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PHENOLIC CONTENT OF *ARTEMISIA ANNUA* L. FROM NATURAL HABITATS IN REPUBLIC OF MOLDOVA

Camelia Paula ŞTEFANACHE[1*], Oana-Crina BUJOR[1], Radu NECULA[1,2], Doina DĂNILĂ[1], Nina CIOCÂRLAN[3], Veaceslav GHENDOV[3], Christoph CARLEN[4], Xavier SIMONNET[5]

Abstract: The aim of our study was to assess the phenolic compounds content and profile of *A. annua* samples harvested from natural habitats in R. Moldova. The samples, consisting in leaves, were harvested in August 2014 (before flowering) from different growing sites in north, centre and south regions. Phenolic extracts were obtained by methanol extraction of the residual plant material resulted from artemisinin separation. The phenolic compounds were identified and quantified by TLC and HPLC-DAD analyses, respectively. In all samples, four phenolic acids (caffeic, p-coumaric and chlorogenic acids, cynarin) and two flavonoids (isoquercitrin and luteolin-7-glucoside) were identified and quantified, cynarin being the major compound. The variations in phenolic composition between samples harvested from the same growing site and also for the samples from different growing areas (south, centre, north), were mostly quantitative. Similar phenolic profiles were obtained for all samples, regardless of the growing site. Phenolic acids were the dominant components in the phenolic extracts.

Keywords: *A. annua* leaves, flavonoids, phenolic acids, HPLC, TLC.

Introduction

Artemisia annua L. is an annual species 30–200 (–250) cm in height. It has a pioneer strategy characterized by a high degree of morphological and reproductive plasticity and massive seed production. This species is native in East Asia, most probably Inner Mongolia in China, where it is part of the grassland and steppe vegetation. *A. annua* has become widespread in temperate regions worldwide [TUTIN & al. 1976; VALLES & al. 2001]. In the flora of the Republic of Moldova *A. annua* is present in all regions of the country, but the distribution is very uneven. It regularly occurs in association with human settlements, ruderal habitats associated with transport infrastructure like roads and railways, rarely occurs in fields, as well as in semi-natural habitats [NEGRU, 2007; TZVELEV, 1994; VISJULINA, 1962].

A. annua was widely studied due to the biological effects of its extracts. The chemical composition of *A. annua* consists of volatile and non-volatile constituents. The volatile components are mainly represented by essential oils (0.23-0.97%) [MOHAMMADREZA, 2008]. The main non-volatile compounds include sesquiterpenoids, phenolic acids, flavonoids and coumarins, steroids [WHO Monograph on GACP for

[1] National Institute of Research and Development for Biological Sciences Bucharest / "Stejarul" Biological Research Centre, Alexandru cel Bun, 6, 610004, Piatra Neamt – Romania
[2] "Alexandru Ioan Cuza" University of Iasi, Faculty of Chemistry, Bd. Carol I 20A, 700505, Iasi – Romania
[3] Botanical Garden (Institute) of ASM, Padurii no. 18, MD-2002, Chisinau – Republic of Moldova
[4] Agroscope, Institute for Plant Production Sciences, 1964, Conthey – Switzerland
[5] Mediplant, Swiss Research Centre in Medicinal and Aromatic Plants, 1964, Conthey – Switzerland
[*] Corresponding author. E-mail: camelia.stefanache@yahoo.com

Artemisia annua, 2006]. *A. annua* is the main source of artemisinin, a sesquiterpene lactone used for the treatment of *falciparum* type malaria in many countries [SUBERU & al. 2013]. In addition, phenolic compounds are an important group of bioactive molecules found in high amounts in *A. annua* plants.

According to the literature, the most representative phenolic compounds in *A. annua* are flavones and their glycosides (luteolin, luteolin-7-glucoside, apigenin), flavonols and their glycosides (kaempferol, quercetin, isoquercitrin, rutin, patuletin), coumarins (coumarin, 6,7-dimethoxy-coumarin) and phenolic acids (ferulic acid) [CAI & AL. 2004, IVANESCU & al. 2010].

The phenolic extracts of *A. annua* showed antioxidant and antitumor activity [IQBAL & al. 2012; ZHU & al. 2013]. It is well known that the radical scavenging capacities of plant extracts is correlated with the phenolic content [FERREIRA & al. 2010; SYTAR & al. 2016].

The antioxidant properties of *A. annua* phenolic extracts were reported in several *in vitro* tests such as ABTS, ORAC, ferric reducing antioxidant power and lipid peroxidation in emulsion model and also *in vivo* mouse models [SKOWYRA & al. 2014; KIM & al. 2014].

Regarding the role of phenolic compounds in the plant, it was demonstrated that they are important molecules in plant stress responses, thus having an adaptive role for environmental parameters such as altitude, temperature, evapotranspiration [BAUTISTA & al. 2016]. Flavonoids are involved in plants interactions with other organisms and their response to the environmental stress, mainly due to their strong antioxidant properties [MIERZIAK & al. 2014].

Thus, the synthesis and accumulation of phenolic compounds is influenced by the environmental factors (biotic and abiotic), which are characteristic to each growing site.

The study aimed at assessing the phenolic compounds content and composition for *A. annua* samples harvested from natural habitats in R. Moldova, in order to identify high yielding plants to be used in breeding programs.

Material and method

Plant material

The plant samples consisted in *A. annua* leaves harvested before flowering stage from several habitats in the southern, central and northern regions of the Republic of Moldova in August 2014. We harvested the samples at this plant development stage, when artemisinin content is higher, since our main goal was to isolate the phenolic compounds from the residual plant material resulted from artemisinin extraction.

For each growing site, a habitat assessment was performed, and the plant associations were described (Tab. 1). Description of the associations was done according to the phytosociological research method of the central European school, based on the traditional ecological-floristic systems developed by TÜXEN (1955) and J. BRAUN-BLANQUET (1964). Voucher specimens of identified species are deposited in the herbarium of the Botanical Garden (Institute) of ASM.

R. Moldova has a temperate-continental climate. The average yearly air temperature is 8-10 °C. The average annual amount of precipitation goes down from 620 mm at the north-west to 490 mm at the south-east (Ministry of Ecology, Constructions and Territorial Development of the Republic of Moldova, National Institute of Ecology. Republic of Moldova – State of the Environment Report 2002).

Chemicals and reagents

Methanol (for analysis and HPLC grade), acetonitril, dichloromethane, formic acid and 2-aminoethyldiphenyl borate (NP reagent) were from Merck (Darmstadt, Germany), ethyl acetate was from SC Chimreactiv SRL (Romania), Kollisolv PEG E 400 (Macrogol 400), quercetin, rutin and cynarin were from Sigma Aldrich (Steinheim, Germany), caffeic acid, p-coumaric acid, isoqercitrin and luteolin-7-glucoside were from Roth (Karlsruhe, Germany), hyperoside and chlorogenic acid were from Hwi Analytik GmbH (Ruelzheim, Germany).

Phytochemical analysis

Extract preparation

The dried and milled plant material was extracted with chloroform in order to isolate the sesquiterpene lactone fraction (especially artemisinin). Afterwards, the residual plant material was extracted 3 times at 40 °C in the ultrasonic bath (40 KHz) with methanol 100% for the isolation of phenolic compounds (phenolic acids and flavonoids).

The extracts were vacuum dried using a rotary evaporator, and stored at -20°C until analyzed. The extraction yields for phenolic compounds were calculated for each sample and the data is presented in Fig. 1.

Thin Layer Chromatography (TLC) analysis

For the TLC analysis the dried extract was re-dissolved in methanol, at a concentration of 35 mg dry extract/ mL. *Stationary phase*: HPTLC 20x10cm, silica gel 60 F_{254}, plates (Merck); *mobile phase*: ethyl acetate/formic acid/water (80/10/10, v/v/v); *development distance:* 8 cm; derivatization: NP solution (10 g/L, in ethylacetate) and PEG solution (Macrogol 400, 50 g/L, in dichloromethane); *visualization:* 366 nm.

High Performance Liquid Chromatography (HPLC) analysis

For the HPLC analysis, the dried extract was re-dissolved in methanol, at a concentration of 3.5 mg dry extract/mL.

The phytochemical analysis was performed using an Agilent 1200 HPLC system coupled with a DAD G1315D detector, G1311A quaternary Pump, G1329A autosampler and G1322A degasser.

The chromatographic conditions where: Nucleodur C18 Isis (250 x 4.6 mm, 5 µm) column; mobile phase water adjusted to pH 2.5 with phosphoric acid (A) and acetonitrile (B); elution gradient 2-14-20-26-43% B for 0-20-40-46-58 min. after which we switched back to the initial conditions for 10 min; flow 1 mL/min. Detection was performed at 320 nm for phenolic acids and 350 nm for flavonoids. The phenolic compounds were identified and quantified according to their UV-VIS spectra and available standards. The quantitative results were expressed as mg/100 g dry plant material (d.w.).

Results and discussion

Habitat assessment

In R. Moldova *Artemisia annua* is tending to populate some anthropogenic habitats with a high number of segetal and ruderalised vascular plants, such as: *Amaranthus deflexus* L., *Anagallis arvensis* L., *Anagallis foemina* Mill., *Anchusa pseudoochroleuca* Shost., *Atriplex tatarica* L., *Atriplex oblongifolia* Waldst. et Kit., *Ballota nigra* L., *Berteroa incana* (L.) DC., *Bidens tripartita* L., *Brachyactis ciliata* (Ledeb.) Ledeb., *Capsella bursa-pastoris* (L.) Medik., *Cuscuta campestris* Yunck, *Cyclachaena xanthiifolia* (Nutt.) Fresen., *Daucus carota* L., *Descurainia sophia* (L.) Webb ex Prantl, *Diplotaxis muralis* (L.) DC. etc., forming

sometimes pure vegetal associations (*Artemisietum annuae* Fijalkowski 1967) (Photo 1) where species becomes mono-dominant in some ruderal phytocoenoses or being a part of floristic component of other phytocoenoses: *Galinsogo-Euphorbietum pepli* Mititelu 1972, *Portulacetum oleracei* Felföldy 1942, *Portulacetum oleracei-Amaranthetosum deflexi* (Grigore 1968) Sanda et al. 2001, *Capsello-Descurainietum sophiae* Mucina 1993 (Photo 2), *Hordeetum murini* Libbert 1939, *Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó (Photo 3), etc.

The description of the samples and their harvest site is presented in Tab. 1.

Photo 1. *Artemisietum annuae* Fijalkowski 1967 pure vegetal association, Rascaieti, Stefan Voda district

Photo 2. *Capsello-Descurainietum sophiae* Mucina 1993 vegetal association (antropogenic habitat), Naslavcea, Ocnita district

Photo 3. *Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó vegetal association, Cosauti, Soroca district

Tab. 1. Description of *A. annua* samples

Samples	Growing site/district		Plant association
1-3	South	Ciumai, Taraclia	*Portulacetum oleracei-Amaranthetosum deflexi* (Grigore 1968) Sanda et al. 2001
4-6		Rascaieti, Stefan Voda	*Artemisietum annuae* Fijalkowski 1967
7-9		Colibasi, Cahul	*Galinsogo-Euphorbietum pepli* Mititelu 1972
10-12	Centre	Bacioi, Chisinau	*Portulacetum oleracei* Felföldy 1942,
13-15		Trebujeni, Orhei	*Artemisietum annuae* Fijalkowski 1967
16-18	North	Naslavcea, Ocnita	*Capsello-Descurainietum sophiae* Mucina 1993
19-21		Cosauti, Soroca	*Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó
22-24		Branzeni, Edinet	*Hordeetum murini* Libbert 1939

Phenolic compounds assessment

Extraction of phenolic compounds was made using 100% methanol under ultrasound assisted extraction. The extraction yield for was calculated for each sample and the data is presented in Fig. 1. It varied from 10.22% (sample 6) to 13.57% (sample 10), with lowest average values for the samples harvested from Rascaieti (Stefan Voda district) and Trebujeni (Orhei district) and the highest average values for the samples harvested from Bacioi (Chisinau).

Identification of phenolic compounds was performed by two chromatographic methods, namely TLC and HPLC.

The TLC fingerprint (Fig. 2) showed the presence of the following phenolic compounds: rutin (Rf=0.26), chlorogenic acid (Rf=0.46), hyperoside (Rf=0.50), luteolin-7-glucoside (Rf=0.54) and cynarin (Rf=0.86). In addition, two other flavonoids (green spot with Rf=0.12 and orange spot with Rf=0.18) and three phenolic acids were separated (blue spots with Rf=0.30; 0.58; 0.68).

By HPLC analysis, in all samples 25 phenolic acids and derivatives were separated (Fig. 3a), four of which being identified and quantified: caffeic acid in amounts of 1.70 – 4.31mg/100 g d.w., *p*-coumaric acid 0.50 – 4.35 mg/100 g d.w., chlorogenic acid 112.64 – 210.48 mg/100 g d.w. and cynarin 307.13 to 617.72 mg/100 g d.w. (Fig. 4).

Furthermore, five flavonoids were separated, among which isoquercitrin and luteolin-7-O-glucoside (Fig. 3b). Isoquercitrin was found in amounts of 5.24 – 30.33 mg/100 g d.w., while luteolin-7-glucoside content was 9.80 – 40.47 mg/100 g d.w. (Fig. 5).

Cynarin was the major phenolic compound in all *A. annua* samples, but in literature there are few reports on it regarding only its presence in *A. annua* species, with no quantitative data [ZAO & al. 2014; ZAO & al. 2015].

Phenolic compounds such as chlorogenic acid, *p*-coumaric acid, cynarin, caffeic acid, hyperoside, isoquercitrin, rutin and luteolin-7-glucoside were identified in previous studies on *A. annua* aerial parts and leaves [CAI & al. 2004; IVANESCU & al. 2010; ZHAO & al. 2015].

In contrast with our study, showing phenolic acids as dominant constituents, in the study of SONG & al. (2016) on fresh leaves harvested at flowering stage, the flavonoid composition was more diverse and the flavonoid content was higher. This difference in phenolic profile can be attributed to the plant development stage of *A. annua*, but further studies are needed to confirm this hypothesis.

The values of the phenolic compounds content for the samples harvested from the north region had a narrower variability range, compared with the samples from the centre

and south. The samples from north were characterized by a higher content of flavonoids. Interestingly, high variations of phenolic compounds content were obtained for the samples harvested from the south and centre regions (Fig. 4 and Fig. 5).

The lowest phenolic compounds contents were determined for the samples harvested from Rascaieti (Stefan Voda district) and Trebujeni (Orhei district) growing sites which are characterised by *Artemisietum annuae* Fijalkowski 1967 plant association.

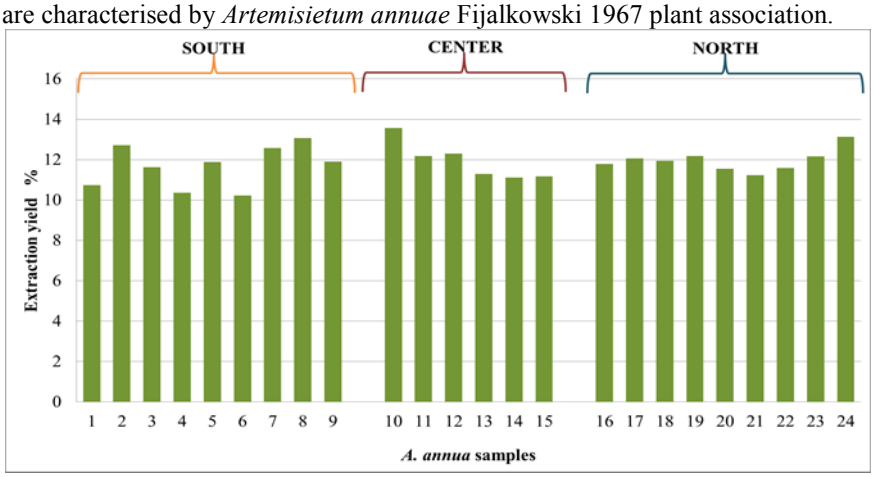

Fig. 1. Extraction yields for methanolic extracts of *A. annua* samples (% dry weight)

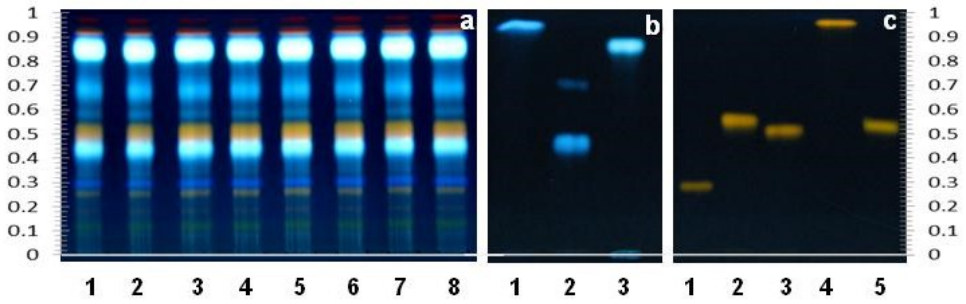

Fig. 2. TLC chromatogram for phenolic acids and flavonoids of *A. annua* samples.
a. *A. annua* samples 1 to 8; **b.** Standards for phenolic acids: 1) caffeic acid, 2) chlorogenic acid, 3) cynarin; **c:** Standards for flavonoids: 1) rutin; 2) isoquercitrin; 3) hyperoside; 4) quercetin; 5) luteolin-7-glucoside

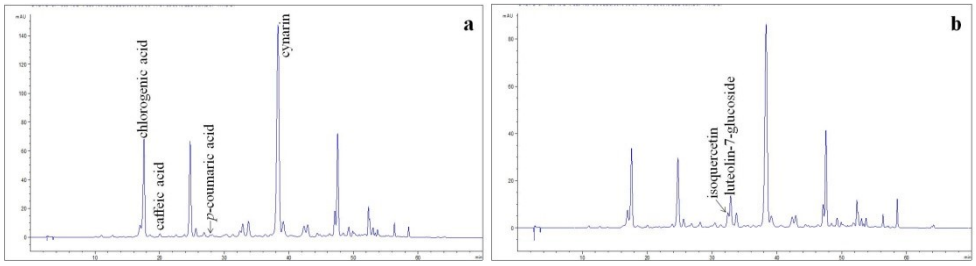

Fig. 3. Chromatographic profile of *A. annua* (sample 1). a. phenolic acids at 320 nm. b. flavonoids at 350 nm

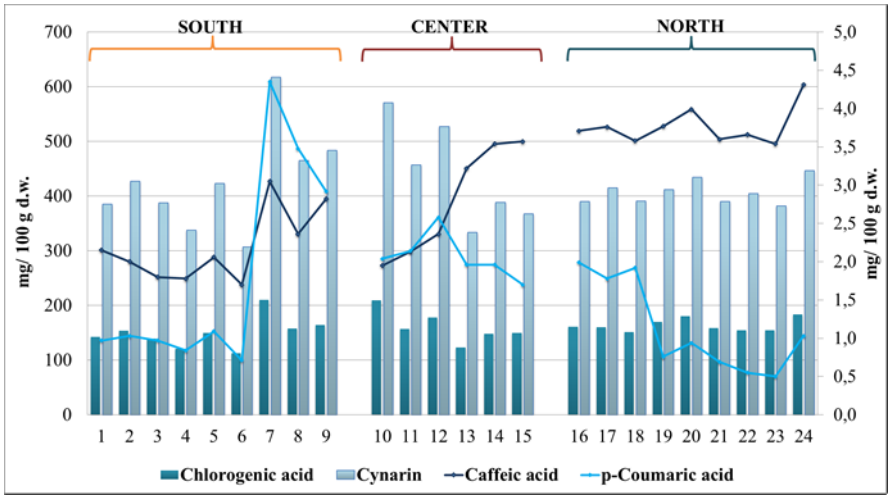

Fig. 4. Phenolic acids conent in *A. annua* samples

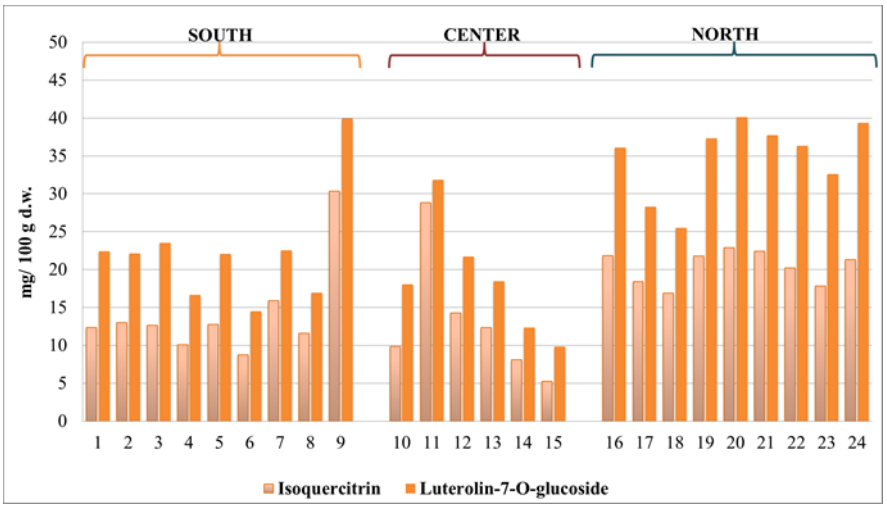

Fig. 5. Flavonoids content in *A. annua* samples

Conclusions

To our knowledge, this study presents the first data on phenolic profile of *A. annua* leaves harvested before flowering. Furthermore, it is the first time when quantitative data on cynarin in *A. annua* were obtained.

The variation on phenolic compound composition between samples, for the samples harvested from the same growing site and also for the samples from different growing areas (south, centre, north), were mostly quantitative. Similar phenolic profile was obtained for all samples, regardless of the growing site.

Phenolic acids were dominant components in the phenolic extracts, both qualitatively and quantitatively.

Several accessions had high amount of phenolic compounds, being promising candidates for breeding programs.

Considering the high amounts of phenolic compounds in our samples, it is feasible to use the residual plant material resulted from artemisinin extraction as a source of phenolic compounds, and thus achieving also the sustainable exploration of raw materials.

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