HPLC ANALYSIS OF PHENOLIC COMPOUNDS FROM ARTEMISIA SPECIES

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The aim of this study was the identification and quantification of biologically active compounds from three *Artemisia* species, used as remedies in Romanian traditional medicine. A new LC-MS method was developed for the analysis of methoxylated flavones and also the quantification of caffeic and chlorogenic acids was performed. The antioxidant activity of plant extracts was evaluated by DPPH radical scavenging assay. We report for the first time the presence of eupatorin and hispidulin in *A. absinthium* and *A. vulgaris*, of eupatilin in *A. annua* and casticin in *A. vulgaris*. Jaceosidin and acacetin were not identified in any sample. Although all extracts showed antiradical scavenging activity, *A. vulgaris* exhibited the strongest antioxidant effect. Chlorogenic acid was found in high amounts in all species, notably in *A. annua* and *A. vulgaris*.

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Introduction

Polyphenolic compounds constitute one of the largest, most widely spread and functionally important groups of secondary plant metabolites. In recent years, these substances, and flavonoids in particular, have attracted great interest due to their antioxidant capacity which confers them a valuable therapeutic potential in treating free-radical mediated diseases. Plant polyphenols are serious candidates in explanations of the protective effects of plants against cancer and cardiovascular maladies.

Although medicinal plants stood the test of time as traditional remedies, their phytochemistry is only partially known. The species selected for this study have long been used in Romania as herbal medicines, but also throughout the world and are included in the pharmacopoeias of different countries.[1] *Artemisia annua* L., *Artemisia absinthium* L. and *Artemisia vulgaris* L. (*Asteraceae* family) are largely spread in nature and employed in the treatment of various conditions, such as hepatitis, inflammation, bacterial and fungal infections, cancer, malaria, helminthiasis and other parasitic infections.[2] *A. annua* became known globally after the identification and isolation of the antimalarial compound artemisinin and today is investigated for its anticancer compounds.[3]

Many of the above mentioned pharmacological activities are due to the phenolic compounds present in the plants and for this reason, the present study focuses on them. This research continues the work from a previously published study that examined the occurrence of 18 phenolic compounds in the same *Artemisia* species.[4] The present paper is centred on the analysis of six methoxylated flavones, valuable bioactive compounds with numerous therapeutic properties, mainly antitumor, anti-inflammatory, antioxidant, antimicrobial and anti-ulcer.[4-7] For this purpose, a new LC-MS method was developed and applied to the plant extracts in order to identify and quantify the methoxylated flavones in the tested species. Because in the previous study, caffeic and chlorogenic acids were only identified, but not quantified, another analysis was performed for the quantitative determination of these compounds.

The antioxidant activity of plant extracts was also investigated by DPPH radical scavenging assay and correlated with the total phenol content and total flavonoid content.

Experimental

Plant material and extraction procedure

The aerial parts of *A. annua*, *A. absinthium* and *A. vulgaris* were harvested at the flowering stage from the countryside around Iasi, Romania, in August-September 2014. The species were authenticated and a voucher specimen of each was deposited in the Herbarium of Pharmaceutical Botany Department from the Faculty of Pharmacy. The plants were air-dried at room temperature and grounded to a fine powder. 10 g of plant material was extracted three times with 200 mL methanol for 1 hour, at room temperature, using a magnetic stirrer. The extract was appropriately diluted before injection in HPLC.

Chromatographic conditions for the analysis of methoxylated flavones

Methoxylated flavonoid aglycones were quantified through high-performance liquid chromatography coupled with mass spectrometry (LC-MS), using six standards: jaceosidin, eupatilin (ALB Technology, China), casticin, acacetin, eupatorin, hispidulin (Sigma, Germany).

The separation of the methoxylated flavones was achieved using a Zorbax SB-C18 reversed-phase analytical column (100 × 3.0 mm i.d., 5 μm particle) fitted with a guard column Zorbax SB-C18, both operated at 48 °C. The mobile phase consisted of 0.1 % (v/v) acetic acid and methanol with the following gradient: beginning with 45 % methanol and ending at 50 % methanol, for 8 minutes with a flow rate of 0.9 mL min^{-1} and an injection volume of 5 μL. For the MS analysis the following optimized conditions were used: electrospray ionization (ESI) interface operating in negative mode, gas (nitrogen) temperature 325 °C at a flow rate of 12 L min^{-1}, nebulizer pressure 60 psi and capillary voltage -2500 V. The full identification of compounds was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions. The MS was operated in the multiple reactions monitoring analysis (MRM) mode instead of single ion monitoring.

Chromatographic conditions for the analysis of caffeic and chlorogenic acids

The two hydroxycynnamic acids (caffeic acid and chlorogenic acid from Sigma, Germany) were separated using a Zorbax SB-C18 reversed-phase analytical column (100 × 3.0 mm i.d., 3 μm particles) fitted with a guard column Zorbax SB-C18, both operated at 42 °C. The separation was achieved under isocratic conditions using a mobile phase consisting of 0.1 % acetic acid and acetonitrile (v/v). The flow rate was 0.8 mL min^{-1} and the injection volume was 5 μL. Mass spectrometry analysis was performed on an Agilent Ion Trap 1100 VL mass spectrometer with electrospray ionization (ESI) interface in negative mode. Operating conditions were optimized in order to achieve maximum sensitivity values: gas (nitrogen) temperature 60 °C at a flow rate of 12 L min^{-1}, nebulizer pressure 60 psi and capillary voltage - 3500 V. The full identification of compounds was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions. To avoid or limit the interference from background, the multiple reactions monitoring analysis mode was used instead of single ion monitoring (e.g., MS/MS instead of MS).

Determination of total flavonoid and total polyphenols content

The concentration of total phenols in plant extracts was estimated by Folin-Ciocalteu procedure.[8] 2 mL of the diluted samples were mixed with 10 mL of a Folin-Ciocalteu reagent dilution (1:10). After a period of time ranging between 30 seconds to 8 minutes, the Na_2CO_3 solution was added. The absorbance was determined after 2 h at 20 ºC, versus a zero-absorbance reagent blank. The blue color produced was measured at 760 nm using an Able Jasco V-550 UV-VIS spectrophotometer. The concentration of total phenolic compounds in extracts was calculated by comparison with a standard curve similarly prepared with 0 to 500 μg gallic acid 100 mL^{-1}. Total phenolic content values were determined using an equation that was obtained from the calibration curve of gallic acid graph (R^2=0.994). The total phenolic content of the sample was expressed as gallic acid equivalents which reflected the phenolic content as the amount of gallic acid (mg) in 1 g of dry material.

The flavonoids content in extracts was estimated by the spectrophotometric method described in Romanian Pharmacopoeia based on the reaction with aluminium chloride solution.[9] The 5 mL sample solution was placed in a 25 mL volumetric flask. Next, we added 5 mL solution of 100 g L^{-1} sodium acetate, followed by 3 mL of 25 g L^{-1} aluminium chloride solution in ethanol and the volume was made up with ethanol. After 15 minutes, the absorbance was determined at 350 nm versus a reagent blank. The flavonoids content in extracts was calculated by using quercetin (20-160 μg mL^{-1}) as a standard. The total flavonoids content values was determined using an equation obtained from the calibration curve of the quercetin graph (R^2 = 0.998). Results were expressed as mg equivalent quercetin per gram dry weight.

DPPH radical-scavenging activity

The free radical scavenging activity of the methanol extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method.[8] For this, the crude methanol extract was evaporated to dryness and dissolved in DMSO at different concentrations ranging from 0.3 to 10 mg mL^{-1}. 0.05 mL from each solution were added to 2.95 mL DPPH solution in methanol (A_{517nm} = 1.00 ± 0.05); the mixture was energetically shaken. After 5 minutes, the absorbance of the mixture was measured at λ = 517 nm. The percent DPPH scavenging ability (φ) was calculated using the formula:

$$\varphi = 100 \frac{A_{control} - A_{sample}}{A_{control}}$$

where

$A_{control}$ is the absorbance of methanol solution of DPPH radical (containing all reagents except the sample) and

A_{sample} is the absorbance of DPPH radical + sample extract, measured 5 minutes after the addition of extract.

Afterwards, a curve of % DPPH scavenging capacity *versus* concentration was plotted and EC_{50} values were calculated. EC_{50} denotes the concentration of sample required to scavenge 50% of DPPH free radicals. Quercetin was used as a positive control. All measurements were carried out in triplicate and results were expressed as mean value ± standard deviation.

Results and Discussion

The analysis of methoxylated flavones

Considering the fact that methoxylated flavones are bioactive compounds widely distributed in *Artemisia* genus, found generally as aglycones in the epicuticular wax, their determination is of interest from the point of view of utility of a medicinal plant. Thus, a new LC-MS method was developed in order to assess the presence of 6 methoxylated flavones in the plant extract i.e., jaceosidin, hispidulin, eupatilin, eupatorin, casticin and acacetin. The analytes eluted in less than 10 minutes in the chosen chromatographic conditions, as shown in Figure 1.

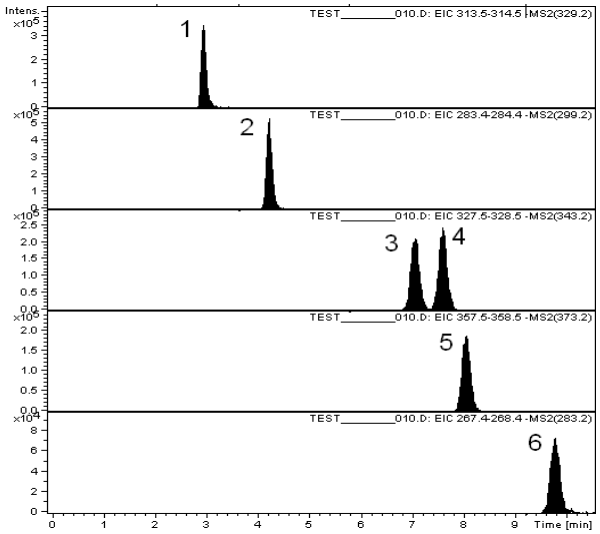

Figure 1. MS chromatograms of analyzed flavones: jaceosidin (1), hispidulin (2), eupalitin (3), eupatorin (4), casticin (5) and acacetin (6).

In the process of MS analysis, the pseudo-molecular ions of the flavones (329.3 for jaceosidin, 299.2 for hispidulin, 343.3 for eupatilin, 343.3 for eupatorin, 373.3 for casticin and 283.3 for acacetin) have been fragmented, and based on their daughter ions from the MS spectrum (Table 1) the extracted chromatograms of each compound were constructed for quantification. The calibration curves of methoxylated flavones were built and they all showed a linear correlation coefficient and a satisfactory level of precision and accuracy.

Table 1. Characteristic ions of standard flavones in full scan and specific ions used in quantification

Compound	R_T, min	M	$[M-H]^-$	Monitored ions/fragments
Jaceosidin	2.9	330.3	329.3	314
Hispidulin	4.2	300.2	299.2	284
Eupalitin	7.05	344.3	343.3	328
Eupatorin	7.6	344.3	343.3	328
Casticin	8.05	374.3	373.3	358
Acacetin	9.8	284.3	283.3	268

Table 2 shows the levels of methoxylated flavones found in the three medicinal plants analyzed.

It can be observed that all species contain casticin, hispidulin and eupatorin and lack jaceosidin and acacetin, although the two latter compounds are present in other *Artemisia* species.[10,11] Eupatilin is present only in *A. annua* in small amounts, while casticin is found in high concentrations in all three samples, most notably in *A. annua*.

Casticin is an active compound of *A. annua* that potentiate the antimalarial activity of artemisinin and exhibits modest antimicrobial activity against *Clostridium perfringens*.[12,13] Casticin also manifest antitumor activity against a large spectrum of cancer cell lines as well as anti-inflammatory activity.[14,15] A previous study lists casticin as one of three major components of *A. absinthium* plants cultivated in Spain, where it reaches a concentration (131 mg 1000 g^{-1} dw) similar to that found in our study.[16]

Hispidulin is a pharmacologically active flavone with anticancer and antiepileptic properties, while eupatorin has a broad spectrum of anticancer activity.[3,17] Eupatilin has potent anti-ulcer effect, strong anti-inflammatory and anti-oxidative activity and it is marketed in South Korea for the treatment of gastric inflammation in the form of standardized extract DA-9601.[18]

Flavones eupatorin and hispidulin were identified for the first time in *A. absinthium* and *A. vulgaris*. Also, the presence of eupatilin in *A. annua* and casticin in *A. vulgaris* is first reported in this study. Acacetin and jaceosidin were not identified in any of the three species, even though acacetin is mentioned in literature as a component of *A. annua*.[3]

Methoxylated flavonoids are promising therapeutic candidates due to their lipophilic nature and increased metabolic stability, that results in high oral bioavailability compared to other polyphenols.[19]

Caffeic and chlorogenic acids analysis

In the previous polyphenols analysis,[4] caffeic and chlorogenic acids - both powerful antioxidants, could not be quantified due to co-elution, so a new LC-MS method was used for their determination in plant extract.

In the aforementioned chromatographic conditions, the retention time of the chlorogenic was 2.2 minutes and of caffeic acid 3.3 minutes, as shown in Figure 2. Because in the ionization conditions both acids lose a proton, the ions monitored by the mass spectrometer are always in the form $[M-H]^-$, so the ions recorded have m/z = 353 for chlorogenic acid and m/z = 179 for caffeic acid. Further, in order to increase the selectivity and sensitivity of the method, for each compound, a second ion was monitored from the MS/MS spectrum: m/z 191 for chlorogenic acid and m/z 135 for caffeic acid.

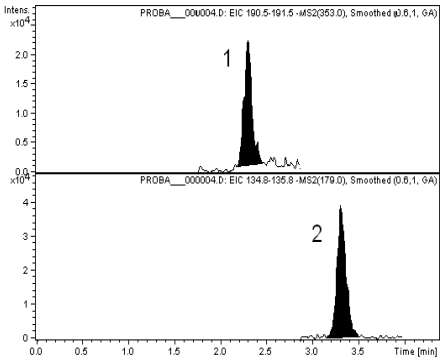

Figure 2. MS chromatograms of chlorogenic (1) and caffeic (2) acids

The ions with m/z = 191 and m/z = 135 were further used for the quantitative determination of these compounds, seeing that the intensity of ions in the mass spectrum is proportional to the concentration of the substance in the sample.

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Table 2. Concentration of methoxylated flavones in plants

Plant	Concentration (μg g^{-1} dry weight plant material)			
	Eupatorin	**Eupatilin**	**Casticin**	**Hispidulin**
A. annua	21.86	2.98	1587.45	23.73
A. vulgaris	0.97	0.00	124.51	1.47
A. absinthium	36.24	0.00	199.10	15.01

Acacetin and jaceosidin were not found in any sample.

Table 3 presents the levels of caffeic and chlorogenic acids in the analyzed species. It can be noticed that chlorogenic acids predominates in all species, the highest concentrations of both the hydroxycinnamic acids were found in *A. annua* and *A. vulgaris* in comparable amounts.

Table 3. Concentration of caffeic and chlorogenic acids in plants

Plant	Concentration, μg g^{-1} dw plant material	
	Caffeic acid	**Chlorogenic acid**
A. annua	50.20	4658.60
A. vulgaris	23.20	3984.20
A. absinthium	11.80	1658.00

Another study found in Romanian *A. absinthium*, comparable amounts of caffeic acid (0.181 mg g^{-1} dry extract), but much lower levels of chlorogenic acid (0.077 mg g^{-1} dry extract) in the ethanol 70 % extracts of aerial parts.[20] The difference may arise from the different method of analysis and type of extract.

The methanol extract of *A. vulgaris* leaves collected from Serbia contains (0.44 mg g^{-1} dw) chlorogenic acid, almost ten times lower than our result, probably due to the difference in the analysis method.[21] An aqueous extract obtained from the aerial parts of *A. annua* cultivated in Italy was reported to contain 9.0 mg g^{-1} dw chlorogenic acid and 3.12 mg g^{-1} dw caffeic acid.[22] Again, the contrasting values may be explained through the variation of extract type, plant origin (cultivated versus wild) and distinct method of analysis.

Total phenols and total flavonoids content

The results are shown in Table 4. Both total flavonoid and total phenols content in plant extracts decreases in the following order: *A. annua* > *A. vulgaris* > *A. absinthium*.

Table 4. Total phenols and total flavonoids content in plants

Plant	Active ingredient content, mg g^{-1} dry weight plant material	
	Phenols	**Flavonoids**
A. annua	80.65	13.88
A. vulgaris	41.67	10.11
A. absinthium	18.14	3.23

Antioxidant activity

The radical scavenging activity (Table 5) of the extracts decreases in the following order *A. vulgaris* > *A. annua* > *A. absinthium*. The antioxidant activity of analyzed extracts is concentration dependent and is influenced by the level of phenolic compounds, but also by the nature of those compounds.

Table 5. Free radical (DPPH) scavenging activity of plants extracts

Plant	EC_{50} (μg mL^{-1})
A. annua	77.73 ± 1.15
A. vulgaris	41.3 ± 0.2
A. absinthium	88.83 ± 0.55

Other researchers reported EC_{50} values of 976 μg mL^{-1} of an ethanol 70 % extract from the whole *A. vulgaris* plant[23] or 16 μg mL^{-1} of a methanol extract from *A. vulgaris* leaves[21], the latter being closer to our result. Another study found a lower antioxidant activity of a methanol extract from *A. annua* leaves (EC_{50} = 190.54 μg mL^{-1}) compared to our results.[24] For *A. absinthium* extract in ethanol 70 %, Craciunescu et al. reported a powerful radical scavenging activity with EC_{50} = 0.57 μg mL^{-1}.[20]

Conclusions

Biologically active compounds were identified and quantified in three *Artemisia* species, commonly used in Romanian traditional medicine. Six methoxylated flavones and two hydroxycinnamic acids were analyzed through LC-MS methods. Occurrence of eupatorin and hispidulin in *A. absinthium* and *A. vulgaris*, eupatilin in *A. annua* and casticin in *A. vulgaris* are reported here for the first time

There was a significant difference in concentration between caffeic acid and chlorogenic acid, in favour of the last one. All extracts showed antioxidant effect, notably *A. vulgaris* extract which exhibited a very good scavenging activity against DPPH radicals. The plant species studied here are important sources of biologically active compounds that can be used for the treatment and prevention of diseases.

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