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Chemical characterization of *Artemisia annua* L. subcritical extract

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The aerial parts of *Artemisia annua* L. from Bulgaria were extracted with 1,1,1,2-tetrafluoroethane. Static two-stage extraction at a pressure of 8 and 12 bar was used at a relatively low temperature. The yield was 2%. The chemical profile of the product was reported for the first time. GC-MS analysis showed that the extract contained the essential oil constituents, but in lower concentrations. In total, 28 components were identified, the main ones being artemisia ketone (26.2%), camphor (10.7%), and eucalyptol (9.1%). Artheanin B (3.7%) and arteannuic acid (3.7%) were found to be co-metabolites and precursors of artemisinin. The content of the sesquiterpene lactone artemisinin, determined spectrophotometrically, was found to be 1.28 ± 0.10 %. The results revealed that the extract is of interest with the presence of biologically active substances used as a modern anti-malaria and potential anti-coronavirus agent.

Keywords: *Artemisia annua* L.; extraction; chemicals; artemisinin.

INTRODUCTION

Artemisia annua L. (Asteraceae), known as wormwood, a highly aromatic annual herb of Asiatic and East European origin, is widely distributed in the temperate and tropical regions throughout the world. The plant is spread wild in Bulgaria and is used in traditional medicine for treatment of gastric insufficiency, in infusion as poison antidote and for activating blood circulation [1]. It is an important medicinal plant, whose biological activity is due to volatile and non-volatile secondary metabolites [2]. The essential oil which is rich in mono- and sesquiterpenes represents another source of potential commercial value. Besides significant variations in its percentage and composition have been reported (major constituents can be camphor, germacrene, artemisia ketone, and 1,8-cineole (eucalyptol)), the oil has been subjected to numerous studies supporting promising antibacterial and antifungal activities [3].

The wormwood is among the ten top pharmaceutical crops and a source of the life-saving drug artemisinin, an active ingredient of most antimalarial medicines, used every year by over 300 million people. It is mainly located in the flowers and leaves of the plant and stored in the glandular trichomes on the surface of leaves, because of its toxicity for the plants cells [2]. The isolation of artemisinin was firstly achieved by the Chinese scientist Tu Youyou and awarded with the 2015 Nobel Prize in Medicine together with William C. Campbell and Satoshi Ō [4]. Recent docking studies indicated that artemisinin and its derivatives artesunate and arteminol could bind the SARS-CoV-2 spike protein in a way that would interfere with its docking onto the human ACE2 receptor protein, which is the required first step in the host infection process of the coronavirus disease (COVID-19) [5].

However, other bioactive compounds in *A. annua* contribute to the overall activity of the extracts: flavonoids, arteannuin B and artemisitene, but also scopoletin and 1,8-cineole act synergically with artemisinin and exhibit beneficial activity [2, 6, 7].

For this reason, in recent years, efforts have been focused on the application of different approaches to obtain a complex end product.

The studies assessed the economic and environmental potential of the extraction by ethanol, supercritical CO_2, ionic liquids, freons, monoether-based solvents as compared to hexane and ethyl acetate [8-14]. Extraction by tetrafluoroethane was found to be the most promising approach among the considered new alternative processes [15]. The solvent (also known as refrigerant R134a) is nontoxic, nonreactive, nonflammable, and nonozone depleting. It has high volatility and boiling point at atmospheric pressure of -25.9°C, which means that it leaves negligible solvent residue in the products. It is in a gas form at room temperature, stable to aqueous acids and bases, immiscible with water and sparingly soluble in water (1500 ppm at 20°C). It is normally handled as a compressed gas under pressure in liquid form

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and has a liquid density of about 1.3 kg/L [16]. The biological products made by this process have extremely low residual solvent - less than 20 parts per billion or frequently below the levels of detection. It is neither acidic nor alkaline and has only minimal potential reaction effects on the botanical materials [11]. It can perfectly extract the essential oil with a particularly cleaner process comparing with other solvents, with contamination-free product and facile downstream processing. Despite the excellent indications for the efficacy of R134a as an extractant, there are no data in the literature for a complete chemical analysis of the product. The authors concentrate and comment on the content of artemisinin and its derivates, but other components are not mentioned [11, 13, 14]. Only two references comment on the camphor content in a supercritical extract with CO_2 [9, 12]. Adding the importance of the origin of *A. annua* for the quantitative and qualitative characteristics of the final product [1, 2, 17, 18], the question of the chemical profile of the subcritical extract of the native plant becomes important.

The theme is very actual and the aim of the present work was to evaluate the chemical composition of the freon extract from *A. annua* L., grown in Bulgaria and its potential to obtain artemisinin.

EXPERIMENTAL

Plant material

A. annua L. herbs (full blooming) were collected in the area of Kazanlak (Bulgaria) in July 2016. The plant material was air dried at room temperature in a shadow place. According to Ferrera *et al.* [19], that kind of drying provided the highest artemisinin yield. Later it was stored in a cool, dark and dry place prior to extraction and analysis. The moisture of the material (12.5%) was determined by drying up to constant weight at 105°C. The plants were grounded immediately prior to extraction.

Extraction

Extraction of wormwood material using tetrafluoroethane was performed in a pilot apparatus. The unit consists of a 1 L extraction vessel, a 5.5 L of collector vessel, equipped with a 200 W electric heater, a compressor, a heat exchanger unit, and a filtration set. The system is equipped with temperature and pressure sensors. It is controlled by a fully automatic Programmable Logic Controller (PLC) screen interface with first level safety functionality and user programmable extraction pressure, number of extractions, separation end pressure and extraction time. A heating mantle was constructed around the collector vessel to maximize the freon transfer rate from liquid to gas state.

The solvent was food grade 1,1,1,2-tetrafluoroethane (CAS number 811-97-2), purchased from Frigo Chem Ltd. (Bulgaria).

The experiments were conducted at conditions, shown in Table 1.

GC/MS analysis

GC/MS analysis was carried out on a 7890A gas chromatograph (Agilent Technologies) interfaced with a 5975C mass selective detector (Agilent Technologies). Separations were performed using a 30 m×0.25 mm (i.d.) DB-5 ms silica-fused capillary column coated with a 0.25 μm film of poly (dimethylsiloxane) as the stationary phase. The flow rate of carrier gas (helium) was maintained at 1.0 mL/min. The injector and the transfer line temperature were kept at 250°C. The oven temperature program used was 40°C for 2 min, then 5°C/min to 300°C for 10 min. The injection volume was 1 μL. The injections were carried out in a split mode 20:1. The mass spectrometer was scanned from 50 to 550 m/z. All mass spectra were acquired in an electron impact (EI) mode with 70 eV.

A mixture of aliphatic hydrocarbons (C_8-C_{40}) (Sigma) was injected into the system under the above temperature program in order to calculate the retention index RI (as Kovàts index) of each compound. Identification of compounds was obtained by comparing the RI and the spectral data from the NIST'08 (National Institute of Standards and Technology, USA).

Table 1. Extraction parameters of subcritical processing of *Artemisia annua* L.

*Variants	Charge weight, g	Pressure, bar	Duration, min	Temperature, °C	Number of extractions
Variant 1	95	6 - 7	60	30	2
Variant 2	95	10 - 12	60	45	2

Spectrophotometric determination of artemisinin

The quantitative determination of artemisinin in the extract was performed according to the procedure developed in our laboratory (unpublished data) based on that described by Zhao and Zeng [20]. Briefly, 50 mg of the extract was dissolved in $CHCl_3$ and applied on a TLC plate (Silica gel 60 $F_{254,}$ glass plate 20 × 20 cm, Merck) on both ends of which the standard (artemisinin) was also added. The plate was developed using n-hexane-diethyl ether (1:1/v:v). After developing, the plate was dried and a small part of it was sprayed with H_2SO_4 in order to locate the artemisinin on the plate. Further, the TLC zone containing artemisinin was scrapped, quantitatively transferred to a small glass column and eluted with diethyl ether (50 mL). Further, the solvent was evaporated under reduced pressure. The artemisinin-enriched sample was dissolved in 10 mL of ethanol, transferred to a 50 mL volumetric flask and made up to the mark with 0.2% aqueous NaOH. The resulting solution was kept in a water bath (50ºC) for 30 min and then the absorbance was measured at 291 nm on a spectrophotometer. Different concentrations of artemisinin (0.2-1.0 mg/50 mL) at the same conditions were used for preparation of the calibration curve ($y = 1.2037x + 0.0454$, $R^2 = 0.9975$). The amount of artemisinin in the extract was calculated using the following equation:

$$\text{Artemisinin (\%)} = (C/P) \times 100,$$

where C was the amount of artemisinin (mg/50 mL), calculated from the calibration curve and P was the amount of the extract (mg) used for the analysis. The analysis was performed in triplicate. The value is presented as a mean ±SD.

RESULTS AND DISCUSSION

After extraction the exhausted raw material seems like before - with the same color and type. This is due to the gentle technology and the specificity of the solvent. The extracts themselves are a green mass with a brownish hue.

Despite the different pressure, the product yield is 2%. This indicates that extraction at lower pressure reaches the extraction limit of the extractable substances. Literature data for the extraction of essential oil vary from 0.30% to 0.66% [3, 18]. A non-polar solvent extracts the essential oil and an additional large number of substances. As regards the yield it is always more efficient than distillation.

The same solvent achieved a yield of 1.66% at a pressure of 10 bar and a duration of 24 h [15]. Supercritical extraction with CO_2 at a pressure of 30 MPa and 2 h realized a total product within 3.90 - 4.46% [6, 12]. According to these data our result was over the average values and indicated the effectiveness of the experiment.

Our results on the chemical composition can be considered as the first ones. The profile of the chromatogram (shown in Fig. 1) revealed its complex picture. The composition of the *A. annua* essential oil with the same origin is the best basis for a parallel with our product. The chemical structure of the subcritical product is shown in Table 2.

Twenty-eight components with concentrations higher than 0.1% were identified, which represents 85.48% of the total detected compounds. Among them, the most abundant class was that of oxygenated monoterpenes, followed by oxygenated sesquiterpenes, monoterpene hydrocarbons, phenylpropanoids and hydrocarbons. The oxygenated terpenoids were the main chemicals with 74% of the composition. Compared with data on Bulgarian wormwood essential oil from the same ecological region, it can be seen that the number of its compounds represents 60% of the whole number in the extract. The main groups in the structure of the essential oil were sesqiterpene hydrocarbones, followed by oxygenated monoterpenes and monoterpene hydrocarbons. Phenylpropanoids were noted by about 1%. The results can be explained by the nature of the solvent and the extraction method.

Artemisia ketone and artemisia alcohol are specific for the genotype and the data showed that their amounts were at average three times over in the extract. The quantity of the camphor in a group with eucalyptol and camphene was also triple in our product.

The main compound in the subcritical extract was artemisia ketone, while in the essential oil it was β-caryophyllene. According to Martinez-Correa *et al.* [8], the volatile fraction of the CO_2 extract consists mainly of camphor, so our product should be classified as different. This result can also be attributed to the different origins of wormwood. The advantage is emphasized in the non-polar low-molecular weight constituents. With increasing molecular weight and reducing volatility, sesquiterpenes exhibit greater amounts in the distillation product (after α-copaene). It is rather due to the relative proportion of ingredients in the two products. The extract contains the valuable germacrene D, arteannuin B, and arteannuic acid in sufficient quantity. This result is a consequence of the specificity of the solvent and emphasizes the advantage of the method.

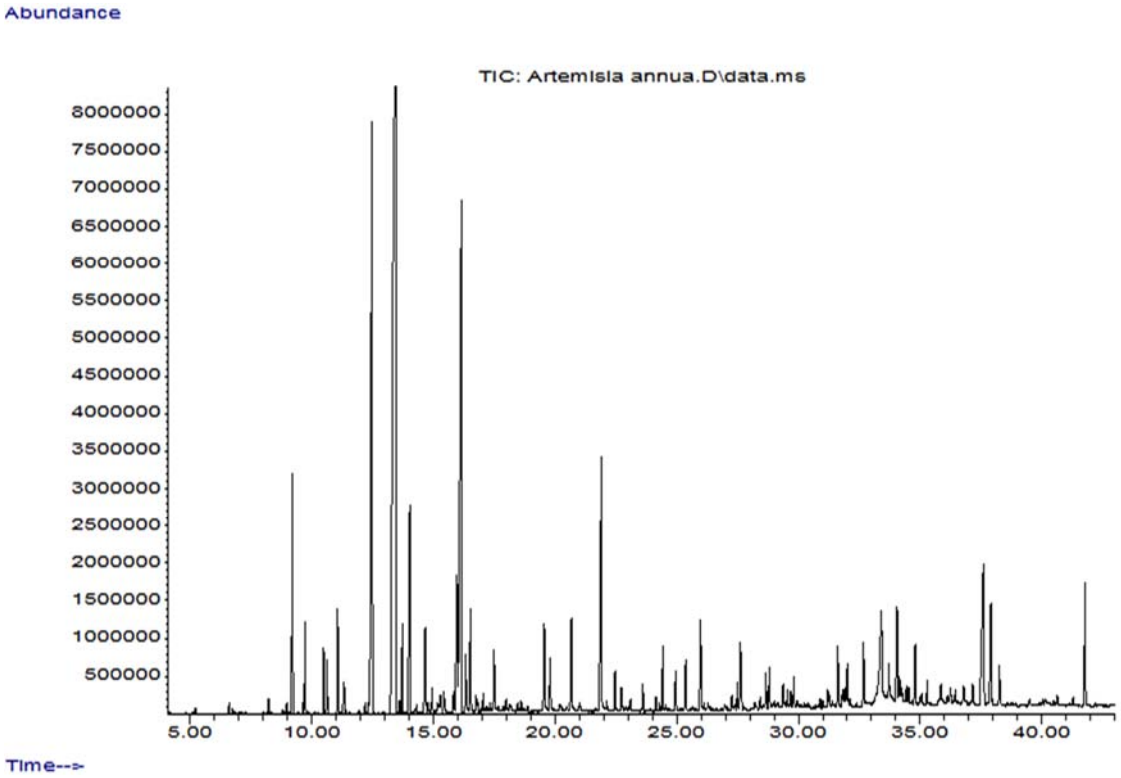

Figure 1. CG-MS profile of the subcritical extract from *A. annua*

Table 2. Chemical composition of the subcritical extract and essential oil from Bulgarian *Artemisia annua* L.

№	Component	Rel. %, as determined by GC/MS		
		KI	Subcritical Extract	Essential oil [1]
1	α-Pinene	939	2.52	1.26
2	Camphene	953	0.91	0.40
3	Sabinene	969	0.67	*
4	β-Pinene	974	0.52	0.32
5	β-Myrcene	991	1.06	*
6	(3E)-2,5,5-Trimethyl-3,6-heptandien-2-ol	1018	0.44	*
7	Eucalyptol	1027	9.06	2.55
8	Artemisia ketone	1051	26.15	8.45
9	Artemisia alcohol	1068	3.02	*
10	trans-Pinocarveol	1136	2.21	0.43
11	trans-Verbenol	1141	1.63	*
12	Camphor	1149	10.68	3.61
13	Pinocarvone	1154	1.29	0.73
14	Terpinen-4-ol	1177	0.86	<0.10
15	Eugenol	1358	4.33	<0.10
16	β-Elemene	1390	0.62	0.26
17	α-Copaene	1376	0.39	7.42

Table 2 (Continued)

№	Component	Rel. %, as determined by GC/MS		
		KI	Subcritical Extract	Essential oil [1]
18	β-Caryophyllene	1419	0.73	24.73
19	γ-Elemene	1433	0.21	0.26
20	α-Humulene	1454	0.37	3.86
21	Eugenyl acetate	1485	1.21	*
22	Germacrene D	1580	0.28	*
23	Caryophyllene oxide	1582	0.92	*
24	Arteannuin B	1692	3.69	*
25	n-Heptacosane	1700	1.41	*
26	n-Octacosane	1800	3.55	*
27	Arteannuic acid	1818	3.70	*
28	Lupenone	2856	3.08	*
Monoterpene hydrocarbons, %			6.67	3.50
Oxygenated monoterpenes, %			64.35	29.10
Sesqiterpene hydrocarbones, %			3.05	67.22
Oxygenated sesquiterpenes, %			9.75	*
Phenylpropanoids, %			6.54	<0.18
Hydrocarbons, %			5.84	*
Other, %			3.80	*
Total			85.48	54.48

Legend: KI - Kovats Index; * - not identified

The amount of artemisinin in the studied extract was determined spectrophotometrically using the known reaction of conversion of artemisinin into a UV-absorbing compound, Q292 (Fig. 2) by treating with 0.2% aqueous NaOH [20].

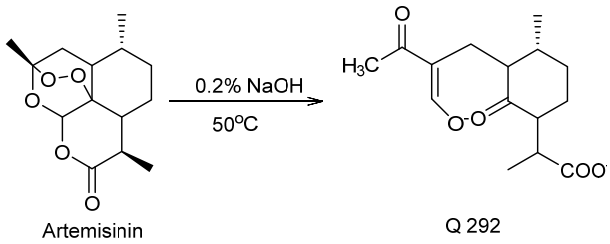

Figure 2. Conversion of artemisinin in Q292 derivative

Then it was subjected to alkaline hydrolysis and the absorbance of the hydrolyzed mixture was measured at 292 nm using a spectrophotometer. Pure artemisinin that underwent the same procedure was used to make a calibration curve. The content of artemisinin in the extract was found to be 1.28 ± 0.10%. With a method efficiency of 60% [17], we can assume with sufficient probability that the initial concentration in the plant is around 0.77%. The literature data for *A. annua* L. of different geographical origin indicated an artemisinin content of 0.33 to 0.97% [17]. The optimized analytical method of Kochler *et al*. [21] showed a maximum content of artemisinin of 0.96% in superctitical CO_2 extract. The high yield and selectivity of the solvent used by us in the intramolecular synthesis of the lactone is also proved in another study [22]. Our results correlate with these data and show a comparatively high potential for the availability and extraction of artemisinin in Bulgarian wormwood plants.

CONCLUSION

Subcritical extraction with 1,1,1,2-tetrafluoroethane of *Artemisia annua* L. from Bulgaria gave a yield of 2%. The chemical composition of the product consists mainly of oxygenated monoterpenes with the major compound artemisia ketone. The potential antimalarial constituents - artemisinin and its derivatives are proved to be in sustainable quantities.

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