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Evaluation of the Main Polyphenolic Compounds in Aromatic Plants of Asteraceae and Solanaceae Families of Bulgarian Origin

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Bulgarian specimens of Asteraceae and Solanaceae families are very scarce studied concerning the polyphenolic compounds. The aim of this investigation was to obtain fingerprint chromatographic profiles and to quantify the main polyphenols. HPLC–PDA on Kromasil and Purospher columns was employed for the separation of the components by applying two methods (TFA and AcAc). Better separation and reliable quantification of the polyphenols in *Achillea* species were obtained by the TFA method on Kromasil column. Chromatographic profiling and quantification of polyphenols in A. *campestris* was successful by using the TFA method on both columns. The polyphenols of A. *vulgaris* were better separated by AcAc method on Purospher column. Caffeoylquinic acids and rutin were present in higher quantities in *Achillea asplenifolia* and tobacco (Djebel). Fingerprint chromatographic profiles and reliable quantification of the polyphenolic compounds were achieved by considering the differences in the polyphenol complex and the specificity of the plant matrix

Keyword: Polyphenols, *Achillea*, *Artemisia*, tobacco, HPLC- PDA

1. Introduction

The aromatic plants of Asteraceae family are well known all over the world because of their different therapeutic applications[8,9]. Tobacco (Solanaceae family), especially the oriental types are used in the traditional medicine [5]. In many cases the healthy effect of the plants has been related with their polyphenolic components. For this reason, in the last years a great number of works has been aimed at finding active polyphenol components in a variety of different plants[2,14,16].

Polyphenols are one of the most widely occurring groups of phytochemicals of considerable physiological and morphological importance in plants. As a large group of bioactive chemicals, they have diverse biological functions. The most abundant polyphenols in plants are the phenolic acids and the flavonoids. The phenolic acids are presented mostly by the caffeoylquinic acids, which are ubiquitous, long recognized as powerful antioxidants. The chlorogenic acid family may provide significant health benefits by protecting against the damaging effects of free radicals. Growing evidence indicates the contributions of dicaffeoyl derivatives to the antioxidant activity of the plants of Asteraceae family[10,12]. Flavonoids are the other most commonly found phytochemicals in the medicinal plants of Asteraceae family [1]. Typically these compounds help to protect the plant against UV radiation, fungal parasites, herbivores, pathogens and oxidative cell injury. The ability of these natural antioxidants to scavenge several oxygen and nitrogen free

radicals has been associated to their health benefits[18]. The polyphenols of *Achillea* specimens include predominantly chlorogenic acid, dicaffeoylquinic acids as well as the flavonoids apigenin, luteolin, quercetin and their glycosides[3,15,17]. Most abundant are the 7-glycosides of the flavonoids apigenin and luteolin. In the polyphenolic profiles of *Artemisia* species a great number of flavonoids have been identified, namely quercetin, kaempferol, patuletin, apigenin, luteolin and their glycosides[4,11,13].

Djebel is a tobacco cultivar traditionally cultivated in Bulgaria. This cultivar is characterized by specific, pleasant aroma of the leaves. The Djebel plants have low nicotine content and are rich in polyphenols[6,7]. In tobacco, the polyphenols are presented with the so called typical five, chlorogenic acid and its isomers- neochlorogenic acid and 4-0-caffeoylquinic acid, and the flavonoids rutin and kaempferol-3-rutinoside[6,19]. Hence, the tobacco polyphenol profile is relatively simple and well defined.

HPLC has become the preferable analytical technique for determination of polyphenols providing qualitative and quantitative data. HPLC especially coupled with photodiode array detector (PDA, DAD) produces multi-wavelength detection. HPLC- PDA is used to facilitate the identification of compounds and to control the peak purity. The separations and the detection modes differ one from other aiming at identifying or quantifying preferable components of the polyphenol complex. Good separation is absolutely necessary to achieve fingerprint chromatographic profile and reliable quantification of the components. It depends on the distribution of the polyphenols and the complexity of the plant matrix. Most of the investigations are directed to identify and quantify the representatives of the phenolic acids or the flavonoid components, taking into account only the chlorogenic acid[3,10]. Hence, studying the polyphenol complex (phenolic acids and flavonoids) requires most complicated technique, such as the application of different chromatographic columns and mobile phases. There are no data on the separation of polyphenol components of *Achillea* and *Artemisia* obtained by different chromatographic conditions of HPLC- PDA method. Fingerprint chromatographic profiles and quantitative results on the polyphenol distribution in these species and Djebel cv. originating from Bulgaria are very scarce.

The aim of the present work was to obtain fingerprint chromatographic profiles of the polyphenolic compounds in *Achillea* and *Artemisia* species and Djebel tobacco cv. and to quantify the main compounds.

2. Materials and Methods

a. Plant Material

For the aim of the investigation two wild species of genus *Achillea* - *A. collina* and *A. asplenifolia* (members of *A. millefolium* group) were studied, each represented by one population, namely *A. collina* 102 and *A. asplenifolia* 9602, two wild species of *Artemisia, A. campestris* and *A. vulgaris*, and Djebel tobacco cv. Seedlings of *Achillea* plants were produced in a greenhouse and then transferred to the experimental field of Institute of Plant Physiology and Genetics, Sofia, in 2009. These species were identified by Assoc. Prof. Dr. Antonina Asenova Vitkova (Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria). The specimens of *Artemisia* species were collected in the region of Shipka town, Balkan Mountain and determined by Assist. Prof. Dr. Katia Uzundjalieva (Institute for Plant Genetic Resources "K. Malkov", Town of Sadovo, Bulgaria). The plant material was collected in July at full flowering stage. Flos (*Achillea*) and herbal (*Artemisia*) material were taken to the purpose of this study. Dry leaves of Djebel cv. were purchased from Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria and identified by Assoc. Prof. Dr. D. Dimanov. To prepare representative samples the material was dried at room temperature, avoiding direct sunlight, then milled, packed into multilayer

paper bags and stored in a dark room at ambient temperature.

b. Reagents

Methanol and acetonitrile used for HPLC analyses were of chromatographic grade (VWR, Austria). Water for HPLC was prepared with Millipore purifier (Millipore, USA). Trifluoroacetic acid (TFA) and acetic acid (AcAc), HPLC grade, Merck, Germany were used to prepare the mobile phases. Neochlorogenic acid (Cas Number 906-33-2), chlorogenic acid (Cas Number 327-97-9), rutin (Cas Number 250249-75-3), quercetin (Cas Number 117-39-5) and apigenin (Cas Number 520-36-5) were purchased from Sigma Aldrich, HPLC standards, purity >95%. Standard solutions with concentrations of 0.1, 0.05, 0.025, 0.0125, 0.0062 mg ml^{-1} were obtained by diluting the stock solution of each component in methanol.

c. Extraction of Polyphenols

The milled plant material was weighed with 0.0001 g precision, taking 0.2 g samples for *Achillea* and *Artemisia* and 0.1 g for Djebel cv. Two replications of every sample were prepared. The flavonoids were analyzed in their glycoside form and therefore nonhydrolysed plant extracts were prepared. The powdered *Achillea* and *Artemisia* samples were extracted with 10 ml 70% (v/v) aqueous methanol. The tobacco powder was extracted with 5 ml, 60 % (v/v) aqueous methanol. All extracts were sonicated for 30 min and then they were filtrated under vacuum. The extract from tobacco was purified by passing it through cartridge C18 according to the method described by Dagnon & Edreva[6]. The volume of all samples was adjusted to 10 ml and passed through a membrane filter 0.45 µm prior to HPLC analysis.

d. HPLC –PDA Analysis

The instrumentation used for HPLC analysis consisted of quaternary mixer Smartline Manager 5000, pump Smartline 1000 and PDA 2800 detector (Knauer, Germany). Two chromatographic columns were used: a Purospher ^{R}star RP-18e 25cm x 4.6mm i.d., 5µm particle size (Merck, Germany) and a Kromasil C18, 15 cm x 4.6 mm i.d., 5 µm particle size (Supelco, USA). Mobile phase flow rate was set by 1.0 ml min^{-1}; sample volume was 20 µl.

d.1 Separation Modes

Two different methods were employed to separate the polyphenols. In the TFA method 0.1% (v/v) trifluoroacetic acid in acetonitrile (AcN) was used as solvent B. A mixture from 90 parts water and 10 parts 0.1% TFA in acetonitrile was used as solvent A with the following gradient elution program: 0-10 min, 100%-90% A (0-10% B), 10-18 min, 89% A (11% B), 18-25 min, 85% A (15% B), 25-40 min, 45% A (55% B).

In the AcAc method the mobile phase composition was A - CH_3OH: H_2O: CH_3COOH= 5: 93: 2; B - CH_3OH: H_2O: CH_3COOH= 86: 12: 2. The elution followed the gradient profile: 0-15 min, 100% - 80% A (0-20% B); 15-35 min, 45% A (55% B); 35-50 min to 0% A (100% B). By this method maximum separation of chlorogenic acid from its isomer 4-0-caffeoylquinic acid was achieved. All samples (*A. collina* 102, *A. asplenifolia* 9602, *A. campestris*, *A. vulgaris* and Djebel tobacco cv.) were analyzed on the two chromatographic columns by using the described methods of separation (TFA and AcAc).

d.2 Detection

The polyphenols were monitored at 320 nm (maximum absorption for caffeoylquinic acids), 352 nm (maximum absorption for rutin and quercetin) and at 340 nm (maximum absorption for apigenin).

d.3 Identification and qualitative analysis

The identity of the chromatographic peaks was confirmed by comparison of the retention times of the samples with those of the standard compounds. The spectral characteristics of the eluting peaks, scanned with diode-array detector (λ=200-400 nm) were compared with those of the authentic standards. Peak assignments for those components where no references were available (4-0-caffeoylquinic acid and kaempferol-3-rutinoside) were based on data for retention time according to the literature and the quantification

was done by using the calibration curves of chlorogenic acid and rutin. The content of the components was determined by the external method using a calibration curves established with five dilutions of each standard with correlation coefficients between 0.997 and 0.999. The recovery of the polyphenols by the established procedures was from 95% to 100%. The relative standard deviation (RSD) of the methods varied between 3% and 18% by the different components. The limit of detection of the polyphenols pointed to 0.5 µg/ml. The limit of quantification differed from 1.0µg/ml to 3.0 µg/ml. Each sample was analyzed several times (min 3), and the mean value was used for calculation. The results are expressed as mean ±SD.

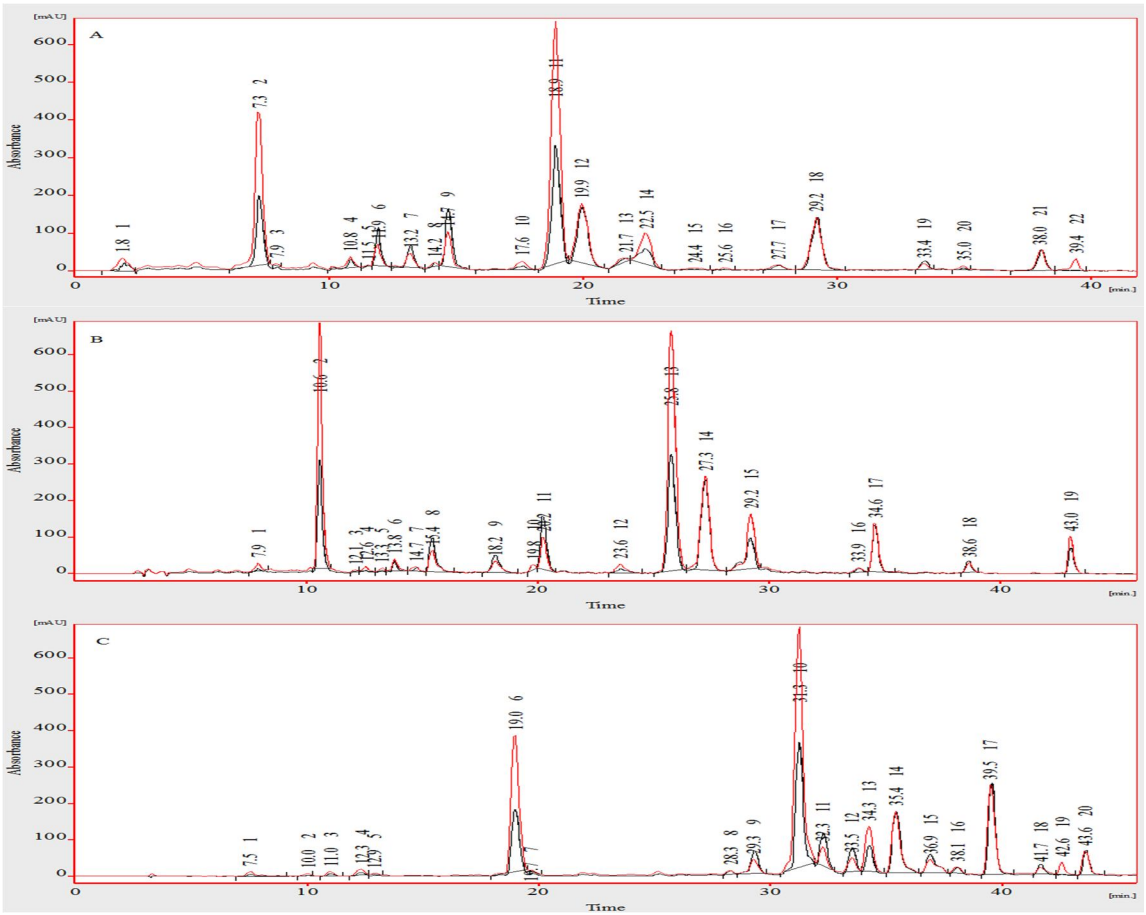

Fig. 1: Chromatographic profiles of polyphenols of *A. asplenifolia* 9602 on Kromasil column, TFA method (A), Purospher column, TFA method (B) and Purospher column, AcAc method (C). Peaks 2, 7, 19 and 21 were identified as chlorogenic acid, rutin, quercetin and apigenin, respectively (A); Peak 2, 9, 18, 19 as chlorogenic acid, rutin, mixed, mixed, respectively (B); Peaks 4, 6 and 7 as chlorogenic acid and isomers, peak 12 as rutin, peak 18 as quercetin and peak 20 as apigenin (C). Red line represents absorbtion at 320 nm. Black line represents absorbtion at 340 and 352 nm.

3. Results and Discussion

3.1 Separation of Polyphenols

In this study, Bulgarian aromatic plants (*Achillea, Artemisia* and *N. tabacum* species) were studied for the distribution and quantification of the main polyphenol compounds. Two chromatographic methods (TFA and AcAc) were employed to separate the polyphenols. They were designed to obtain fingerprint profiles of the polyphenol complex in the studied plants. The qualitative and quantitative differences in the polyphenols of the three groups of plants, the specificity of the plant

matrix and the need to obtain reliable data for the Bulgarian specimens motivated the development of this study. The use of chromatographic column Kromasil RP 18 and trifluoroacetic acid in the mobile phase were the most applied chromatographic conditions for the determination of flavonoids in *Achillea* species[3,15]. The application of RP 8 or RP 18 columns with other acids in the mobile phase was successful for the analysis of caffeoyl derivatives[10, 12]. Thus, there are few data concerning the simultaneously separation and quantification of the phenolic acids and flavonoids in *Achillea* and *Artemisia* species. Such data obtained by employing different chromatographic conditions will contribute to increase the reliability and accuracy of the phytochemical methods.

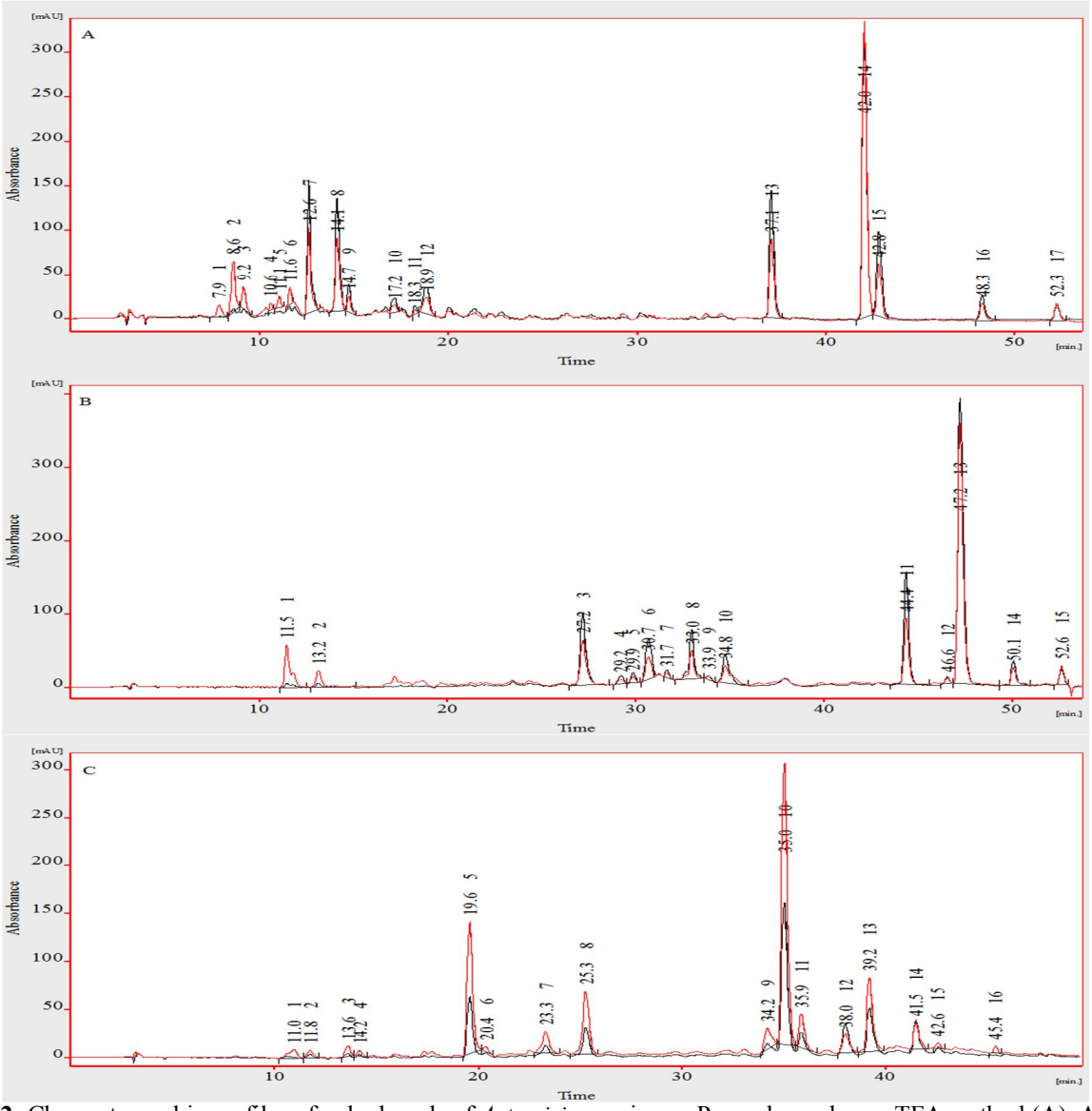

Fig. 2: Chromatographic profiles of polyphenols of *Artemisia* species on Purospher column, TFA method (A), AcAc method (B) and AcAc method (C); *A. campestris* (peak 9-rutin, peak 13-quercetin, peak 14-apigenin) (A); *A. campestris* (peak 2- neochlorogenic acid, peak 8- rutin, peak 11-quercetin, peak 13-mixed) (B); *A. vulgaris* (peaks 3, 5 and 6-chlorogenic acid and isomers, peak 12- rutin) (C). Red line represents absorbtion at 320 nm. Black line represents absorbtion at 340 and 352 nm.

The chromatographic profiles of the polyphenols in *A. asplenifolia* 9602 are presented on Figure 1. Figures 1A and 1B represent the separation of the polyphenol components by the chromatographic conditions of the TFA method on Kromasil and Purospher columns, respectively. They reveal some differences in the chromatographic shape, mostly in the area of flavonoid aglycones and one of them was critical for the quantification of apigenin. As shown on Figure 1A (TFA method, Kromasil column) the peak of apigenin (21) is followed by a well-defined peak(22) with an absorption maximum at 320 nm. This component was not separated from the peak of apigenin (19) by the same mobile phase conditions on Purospher column (Figure 1B). Figure 1C represents the chromatographic profile of polyphenols of *A. asplenifolia* 9602 obtained by using AcAc method on Purospher column. By this method the peak of apigenin (20) and the peak with an absorbtion maximum at 320 nm (19) were clearly separated, which allowed the successful quantification of the flavonoid apigenin (Figure 1C).

The chromatographic profile of *A. asplenifolia* 9602 obtained by the TFA method, Kromasil column compared to the profiles of *Achillea* species in previous studies differ in relation of quercetin, obviously presented in the Bulgarian specimens[3, 15]. The chromatographic patterns are similar and they confirm the presence of the three main flavonoids – luteolin, quercetin and apigenin in the studied *Achillea* specimens.

On Figure 2 the chromatographic profiles of *Artemisia* species are shown. They were obtained on Purospher column by using the TFA (2A) and AcAc (2B) methods. The separation of the polyphenols in the specimen of *A. campestris* pointed to the predominant presence of two flavonoid aglycones. The highest peak on the profile of *A. campestris* was identified as apigenin (14) and the peak 13 as quercetin (Figure 2A). The peak of apigenin (14) is well separated from the following peak (15) as shown on Figure 2A. The same shape of the polyphenols was obtained by the TFA method on the Kromasil column. On the contrary both peaks overlapped in the conditions of AcAc method, Purospher column, as shown on Figure 2B. Hence the quantification of the flavonoid apigenin in *A. campestris* was possible by using TFA method on both columns.

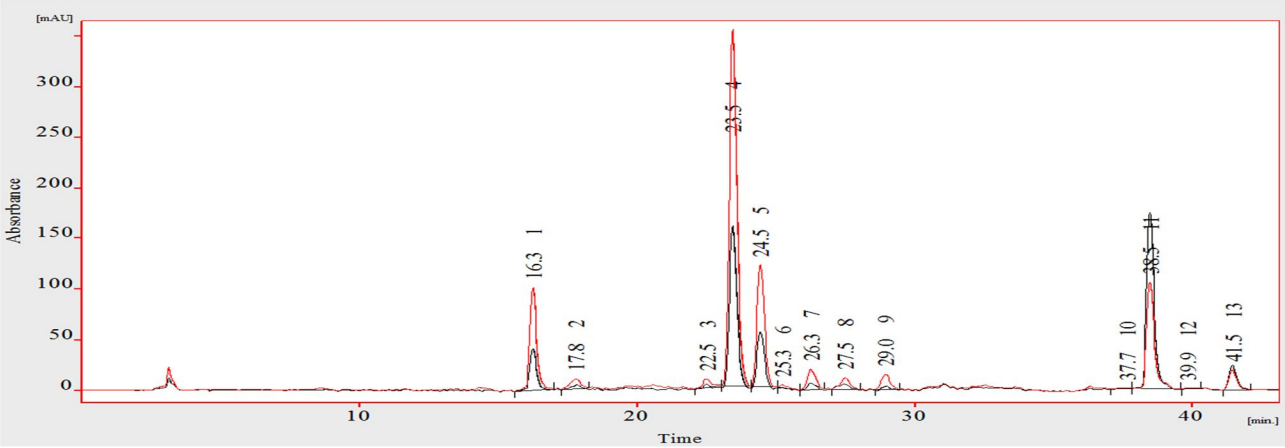

Fig. 3: Chromatographic profile of polyphenols in Djebel cv. tobacco on Purospher column, AcAc method. Peaks 1, 4 and 5 were identified as neochlorogenic acid, chlorogenic acid and 4-caffeoylquinic acid, peak 11 as rutin and peak 13 as kaempferol-3-rutinoside, respectively. Red line represents absorbtion at 320 nm. Black line represents absorbtion at 340 and 352 nm.

In the chromatographic profile of the specimen of *A. vulgaris* (Figure 2C) many differences in comparison with those of *A. campestris* obtained by the same conditions (AcAc method, Purospher column) have been observed. Obviously, most of the polyphenol components (peaks 3, 5, 6, 8, 10, 13) have absorption maximum at 320 nm (Figure 2C). This fact points to the predominant presence of the phenolic acids in the specimen of *A. vulgaris* (Figure 2C) in contrast to *A. campestris* (Figure 2B).

The chromatographic profile of Djebel tobacco cv. is presented on Figure 3. It shows the clear separation of the chlorogenic acid from its isomer 4-caffeoylquinic acid achieved by AcAc method on Purospher column. Moreover, it reveals the abundant presence of the caffeoylquinic acids (neochlorogenic, chlorogenic and 4-0-caffeoylquinic acids) and the flavonoid glycosides (rutin and kaempferol-3-rutinoside) (Figure 3).

3.2 Quantification of Polyphenols

The content of the main polyphenols in the specimens of *Achillea*, *Artemisia* and Djebel tobacco cv. was determined. The amount of the identified compounds is given in Table 1. Chlorogenic acid was presented in all specimens in being of lowest amount in *A. campestris* (Table 1). The neochlorogenic acid was the major caffeoylquinic acid in the specimen of *A. campestris*. Quercetin and apigenin were the most abundant flavonoid aglycones in the polyphenol complex of *A. campestris,* 2.12 mg g^{-1} DM and 2.81 mg g^{-1} DM, respectively. In comparison, these flavonoids were not found in the specimen of *A. vulgaris* (Table 1).

Table 1: Content of main polyphenolic compounds (mg g^{-1} DM) in flos of *Achillea*, in herbal material of *Artemisia* species and in dry leaves of Djebel tobacco cv. Caffeoylquinic acids and flavonoid glycosides are calculated by using method AcAc. Flavonoid aglycones are calculated by using method TFA. Data are means of three replicates ±SD.

Sample description	Caffeoylquinic acids		Rutin	Kaemp-ferol-3-rutinoside	Quercetin	Apigenin
A. collina 102	Neochloro-genic Chlorogenic 4-0-caffeoyl-quinic	0.19±0.013 4.49±0.22 0.02±0.009	n.d.	n.d.	1.03±0.12	0.29±0.03
A. asplenifolia 9602	Neochloro-genic Chlorogenic 4-0-caffeoylquinic	0.27±0.03 6.54±0.18 0.07±0.009	2.42±0.22	n.d.	0.42±0.08	0.55±0.02
A. campestris	Neochloro-genic Chlorogenic 4-0-caffeoylquinic	0.33±0.05 0.078±0.008 n.d.	1.26±0.20	n.d.	2.12±0.15	2.81±0.15
A. vulgaris	Neochloro-genic Chlorogenic 4-0-caffeoylquinic	0.13±0.014 1.70±0.10 0.05±0.009	0.87±0.06	n.d.	n.d.	n.d.
Djebel cv.	Neochloro-genic Chlorogenic 4-0-caffeoylquinic	2.41±0.15 10.27±0.28 3.23±0.25	9.20±0.32	1.62±0.20	n.d.	n.d.

The caffeoylquinic acids were the dominating components of the polyphenol complex in the *A. vulgaris* (Table 1 and Figure 2C). The *Achillea* species and Djebel cv. were rich of caffeoylquinic acids (Table 1). The data in Table 1 reveal that the highest quantities of the three isomers of the caffeoylquinic acid (neochlorogenic 2.41 mg g^{-1}, chlorogenic 10.27 mg g^{-1} and 4-0-caffeoylquinic 3.23 mg g^{-1}) and the highest amount of rutin, 9.20 mg g^{-1} DM were determined in the sample of tobacco. In lower amount rutin was quantified in the two *Artemisia* species and in *A. asplenifolia* 9602. The content of rutin was 1.26 mg g^{-1} DM (*A. campestris*), 0.87 mg g^{-1} DM (*A. vulgaris*) and 2.42 mg g^{-1} DM (*A. asplenifolia* 9602) (Table 1). Rutin was not detected in the specimen of *A. collina* 102.

The results of this study show undoubtedly that for reliable quantification of the polyphenol compounds it is necessary to perform good separation considering the different distribution of the polyphenols in the plant species. The separation and quantification of flavonoid aglycones were better achieved by using method TFA. Method AcAc was most suitable to separate the caffeoylquinic acids and flavonoid glycosides. Thus, it is advisable to analyze the polyphenols in the species of *A. millefolium* by using method TFA on Kromasil column. The polyphenols of *Artemisia campestris* can be analyzed successfully employing the same method on Kromasil or Purospher column. Chromatographic profiling of polyphenol components in *Artemisia vulgaris* requires applying the method AcAc to achieve good separation of the phenolic acids. Changing the mobile phase composition and monitoring the UV spectrum of the peaks are very helpful analytical practice to improve the separation and for accurate quantification.

4. Conclusions

The data presented in this investigation have revealed a considerable variation in the chromatographic profiles of the polyphenols in the studied *Achillea* and *Artemisia* species, and in the Djebel cv. Thus, the separation and quantification of the polyphenolic compounds in the different plant species require employing a specific chromatographic approach. Tobacco polyphenol profile may be regarded as a model for caffeoylquinic acid distribution in the plant kingdom and can help to distinguish the chlorogenic acid isomers.

5. Acknowledgements

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