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Chemical profile of *Artemisia annua* from the region of Sliven, Bulgaria. A preliminary NMR study

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

A preliminary study on the chemical profile of hexane extracts of the areal part of *Artemisia annua* from the region of Bulgarian town Sliven by NMR spectroscopy is performed. The presence of a number of main components is found using NMR spectra of authentic samples and comparison with literature data. Estimation of the artemisinin quantity is made.

Key words: *Artemisia annua*; Sliven region; artemisinin; sesquiterpenes; NMR

INTRODUCTION

Artemisia annua L. (Chinese sweet wormwood, family Asteraceae) is indigenous to China. The species is also found as native to Korea, Japan, Myanmar, Northern India, Vietnam, and Southern Siberia throughout Eastern Europe and is cultivated in many parts of the World as well, like equatorial Africa, Argentina, Europe and India [1]. *A. annua* L is used in traditional medicine for the treatment of malaria, cough, cold, and diarrhea. Whole flowering plant parts are also known to possess anthelminthic, antipyretic, antiseptic, carminative, antispasmodic, stimulant, tonic, and stomachic properties. World Health Organization has recommended *A. annua* as antimalarial drug [1].

A. annua has become the subject of intensive phytochemical evaluation following the discovery of the antimalarial drug artemisinin [1–3]. Phytochemical studies of *A. annua* have resulted in identification of more than 600 compounds including terpenoids (like sesquiterpene lactones), coumarins, phenolics, flavonoids, essential oils, etc. [2]. *A. annua* is the only known source of artemisinin (Qinghaosu). This sesquiterpene lactone is also effective in other infectious diseases such as schistosomiasis, HIV, hepatitis-B, leishmaniasis, and against a variety of cancer cell lines [1, 4–7].

Analysis of artemisinin is a challenging problem since the compound is present in low concentration in the plant; it is thermolabile, acid sensitive and unstable and lacks chromophoric groups. Various conventional and unconventional methods have been developed in order to detect and quantify artemisinin [8], including NMR spectroscopy [9]. The popularity of quantitative NMR (qNMR) has grown substantially over the past two decades, as it can provide absolute or relative quantification of multiple metabolites within a sample without prior separation of components [10, 11].

Literature survey for wild growing *A. annua* from Bulgaria revealed only few articles on essential oil composition [12–15] and surface flavonoid aglycones [16]. Surprisingly, the content of artemisinin in Bulgarian species has not been investigated so far. The aim of this study was to determine the amount of artemisinin in *A. annua* from the region of Sliven in Bulgaria as well as to identify its accompanying compounds.

EXPERIMENTAL

Materials and methods

All solvents were purchased from Aldrich and LabScan and were used without purification. Merck Silica gel 60 (0.040-0.063 mm) was used for flash chromatography fractionation of the total extract. Salophene, a calibration substance of Reichert (Vienna) Kofler block for determination of melting points, was found to be a suitable standard for NMR quantitation of artemisinin.

Plant material

Plant material was collected in full flowering stage from the region of Sliven town in 2015. The aerial parts were air-dried and kept in dark place.

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Extraction and Fractionation

Air-dried aerial parts (300 g) were extracted in hexane (1 L) at room temperature for 48 hrs. The solid phase was removed by filtration and the solvent was evaporated under vacuum at 25 °C to give the yellowish oily residue (305 mg from 200 ml extract; 0.51%; average from three independent experiments collecting 17-19 fractions each). The crude residue was fractionated by flash chromatography on silica gel (1:100) by using a mobile phase with a gradient of polarity from hexane through dichloromethane to 5% acetone in dichloromethane.

NMR spectra

The NMR spectra were recorded on a Bruker Avance II+ 600 spectrometer (Rheinstetten, Germany) using Topspin 3.5pl6 in $CDCl_3$ at 20 °C. The chemical shifts are quoted as δ-values in ppm against internal tetramethylsilane (TMS). Spectral width of 16 ppm on 32K points was acquired and processed with zero filling on 64K points. 64 scans were acquired for all individual fractions.

For estimation of the artemisinin quantity to a solution of 16 mg artemisinin containing fraction in 0.5 ml in $CDCl_3$ 1.6 mg salophene ($5x10^{-3}$ mmol) was added. In the proton spectrum of the mixture the integral of the artemisinin signal at 3.4 ppm amounted 0.62 as compared to the salophene azomethine protons at 10.5 ppm (equal to 2) providing estimation for the quantity of artemisinin as 0.88 mg ($3x10^{-3}$ mmol, 62% in respect to salophene).

RESULTS AND DISCUSSION

The areal part of *Artemisia annua*, collected in full flowering stage from the region of Bulgarian town Sliven, was extracted with hexane, fractionated by flash chromatography and analysed by NMR spectroscopy. The proton NMR spectrum of the total extract provides a complicated picture with a number of overlapped signals in many areas, which hampers a reliable components' assignment. The anti-malarial drug artemisinin was detected only in a single fraction eluted by 2% acetone in dichloromethane. In the ^{1}H NMR spectrum of the latter a characteristic doublet of quartets in an area clear of other signals at 3.4 ppm was found, while this area is quite overlapped in the total extract, analogously no free area for the other expected artemisinin signals was found [17]. A selective TOCSY experiment starting at the proton at 3.4 ppm proved that it does belong to artemisinin via the coincidence of the chemical shifts of several neighbouring protons (methyls at 1.21/1.00 ppm, methynes at 1.78/1.25 ppm and methylenes at 1.87/1.08 ppm and 1.78/1.07 ppm) with literature data (Fig. 1). The amount of artemisinin was roughly estimated by comparison with salophen as an internal standard (Fig. 2). The integral intensity of the signal at 3.4 ppm was determined as 0.62 to the two salophen imine protons, which represents 5.5 wt.% of the fraction, 0.29 wt.% of the total extract, and 0.0015 wt.% of the dry plant. The obtained results showed very low amount of artemisinin in *A. annua* from Bulgaria when compared with the data published in the literature,

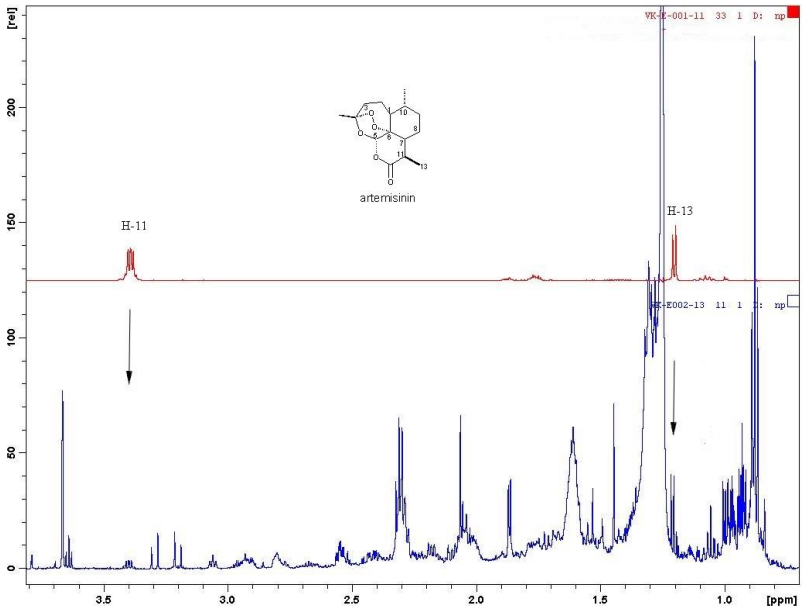

Fig. 1. Aliphatic part of ^{1}H (down) and selective ^{1}H-^{1}H TOCSY (up) spectra of artemisinin containing fraction.

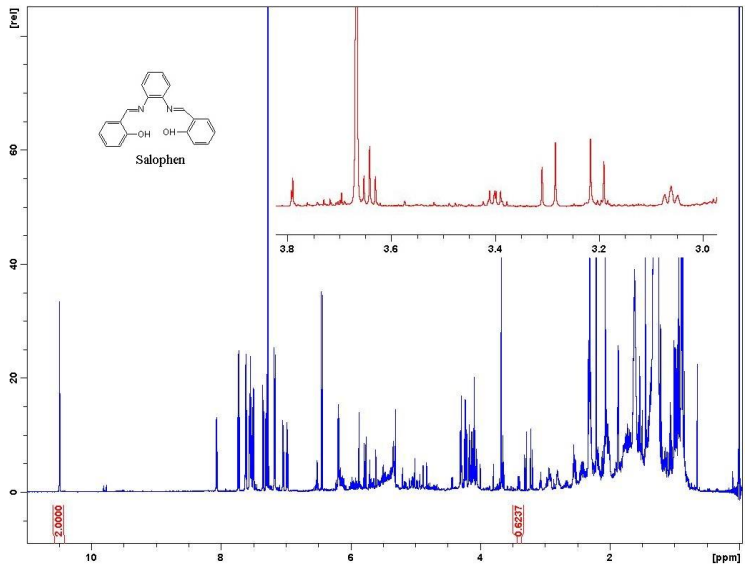

Fig. 2. ^{1}H NMR spectrum of artemisinin containing fraction with addition of salophene.

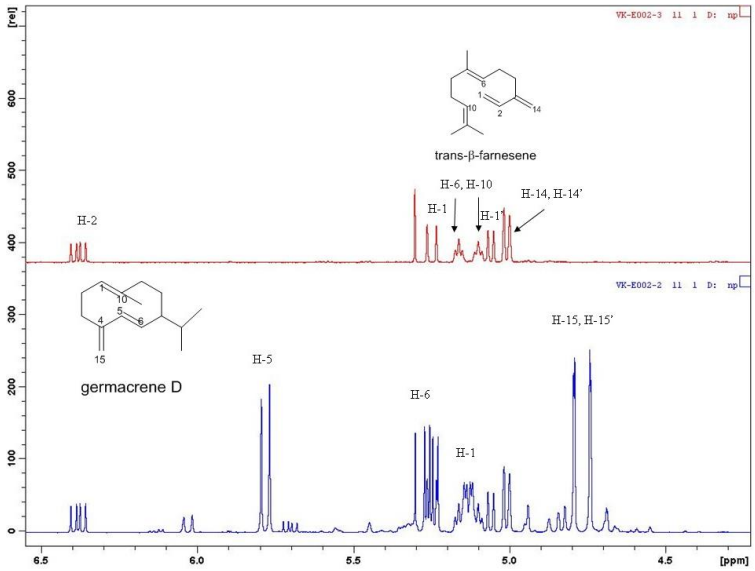

Fig. 4. Partial ^{1}H NMR spectra of *trans*-β-farnesene and germacrene D containing fraction (down) and *trans*-β-farnesene (up) [21].

indicating highly variable content of this lactone (0.01% – 1% depending on variety) [1, 2].

In addition to artemisinin, several other components were determined in the hexane extract (Fig. 3). Their structures were principally elucidated by comparison of their ^{1}H NMR spectra with those published in the literature.

Two sesquiterpene hydrocarbons were found in the less polar fractions, eluted by pure hexane. *Trans*-β-farnesene was identified by characteristic signals for a vinyl group (δ 6.36 dd, 5.24d, 5.06 d), exometylene double bond (δ 5.01 brs and 4.99 brs) and two olefinic protons at δ 5.17 and 5.10 (Fig. 4) [21]. The structure of the other compound, germacrene D was deduced from the characteristic signals for double bonds in 10-membered ring: trisubstituted C-1/C-10 (δ 5.13, H-1 and 1.52, H-14), *trans*-disubstituted C-5/C-6 (δ 5.78, d, H-5 and 5.26, dd, H-6, $J_{5,6}$ = 16 Hz), and exomethylene C-4/C-15 (δ 4.74 brs and 4.79 brs) [22].

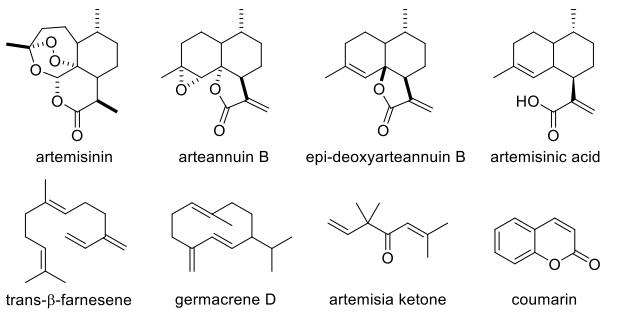

Fig. 3. Detected components in hexane extract of *Artemisia annua* from Sliven region.

The presence of monoterpene ketone artemisia ketone in a fraction eluted by dichloromethane /hexane 1:1 mixture was confirmed by comparison with the ^{1}H NMR spectrum of the authentic standard (Fig. 5). *Trans*-β-farnesene, germacrene D and artemisia ketone are typical volatile components of *A. annua* essential oils and their presence in hexane extracts is not surprising.

Three sesquiterpenes with cadinane skeleton, arteannuin B, *epi*-deoxyarteannuin B and artemisinic acid, were detected in fractions eluted with 2% acetone in dichloromethane. ^{1}H NMR spectra (Fig. 6) of arteannuin B and *epi*-deoxyarteannuin B contain signals characteristic for α-exomethylene-γ-lactones (H-13/13', δ 6.16/5.43 d and 6.17/5.56 d, respectively). Their spectra differ in the multiplicity and chemical shifts of some other signals. Thus, H-7 methyne at δ 2.74 in the spectrum of arteannuin B exhibits allylic coupling with H-13/13' (J = 3.1 Hz), which requires *trans*-fusion of the lactone ring, while the observed smaller allylic constants ($J_{7,13}$ =$J_{7,13'}$ =1.2 Hz) in the spectrum of *epi*-deoxyarteannuin B corresponds to *cis*-fusion of the lactone ring. The signals at δ 5.28 brs and 1.67 s (CH_3) confirmed the presence of a trisubstituted C-4/C-5 double bond in the structure of *epi*-deoxyarteannuin B. These signals are missing in the spectrum of arteannuin B. Instead, two new singlets appear at δ 2.68 and 1.34 (CH_3), indicating the presence of a methyl-substituted epoxy ring. All these data are in agreement with those reported previously [18, 19].

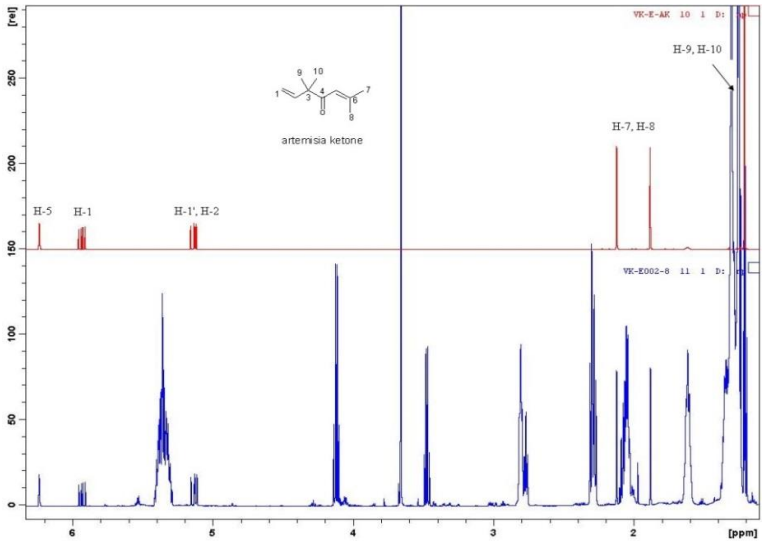

Fig. 5. ^{1}H NMR spectra of artemisia ketone containing fraction (down) and authentic sample of artemisia ketone (up).

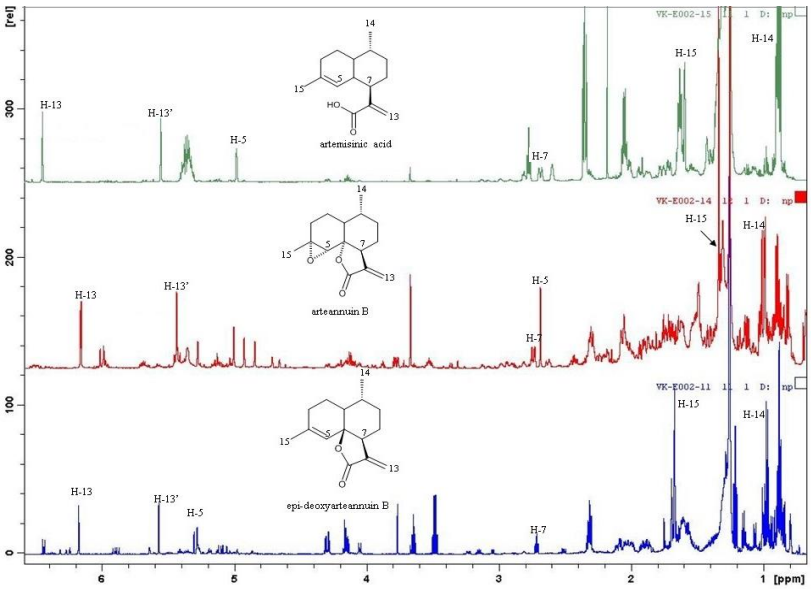

Fig. 6. ^{1}H NMR spectra of *epi*-deoxyarteannuin B (down), arteannuin B (middle), and artemisinic acid (up) containing fractions.

Table 1. Main constituents in the hexane extract of *Artemisia annua* from the region of Sliven, Bulgaria, detected by relevant 1D and 2D NMR spectra and literature comparison.

Component	Selected $^{1}H/^{13}C$ signals in ppm (multiplicity, coupling constants)
artemisinin	3.40 (dq, 5.2,7.2), 1.21 (d, 7.2)
arteannuin B	6.16 (d, 3.1), 5.43 (d, 3.1), 2.74 (dq, 12.4, 3.1), 2.68 (s), 1.34 (s), 0.99 (d, 6.0)
epi-deoxyarteannuin B	6.17 (d, 1.2), 5.57 (d, 1.2), 5.28 (brs), 2.71 (tt, 6.8, 1.2), 1.67 (s)
artemisinic acid	6.45 (brs), 5.55 (t, 1.5), 4.98 (s), 2.69 (dt, 12.3, 3.6), 2.60 (brs), 1.59 (brs),
trans-β-farnesene	6.38 (dd, 10.9, 17.6), 5.25 (d, 17.6), 5.16 (brt, 6.6), 5.10 (brt, 6.9), 5.06 (d, 10.9), 5.02 (brs), 5.00 (brs)
germacrene D	5.13 (m), 5.78 (d, 15.8), 5.25 (dd, 15.8, 10.0) , 4.74 (d, 2.3), 4.79 (d, 2.3), 1.51 (s), 0.86 (d, 6.7), 0.81(d, 6.8)
artemisia ketone	6.24 (quint, 1.3), 5.93 (dd, 17.5, 10.7), 5.15 (dd, 17.4, 0.9), 5.13 (dd, 10.6, 0.9), 2.12 (d, 1.3), 1.89 (d, 1.3), 1.22 (s)
coumarin	7.72 (d, 9.5), 7.54 (ddd, 8.4,7.4,7.2), 7.50 (dd, 7.7,1.5), 7.34 (d, 8.3), 7.29 (td, 7.5, 1.0), 6.43 (d, 9.5)
fatty acids and esters	4.12 (q, 7.1), 3.66 (s) 2.81/2.77 (t, 7.0), 2.35/2.30/2.28 (t, 7.5), 2.06 (m), 1.62 (m), 0.89 (t, 6.8), 0.88 (t, 7.1),
ω-3 fatty acids and esters	0.98 (t, 7.5), 2.08 (m), 2.81 (brt, 6.2)

The ^{1}H NMR spectrum of the biogenetic precursor of artemisinin, artemisinic acid (Fig. 6), is very similar to that of *epi*-deoxyarteannuin B. However, H-5 is shifted upfield (δ 4.98) and H-13 and H-13' - downfield (δ 6.44 and 5.55) suggesting the presence of an α,β-unsaturated acid instead of a lactone ring [20].

Finally, sizeable amounts of coumarin are easily identified in several fractions eluted by 2% acetone in dichloromethane by their characteristic signals in the aromatic area (δ 6.40 – 7.80) [22].

Main components in the hexane extract constitute a mixture of fatty acids (FA), methyl and ethyl esters of fatty acids. The NMR spectra show the presence of both saturated and differently unsaturated fatty acids, including approximately 25% of ω-3 polyunsaturated FA, determined from the ratio of the integral intensities of the methyl groups at 0.97 and 0.88 ppm.

The characteristic signals described above are listed on Table 1.

CONCLUSION

A preliminary study on the chemical profile of hexane extracts of the areal part of *Artemisia annua* from the region of Sliven town performed by NMR spectroscopy show the presence of several components: fatty acid and their methyl and ethyl esters, sesquiterpenes *trans*-β-farnesene, germacrene D, artemisinin, arteannuin B, *epi*-deoxyarteannuin B, and artemisinic acid, irregular terpene artemisia ketone, and the fragrant compound coumarin. The amount of artemisinin in the extract of the Bulgarian plant is estimated and is found to be very low in respect to its content in plants from other regions.

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ХИМИЧЕН ПРОФИЛ НА *ARTEMISIA ANNUA* ОТ РЕГИОНА НА ГР. СЛИВЕН, БЪЛГАРИЯ. ПРЕДВАРИТЕЛНО ЯМР ИЗСЛЕДВАНЕ

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(Резюме)

Проведено е предварително изследване на химичния профил на хексанов екстракт на надземните части на *Artemisia annua* от региона на град Сливен, България, посредством ЯМР спектроскопия. Идентифицирани са основните компоненти чрез сравнение с автентични проби и литературни данни. Направена е оценка на количеството на артемизинин в екстракта.