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Determination of Artemisinin in Selected *Artemisia* L. Species of Turkey by Reversed Phase HPLC

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Abstract: Artemisinin (quinghaosu) is a natural compound, isolated from *Artemisia annua* L. (Compositae), of current interest in treatment of drug-resistant malaria. Aim of the current study was to hit upon novel artemisinin sources as alternative to *A. annua*. Therefore, ten species of the genus *Artemisia* (*A. santonicum* L., *A. taurica* Willd., *A. spicigera* K. Koch, *A. herba-alba* Asso, *A. haussknechtii* Boiss., *A. campestris* L., *A. araratica* Krasch., *A. armeniaca* Lam., *A. austriaca* Jacq., and *A. abrotanum* L.) collected from different localities throughout Turkey were analyzed with respect to artemisinin amount using a reliable and fast Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) analysis technique. HPLC conditions used for determination of the artemisinin content were established as follows; ACE-5 C18 column (250 x 4.6 mm, 5 µm) was employed with the mobile phase of formic acid (% 0.2 v/v): acetonitrile (50:50 v/v) mixture at the flow rate of 1 mL/min. The good linearity of artemisinin was found within the range of 0.1 – 100 µg/mL (r^2= 0.99908). However, our study showed that artemisinin was not detected in all investigated *Artemisia* species of Turkish origin.

Key words: *Artemisia* L., *Artemisia annua* L., Compositae, HPLC

1. Introduction

Malaria is a vector-borne infectious disease, which occurs in approximately 400 million people every year, especially in Sub-Saharan Africa. Malaria infections are treated through the use of antimalarial drugs, such as chloroquine, pyrimethamine and mefloquine, although drug resistance is increasing drastically. On the other hand, extracts of the plant *Artemisia annua*, containing the compound artemisinin (Figure 1) or its semi-synthetic derivatives, offer over 90 % efficacy rates, however, their supply is not meeting demand. Thus, there is a definite need for the discovery of innovative sources of artemisinin (quinghaosu), a sesquiterpene lactone endoperoxide, isolated from

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Artemisia annua L. (Compositae). Artemisinin represents a class of antimalarials that have successfully been used in the treatment of drug-resistant malaria for more than two decades [1,2].

A number of analytical methods have been developed for detection and quantification of artemisinin, e.g., thin layer chromatography (TLC) [3,4], TLC with visible light densitometric detection (TLC) [5-7], high-performance liquid chromatography with UV detection (HPLC–UV) [8-10], HPLC with electrochemical detection (HPLC–ECD) [11-12], HPLC with evaporative light-scattering detection (HPLC–ELSD) [13-16], HPLC with peroxyoxalate chemiluminescence detection (HPLC-PO-CL) [17], HPLC/tandem mass spectrometric (LC/MS/MS) method [18], liquid chromatography/mass spectrometry (LC/MS) [19], high performance capillary electrophoresis using self-designed conductivity detection system [20], gas chromatography with mass spectrometric detection (GC–MS) [21], GC with flame ionization detection (GC–FID) [22], and enzyme-linked immunosorbant assay (ELISA) [23].

In our recent study, we have examined the artemisinin quantity of *A. annua* L. grown under four nitrogen dose applications (0, 40, 80, and 120 kg ha^{-1}) in Çukurova ecological conditions in Turkey [24]. Therefore, our attempt herein was to hit upon novel artemisinin sources as alternative to *A. annua*. The genus *Artemisia* L. is represented by 23 species in the Flora of Turkey and the East Aegean Islands [25,26]. Among them, ten species of the genus *Artemisia* (*A. santonicum* L.*, A. taurica* Willd.*, A. spicigera* K. Koch*, A. herba-alba* Asso*, A. haussknechtii* Boiss.*, A. campestris* L., *A. araratica* Krasch., *A. armeniaca* Lam., *A. austriaca* Jacq. and *A. abrotanum* L.) collected from various regions in Turkey were analyzed with respect to artemisinin using reversed phase-high performance liquid chromatography (RP-HPLC) analysis technique.

2. Materials and Methods

2.1. Plant Materials

Plant materials were collected from different localities throughout Turkey in their natural habitats. The plants were collected and identified by *N. Adıgüzel* and *B. Bani*, Ph.D. Authenticated voucher specimens were deposited in the Herbarium of Gazi University (GAZI). Table 1 shows the collection sites and times for each plant species.

2.2. Preparation of Extracts

Approximately, 5 g of each plant sample was weighed accurately and macerated with 250 mL of *n*-hexane at room temperature for 2 days using a laboratory-scale shaker. Then, the *n*-hexane phases were filtrated and evaporated under vacuum until dryness. The residue was dissolved again in 100 mL of *n*-hexane and the *n*-hexane phase was washed in a separatory funnel with 2 % NaOH solution to get rid of the impurity, which is soluble in NaOH. After abandoning the alkali solution present in the lower layer, the upper solution was washed with distilled water several times until it was neutralized. The extract, obtained after distillation under vacuum at 45 ºC in rotary evaporator, was dissolved with 95% ethanol and then filtrated in 250 mL measuring flask. Then, 10 mL of filter liquor was transferred into a 100 mL measuring flask. 40 mL of 0.2 % NaOH solution was added in the flask, and then, let it react at 50 ºC for 30 min. After that, 0.08 mol/L acetic acid solution was filled up to the mark [27]. The procedure described herein was applied to all of the samples.

2.3. Equipment

The analysis was performed with an LC system consisting of an HPLC Agilent 1100 series quaternary pump with degasser and a photodiode array detector. Samples were injected with an HPLC Agilent 1100 Autosamplers with thermostatted column compartment on an ACE-5 C18 column (5 μm;

250 mm x 4.6 mm), at 30 °C. The system was controlled and data analysis was performed with Agilent ChemStation. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

2.4. Chemicals

Standard artemisinin (361593, 98%, CAS 63968-64-9) was purchased from Sigma-Aldrich Co. (USA). All the solvents used were of analytical-grade of Merck quality.

2.5. Standard Stock Solutions

100 mg of standard artemisinin was dissolved by 95 % ethanol in a 100 mL measuring flask. 2 mL of artemisinin solution and 8 mL of 95 % ethanol were transferred into another 100 mL measuring flask. This was coded as "Solution-1". "Solution-2" was made with 5 mL of artemisinin solution and 5 mL of 95% ethanol and "Solution-3" was prepared with 10 mL of artemisinin solution. 40 mL of 0.2% NaOH solution was added in the three flasks, respectively, and then, let them react at 50 ºC for 30 min. After that, 0.08 mol/L acetic acid solution was filled up to the mark [27]. Three standard solutions were prepared and applied to HPLC.

2.6. Calibration Solutions

In order to establish the linear detection range for each compound, individual standard stock solutions were prepared in mobile phase in 100 mL-measuring flasks. Aliquots of these solutions were diluted and analyzed to determine method linearity. Limit of quantification (LOQ) values were estimated from serial dilution and analyzed for each sample. Calibration ranges for artemisinin 0.1 – 100 µg/mL were prepared. Triplicate 10 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The five concentrations of each compound were subjected to regression analysis to calculate calibration equation and correlation coefficients.

2.7. Procedure for HPLC Analysis

A mobile phase consisting of formic acid (% 0.2 v/v): acetonitrile (50:50) by isocratic elution was chosen to achieve maximum separation and sensitivity. Flow rate was 1.0 mL/min. Column temperature was set at 30 °C. The samples were detected at 254 nm using photodiode array detector. Results of artemisinin quantities in *Artemisia* samples were expressed as the mean of three determinations.

2.8. Linearity

Equation of the regression line formula and correlation coefficient were Y= 10.0031X + 14.8232, and r^2= 0.99908 for artemisinin. Excellent linearity was obtained for compounds between peak areas and concentrations of 0.1 – 100 µg/mL.

2.9. Limits of Detection and Quantification

Limit of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limit of quantification (LOQ) were established at signal-to-noise ratio (S/N) of 10. LOD and LOQ were

experimentally verified by six injections of artemisinin at the LOD and LOQ concentrations. The LOD was calculated to be 0.03; the LOQ was calculated to be 0.1 µg/mL for artemisinin.

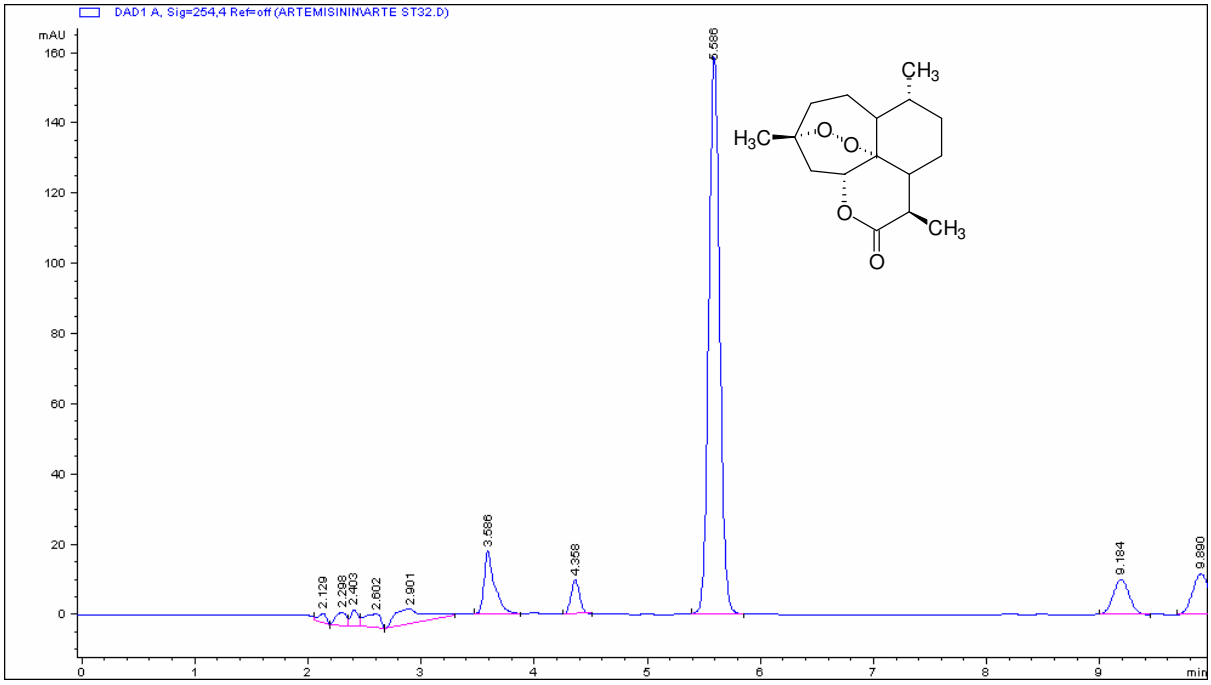

Figure 1. Chromatogram of a standard solution of artemisinin after process prior to analysis (RT= 5.58)

3. Results and Discussion

Numerous species of the genus *Artemisia* have been used in folk medicine all over the world. Besides, several species of *Artemisia* genus are well known due to their antiparasitic activity [28]. Many *Artemisia* species have been examined to find the sesquiterpene lactone, artemisinin, which so far has been isolated only from *A. annua* growing in China [3,28]. *A. annua* is cultivated in China, Vietnam, and several other East Asian countries to obtain artemisinin as an antimalarial drug. Although its total synthesis has been achieved, the synthesis gives low yields and is uneconomical due to its complex structural properties. Therefore, the isolation of artemisinin from the plant still represents the best alternative [1, 29,30].

Artemisinin levels vary considerably along with low amounts in the naturally growing *A. annua*. The content of artemisinin (Figure 1), found in the leaves and flowering tops of the plant, but not in the roots, is affected by some factors such as growth conditions, seasonal and geographical variations as well as breeding [31]. *A. annua* growing in the natural habitats contain 0.06-0.5 % artemisinin, but breeding has yielded plants in which artemisinin amount raised up to 2 % [30]. In other study, Peng et al. reported that the artemisinin content of the sixteen seed-generated lines of the cultivar *A. annua* ranged from 0.2 % to 0.9 % by both GC-FID and HPLC-ELSD [15]. ElSohly et al.'s work stated that artemisinin (% 0.138, 0.140, and 0.153) in *A. annua* cultivars analyzed by HPLC analysis reached to its maximum amounts prior to flowering period [8]. In Qian et al.'s study, the artemisinin content in the herb of *A. annua* was found 0.652 % using RP-HPLC [10]. In a previous study, different extracts of the aerial parts of two samples of *A. annua*, a commercial sample from China and a selected cultivar from Brazil, were analyzed in order to determine which solvents

(dichloromethane and hexane) provide the best yields of artemisinin by HPLC/DAD/MS and yields and contents of artemisinin were found to be quite different in the tested extracts. For instance; the selected Brazilian cultivar contained a higher level of artemisinin as compared to the commercial Chinese herbal drug and *n*-hexane was more selective in isolating artemisinin [31]. For the artemisinin extraction from plants, liquid solvent extraction is currently used using miscellaneous solvents such as toluene [32], *n*-hexane [8,15], petroleum ether [3,10], and chloroform [21]. In our study, *n*-hexane was used effectively for the extraction of artemisinin. After this compound was processed prior to analysis as mentioned in Hao et al.'s method since it is easily degradable.

Table 1. The collected plant materials and collection sites

Plant name (specimen number)	Collection sites
A. abrotanum L. (6028)	Erzurum: Akşar-Şenkaya, 1350 m, bushes, 30.vii.2004
A. araratica Krasch. (4237)	Muş: Malazgirt, 1410m, margines of Murat river, 22.ix.2001
A. armeniaca Lam. (6205)	Kars: Boğatepedüzü, 2280 m, mountain pasture, 03.viii.2004
A. austriaca Jacq. (5550)	Kars: Sarıkamış, Karakurt-Kağızman, 1360 m, bushes, 06.vii.2004
A. austriaca Jacq. (5938)	Kars: Sarıkamış, Gözebaşı-Gaziler, 1700 m, stony steppe, 28.vii.2004
A. austriaca Jacq. (5906)	Kars: Sarıkamış, Çamyazı village, 2300 m, *Astragalus* steppe, 27.vii.2004
A. campestris L. (4205)	Kahramanmaraş: around Kabaktepe village, 1270m, stony places, 19.viii.2001
A. haussknechtii Boiss. (4247)	Hakkari: Between Hakkari and Çukurca, 1200m, roadside, 28.ix.2001
A. herba-alba Asso (4471)	Van: Kampüs, 1700m, barren places, 28.ix.2002
A. herba-alba Asso (4475)	Van: Güzelsu, 2000m, marly slopes, 01.x.2002
A. santonicum L. (2746)	Karaman:Between Ereğli and Ayrancı, 1280m, *Artemisia* steppe, 12.x.1996
A. santonicum L. (4245)	Van: Patnos-Erciş, 1780m, roadside, 22.ix.2001
A. santonicum L. (4265)	Iğdır: Aralık, 800m, salty places, 20.x.2001
A. santonicum L. (4468)	Van: Muradiye waterfall, 2050m, stony places, 28.ix.2002
A. santonicum L. (6395)	Karaman: Ereğli-Karaman, 1000m, *Artemisia* steppe, 24.x.2005
A. santonicum L. (6412)	Kars: Between Kağızman and Tuzluca, 1100m, salty places, 09.xi.2003
A. spicigera K. Koch (2751)	Ankara: Çubuk barrage, 1000m, steppe, 16.xi.1996
A. taurica Wild. (4477)	Van: Güzelsu, 2000m, marly slopes, 01.x.2002
A. taurica Wild. (6384)	Ankara: Polatlı, 800m, salty soils, 30.ix.2005
A. taurica Wild. (6371)	Konya: Cihanbeyli, 930m, salty soils, 23.ix.2005

During our literature survey, we have encountered a number of analytical methods such as HPLC–UV, HPLC–ECD, HPLC–ELSD, HPLC-PO-CL, LC/MS/MS, and GC–MS for evaluation of artemisinin in plant extracts as above-mentioned. Among them, we have chosen the analytical procedure by HPLC performed in this study, which is a simple, fast, and reliable technique, and successfully applied to determine the contents of artemisinin in the aerial parts of investigated *Artemisia* species. HPLC analyses of artemisinin were achieved on an ACE-5 C18 column (250 x 4.6 mm, 5 µm) by using formic acid (% 0.2 *v/v*): acetonitrile (50:50 *v/v*) mixture as a mobile phase at the flow rate of 1 mL/min. This method was applied to ten *Artemisia* species (*A. santonicum* L., *A. taurica* Willd., *A. spicigera* K. Koch, *A. herba-alba* Asso, *A. haussknechtii* Boiss., *A. campestris* L., *A. araratica* Krasch., *A. armeniaca* Lam., *A. austriaca* Jacq. and *A. abrotanum* L.) collected from different localities throughout Turkey (Table 1).

HPLC chromatograms of the plant samples showed many resolved peaks. The peaks were identified by comparison of their retention times to that of standard artemisinin (Figure 1). Linear regression was used to establish the calibration curve. The good linearity of artemisinin was found within the range of 0.1 – 100 µg/mL (r^2= 0.99908). The regression equation and correlation coefficient were determined as Y= 10.0031X + 14.8232, and r^2= 0.99908 for artemisinin. The limit of detection (LOD) was calculated to be 0.03, while the limit of quantification (LOQ) was calculated as 0.1 µg/mL for artemisinin.

According to the outcomes obtained from our study on a number of *Artemisia* species, artemisinin was not detected in all investigated *Artemisia* species of Turkish origin. In other words, it was found lower than LOD value calculated.

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