | 48.33 | – | – |
| 55 | 20.66 | Salvial-4(14)-en-1-one | 0.49 | – | – | – | – |
| 56 | 20.95 | Bornyl angelate | – | – | – | – | 0.5 |
| 57 | 20.99 | Ledol | – | – | 0.59 | – | – |
| 58 | 21.11 | Humulene epoxide II | – | – | 0.81 | – | – |
| 59 | 21.48 | Ledeneoxide II | – | – | – | 0.3 | – |
| 60 | 21.65 | Cadin-4-en-7-ol | – | – | – | – | 0.74 |
| 61 | 21.77 | Caryophylla-4(12),8(13)-dien-5α-ol | – | – | – | – | 0.73 |
| 62 | 21.82 | α-epi-Cadinol | – | 1.29 | – | – | – |
| 63 | 22.15 | α-Cadinol | – | 0.49 | – | – | – |
| 64 | 22.25 | neo-Intermedeol | 0.82 | – | – | 0.38 | – |
| 65 | 22.43 | Eudesm-7(11)-en-4-ol | – | – | – | 1.81 | – |
| 66 | 22.57 | epoxide Aromadendrene | – | – | 0.73 | – | – |
| 67 | 22.59 | Iso-Aromadendrene epoxide | – | – | – | – | 1.11 |

(*continued on next page*)

**Table 2** (*continued*)

| No. | RT (min) | Compounds name | Area (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | A. fragrans | A. absinthium | A. scoparia | A. biennis | A. annua |
| 68 | 22.63 | Isospathulenol | – | – | 1.39 | – | – |
| 69 | 24.98 | Diazinone | – | – | 0.65 | 3.11 | – |
| 70 | 25.31 | (2Z,6E)-Farnesyl acetate | – | – | – | 0.43 | – |
| 71 | 25.95 | Hexahydrofarnesyl acetone | 0.4 | – | 0.67 | 0.75 | 0.65 |
| 72 | 27.35 | Z-Spiroether | – | – | – | 28.53 | – |
| 73 | 27.58 | E-Spiroether | – | – | – | 0.86 | – |
| 74 | 27.72 | Methyl palmitate | – | – | – | 0.57 | – |
| 75 | 28.63 | Palmitic acid | – | – | 4.47 | 3.43 | 0.58 |
| 76 | 30.94 | Linoleic acid, methyl ester | – | – | – | 0.72 | – |
| 77 | 31.07 | Methyl linolenate | – | – | – | 1.94 | – |
| 78 | 31.29 | Phytol | – | – | – | 0.77 | – |
| 79 | 32.05 | oleic acid | 0.87 | – | 4.31 | – | – |
| 80 | 32.07 | 9-Octadecenoic acid, (E) | – | – | – | 3.63 | – |

**Table 3**
Analysis of variance was conducted for the relative expression data of eight investigated genes in the five Iranian *Artemisia* species.

| Gene | Source of variation | | Gene | Source of variation | |
|---|---|---|---|---|---|
| | Species ($df = 4$) | Error ($df = 10$) | | Species ($df = 4$) | Error ($df = 10$) |
| *4FPSF* | 18.207 ** | 1.973 | *WIRKY* | 9.702 ** | 0.989 |
| *DBR2* | 5.318 ** | 0.822 | *ADS* | 14.627 ** | 0.419 |
| *HMGR1* | 4.919 $^{ns}$ | 2.173 | *DXS* | 42.919 ** | 1.068 |
| *HMGR2* | 4.406 $^{ns}$ | 1.169 | *SQS* | 50.790 ** | 0.391 |

ns, *, and **: Non-significant, and significant at $P < 0.05$ and $P < 0.01$, respectively.

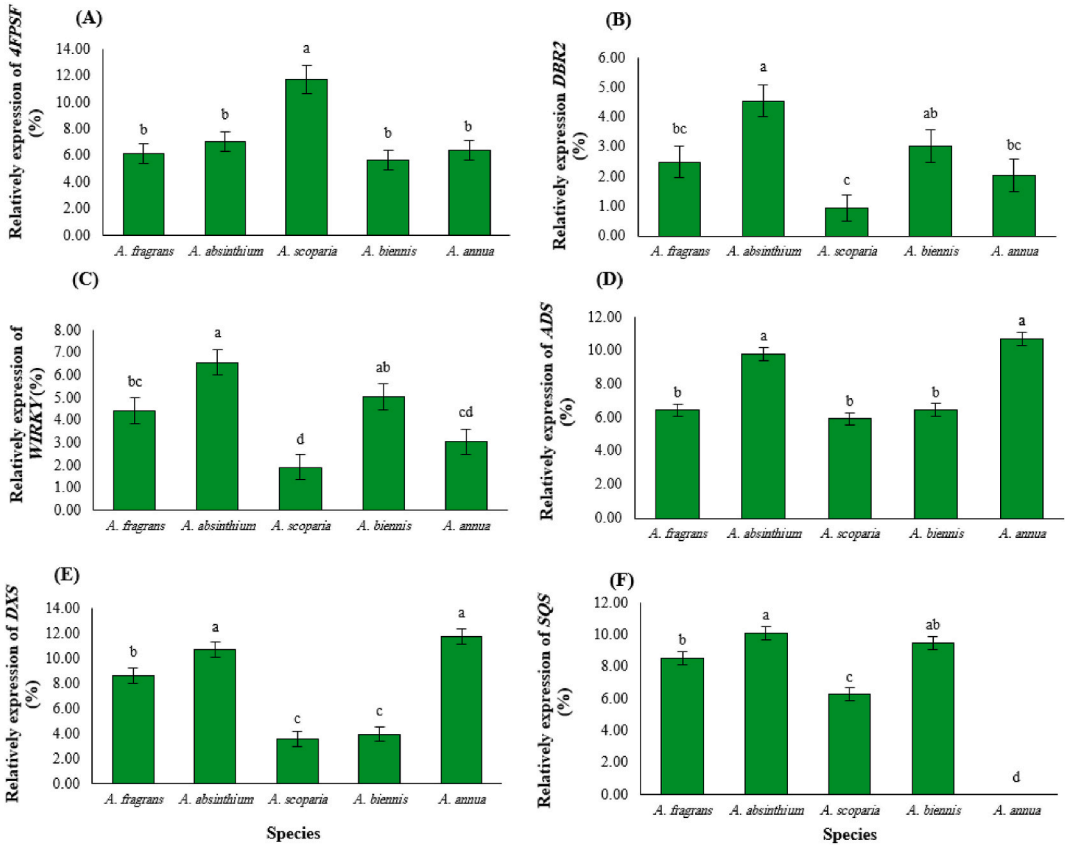


**Fig. 5.** The relative expression of genes including *4FPSF*, *DBR2*, *WIRKY*, *ADS*, *DXS*, and *SQS* in different *Artemisia* species. Different letters show significant differences among species at 0.01 the probability level.

**Table 4**
Correlation coefficients among artemisinin content and the relative expression of studied genes in different *Artemisia* species.

| Species | Variables | Artemisinin | *4FPSF* | *DBR2* | *WIRKY* | *HMGR1* | *HMGR2* | *ADS* | *DXS* | *SQS* |
|---|---|---|---|---|---|---|---|---|---|---|
| *A. fragrans* | Artemisinin | | | | | | | | | |
| | *4FPSF* | 0.23 | | | | | | | | |
| | *DBR2* | 0.97 | 0.46 | | | | | | | |
| | *WIRKY* | −0.06 | 0.95 | 0.18 | | | | | | |
| | *HMGR1* | 0.96 | 0.48 | 1** | 0.21 | | | | | |
| | *HMGR2* | 0.57 | 0.93 | 0.75 | 0.78 | 0.77 | | | | |
| | *ADS* | 0.87 | 0.67 | 0.97 | 0.42 | 0.97 | 0.89 | | | |
| | *DXS* | 0.75 | 0.81 | 0.89 | 0.60 | 0.91 | 0.96 | 0.98 | | |
| | *SQS* | 0.91 | 0.62 | 0.98 | 0.36 | 0.99 | 0.86 | 0.99 | 0.96 | |
| *A. absinthium* | Artemisinin | | | | | | | | | |
| | *4FPSF* | 0.34 | | | | | | | | |
| | *DBR2* | 0.99 | 0.46 | | | | | | | |
| | *WIRKY* | 0.05 | 0.96 | 0.18 | | | | | | |
| | *HMGR1* | 0.98 | 0.48 | 1** | 0.21 | | | | | |
| | *HMGR2* | 0.66 | 0.93 | 0.75 | 0.78 | 0.77 | | | | |
| | *ADS* | 0.93 | 0.67 | 0.97 | 0.42 | 0.97 | 0.89 | | | |
| | *DXS* | 0.83 | 0.81 | 0.89 | 0.60 | 0.91 | 0.97 | 0.97 | | |
| | *SQS* | 0.95 | 0.62 | 0.98 | 0.36 | 0.98 | 0.86 | 0.99** | 0.96 | |
| *A. scoparia* | Artemisinin | | | | | | | | | |
| | *4FPSF* | 0.59 | | | | | | | | |
| | *DBR2* | 0.99** | 0.63 | | | | | | | |
| | *WIRKY* | 0.05 | 0.83 | 0.08 | | | | | | |
| | *HMGR1* | 1** | 0.61 | 1** | 0.06 | | | | | |
| | *HMGR2* | 0.66 | 0.99 | 0.68 | 0.78 | 0.67 | | | | |
| | *ADS* | 0.93 | 0.85 | 0.94 | 0.42 | 0.93 | 0.89 | | | |
| | *DXS* | 0.83 | 0.94 | 0.85 | 0.60 | 0.83 | 0.97 | 0.98 | | |
| | *SQS* | 0.95 | 0.82 | 0.96 | 0.36 | 0.95 | 0.86 | 0.99** | 0.96 | |
| *A. biennis* | Artemisinin | | | | | | | | | |
| | *4FPSF* | −0.34 | | | | | | | | |
| | *DBR2* | −0.99 | 0.46 | | | | | | | |
| | *WIRKY* | −0.05 | 0.95 | 0.17 | | | | | | |
| | *HMGR1* | −0.98 | 0.48 | 1** | 0.21 | | | | | |
| | *HMGR2* | −0.66 | 0.93 | 0.75 | 0.78 | 0.77 | | | | |
| | *ADS* | −0.92 | 0.67 | 0.96 | 0.42 | 0.97 | 0.89 | | | |
| | *DXS* | −0.82 | 0.81 | 0.89 | 0.60 | 0.91 | 0.97 | 0.98 | | |
| | *SQS* | −0.95 | 0.62 | 0.98 | 0.36 | 0.98 | 0.86 | 0.99** | 0.96 | |
| *A. anuua* | Artemisinin | | | | | | | | | |
| | *4FPSF* | 0.34 | | | | | | | | |
| | *DBR2* | 0.99 | 0.46 | | | | | | | |
| | *WIRKY* | 0.05 | 0.96 | 0.18 | | | | | | |
| | *HMGR1* | 0.98 | 0.49 | 1** | 0.21 | | | | | |
| | *HMGR2* | 0.66 | 0.93 | 0.75 | 0.78 | 0.77 | | | | |
| | *ADS* | 0.93 | 0.67 | 0.97 | 0.42 | 0.97 | 0.89 | | | |
| | *DXS* | 0.83 | 0.81 | 0.89 | 0.60 | 0.91 | 0.97 | 0.98 | | |
| | *SQS*[†] | | | | | | | | | |

** significant at $P < 0.01$.

we found a positive association between expression of *WIRKY* and *ASD* genes across all investigated species (Fig. 5C and D). In general, our findings indicated that a large class of phytochemical compounds are available in various *Artemisia* species, which may be responsible for many pharmacological activities. Hence, further work to discover pharmacological activities in various species is required.

## 5. Conclusions

According to the present study, the results conclude that artemisinin content depends on genetic background. In contrast to numerous studies that reported *A. annua* has a considerable artemisinin content, *A. fragrans* showed the highest amount of artemisinin than other species. Moreover, we found a direct association between artemisinin accumulations in different species with the relative expression of artemisinin accumulation-related genes in them. The result of GC-MS analysis identified 80 phytochemical compounds in five investigated species, which in turn emphasized more attention to these species in search of novel medicinal properties.

## Data availability statement

Data will be made available on request.

## CRediT authorship contribution statement

**Bita Jamshidi:** Writing – original draft, Software, Investigation. **Alireza Etminan:** Methodology, Conceptualization. **Alimehras Mehrabi:** Methodology, Conceptualization. **Lia Shooshtari:** Investigation, Conceptualization. **Alireza Pour-Aboughadareh:** Writing – review & editing, Software.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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