

# Chemical composition of various *Ephedra* species

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## ABSTRACT

The medicinal significance of *Ephedra* is based on the sympathomimetic properties of ephedrine (E) alkaloids. Pharmacological effects depend on the phytochemical composition of individual *Ephedra* species. The aim of this study was to measure the total alkaloids content (TAC), total phenolics content (TPC), and total flavonoids content (TFC) and determine their relationship in dry herb of *Ephedra major*, *Ephedra distachya subsp. helvetica*, *Ephedra monosperma*, *Ephedra fragilis*, *Ephedra foeminea*, *Ephedra alata*, *Ephedra altissima* and *Ephedra foliata*. Nowadays, medicinal use of *Ephedrae herba* is limited, but the abuse of its psychostimulants is rising. In this study, TAC, TPC and TFC were determined using spectrophotometric methods. For the first time, ultra-performance liquid chromatography with ultraviolet detection (UPLC-UV) was used for separation and quantification of E-type alkaloids of various *Ephedra* species. The highest TPC and TFC were found in *E. alata* (53.3 ± 0.1 mg Gallic acid equivalents/g dry weight, 2.8 mg quercetin equivalents/g dry weight, respectively). The total content of E and pseudoephedrine determined by UPLC-UV varied between 20.8 mg/g dry weight (*E. distachya subsp. helvetica*) and 34.7 mg/g dry weight (*E. monosperma*). The variable content and ratio between secondary metabolites determined in different *Ephedra* species reflects their metabolic activities. Utilization of UPLC-UV unveiled that this technique is sensitive, selective, and useful for separation and quantification of different alkaloids in complex biological matrixes. The limit of detection was 5 ng. Application of UPLC-UV can be recommended in quick analyses of E-type alkaloids in forensic medicine and quality control of pharmaceutical preparations.

KEYWORDS: *Ephedra*; alkaloids; phenolics; flavonoids; spectrophotometry; ultra-performance liquid chromatography with ultraviolet detection

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## INTRODUCTION

*Ephedra* is one of the oldest known drugs, having been used by the Chinese for at least 5000 years [1]. It contains alkaloids of the ephedrine (E)-type which act as sympathomimetics. The pharmacological and toxicological effects depend on the individual E alkaloid type, its enantiomeric form and receptor binding characteristics. Ephedrine stimulates the heart rate, increases blood pressure, promotes bronchodilatation, and exhibits pronounced effects on the central nervous system (CNS) by binding to adrenergic receptors. Pseudoephedrine (PE) acts similarly, yet, with fewer CNS effects [2]. Many plants, which have a stimulatory effect on the CNS, synthesize substances that contain phenylethylamine or xanthine structures that are able to enhance catecholaminergic effects

and/or to act on adrenoreceptors [3]. In the past, E alkaloids were used in the treatment and/or prophylaxis of various conditions such as asthma, nasal congestion, and hypotension caused by spinal anesthesia and urinary incontinence. Today, there is a rising interest for the application of *Ephedra* in enhancing performance and appetite suppression [4]. Contrary to most other herbal supplements, *Ephedra* products carry a remarkable health risk, which is aggravated by their misuse and/or abuse. According to the Food and Drug Administration (FDA) assessment in 2004, food supplements containing E-type alkaloids represent an unacceptable health risk, bearing in mind the conditions of use. Consequently, FDA banned all over the counter drugs containing ephedrine. Reviews of human case reports described adverse cardiovascular and cerebrovascular events as possibly associated with the use of dietary supplement preparations containing E-type alkaloids [2]. The World Anti-Doping Agency prohibited E, PE and methylephedrine as stimulants. Additionally, E and PE are worldwide monitored as precursors for the chemical synthesis of the methamphetamine. Apart from consumer abuse, there is also manufacturer abuse, which is reflected in

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spiking of *Ephedrae herba* or its preparations with synthetic E alkaloids. Therefore, the identity and origin of the alkaloids in herbal preparations is often questionable and analytical methods which ensure and verify safety and quality control are of high importance and require improvements. The phytochemical composition of various *Ephedra* species is not completely elucidated. Secondary metabolites originating from *Ephedra* species comprise alkaloids, amino acids and derivatives, volatiles, and phenolic compounds [5]. *Ephedra* species contain alkaloids of biological relevance: E, PE, norephedrine, norpseudoephedrine, methylephedrine, and methylpseudoephedrine. Beside the E-type alkaloids, ephedroxane, and macrocyclic spermidines called Ephedradine A-D, have been found in some Eurasian *Ephedra* species [5]. Other phytochemical compounds include kynurenates, citric, malic and oxalic acid [6], saponins, crystals of calcium oxalate, and trace minerals. According to several reports, volatile compounds present in this plant are mainly represented by terpenoids and may be used as chemotaxonomic markers [7-9]. Phenolic compounds and their most important subgroup flavonoids are aromatic compounds widely distributed in the plant kingdom. They are present in the phytochemical composition of *Ephedra*, however, to our best knowledge, literature lacks data on their total contents in the majority of the *Ephedra* species used in this study. Therefore, the aim of the study is to determine the total alkaloids content (TAC), total phenolics content (TPC) and total flavonoids content (TFC) and their ratio in different *Ephedra* species using spectrophotometric methods; to quantify E and PE, as well as to separate individual E-type alkaloids using ultra-performance liquid chromatography with ultraviolet detection (UPLC-UV). Additionally, the objective is to recommend and justify UPLC-UV as a method for quick analyses in forensic medicine in cases when it is necessary to prove the presence or absence of E-type alkaloids. Possessing *Ephedra* species, which are avoid of E alkaloids is not considered illicit as opposed to species, which contain E alkaloids. The quantitative data can be used in quality control of herbal and synthetic drugs containing E alkaloids which is essential for ensuring their safety and efficacy.

## MATERIALS AND METHODS

### Plant materials

Dried aerial parts of the listed *Ephedra* species were used to determine the TAC, TPC and TFC using spectrophotometric methods; and to separate individual E-type alkaloids and quantify E and PE by UPLC-UV:

- *Ephedra fragilis* Desf. (Botanical Garden of the University of Vienna, Austria) IPEN number: XX-o-WU-EPH130013; garden reference number 22102.

- *Ephedra major* Host (Botanical Garden of the University of Vienna, Austria; identified by I. Racz and F. Lauria) IPEN number: XX-o-WU-EPH120009; garden reference number 23026.
- *Ephedra distachya subsp. helvetica* (C.A. Mey.) Asch. & Graebn. (Botanical Garden of the University of Vienna, Austria) IPEN number: XX-o-WU-EPH130012; garden reference number 22015.
- *Ephedra foeminea* Forssk. (Botanical Garden of the University of Vienna, Austria; identified by I. Racz and F. Lauria) IPEN number: XX-o-WU-EPHE120010; garden reference number 23028.
- *Ephedra monosperma* J.G. Gmel. ex C.A. Mey. (Botanical Garden of the University of Vienna, Austria) IPEN number: XX-o-WU-EPH1300014; garden reference number 22104.
- *Ephedra alata* Decne. (Botanical Garden of the University of Hamburg, Germany; identified by Finckhi Staudinger), garden reference number: 500392, 2002; project Biota Maroc 01LL0601A.
- *Ephedra altissima* Desf. (Botanical Garden of the University of Hamburg, Germany; identified by Finckhi Staudinger), garden reference number: 500044, 2001; project Biota Maroc 01LL0601A.
- *Ephedra foliata* Boiss. ex C.A. Mey. (Botanical Garden of the University of Hamburg, Germany; identified by Finckhi Staudinger), garden reference number: 500882, 2002; project Biota Maroc 01LL0601A.

### Determination of TPC

Total phenolics were determined by Folin–Ciocalteu reagent using the method by Slinkard and Singleton [10].

### Chemicals

Methanol (min. 99.5%, p.a.), glacial acetic acid (min. 99%, p.a.), ethanol 96%, and sodium carbonate decahydrate (p.a.) were purchased from Alkaloid Skopje, Macedonia. Water, Chromasolv®Plus (High-performance liquid chromatography [HPLC] grade, Sigma-Aldrich, USA), gallic acid monohydrate (>98%, for HPLC, FlukaChemika, Germany), Folin-Ciocalteu reagent (Semikem, Bosnia and Herzegovina).

### Preparation of the standard solution

Gallic acid (50 mg) was dissolved in 300 µl 96% ethanol and diluted with purified water (HPLC grade) to 100 ml. The stock solution was further diluted (0.5, 1, 2, 3, 4, 5 µg/ml) to prepare a standard curve.

### Sample preparation

Finely ground, dried plant material (0.5 g) was weighed on an analytical balance and suspended in 4 ml of methanol,

4 ml of purified water (HPLC grade), and 1.5 ml glacial acetic acid. The mixture was shaken several times and heated in a water bath for 30 minutes, then centrifuged at 15000 rpm, for 20 minutes at 15°C. The supernatants were used for the analysis.

### Procedure

Sample or standard (2 ml), diluted 1/10 from the above given concentrations, were mixed with 10 ml 1/10 diluted Folin–Ciocalteu reagent. The solution was left to stand for 0.5–8 minutes before adding 8 ml Na<sub>2</sub>CO<sub>3</sub> (75 g/l). After standing for 30–45 minutes at room temperature, the absorbance of the solution was measured at 743 nm (Ultraviolet-Visible [UV/VIS] spectrophotometer Perkin Elmer Precisely LAMBDA 25, CA, USA).

### Determination of TFC

The aluminum chloride colorimetric method was used for flavonoids determination [11].

### Chemicals

Aluminum hexahydrate (p.a. and for HPLC) and potassium acetate (min 99%, p.a.) were purchased from Kemika, Croatia; potassium chloride (p.a. Destilacija Teslic, Bosnia, and Herzegovina), quercetin anhydride (Sigma-Aldrich, USA), distilled water.

### Preparation of the standard solution

Quercetin standard (30 mg) was dissolved in 100 ml water (HPLC grade). This stock solution was used to prepare serial dilutions (0.3, 0.2, 0.1, 0.08, 0.05, 0.025, and 0.0125 mg/ml) for constructing the standard curve.

### Sample preparation

Samples are prepared in the same way as described in determination of total phenolics.

### Procedure

Each of the plant extracts was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. It remained at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 434 nm (UV/VIS spectrophotometer Perkin Elmer Precisely LAMBDA 25, CA, USA).

### Determination of TAC

The determination of total alkaloids is based on the reaction with ninhydrin, a strong oxidative agent that reacts with amino acids and yields a violet-blue product, which has the maximum absorption at 570 nm.

### Chemicals

Ninhydrin (p.a., Kemika, Croatia), E standard (>99.9%, BASF, Germany), sodium carbonate decahydrate (p.a., Alkaloid Skopje, Macedonia), ethanol (96%, Semikem, Bosnia and Herzegovina), water (HPLC grade, Panreac, Spain), hydrochloric acid (37%, p.a. Carlo Erba Reagents, Italy), sodium hydroxide (min 99%, Semikem, Bosnia and Herzegovina), dichloromethane (min 99.8%, HPLC grade, Merck, Germany), methanol Chromasolv (HPLC grade, Sigma Aldrich, Germany).

### Preparation of working solutions and the standard curve

On analytical balance 2 g of ninhydrin was weighed, then dissolved in 3 ml ethanol, diluted with water to 100 ml, adjusted to pH 7.8–7.9 with 1% Na<sub>2</sub>CO<sub>3</sub>. The E standard was prepared by dissolving 5 mg of the standard in 25 ml water. The standard curve had a concentration range between 0.01 and 0.04 mg/ml and was prepared by following this procedure: 100 µl ninhydrin solution was added on a series of volumes of the E standard solution (60, 80, 100, 120, 140, 160, 180, 200 µl), heated in a water bath at 100–110°C for 10 minutes, immediately diluted with water to 1 ml. Absorbance was measured at 570 nm.

### Sample preparation

On analytical balance 1 g of finely ground dried plant material was weighed, put into an Erlenmeyer flask, added 20 ml of hydrochloric acid (6.2%, v/v). The mixture was sonicated for 15 minutes. The solid residue was allowed to settle and filtrated. The pH of the remaining solution was adjusted to about 12 with sodium hydroxide solution (20%, w/v) and transferred to a separation funnel. Ephedrine alkaloids were extracted by adding 20 ml of dichloromethane. The lower layer was collected and evaporated to dryness on a warm water bath (50–60°C). Next day, the residue was dissolved in 500 µl of methanol.

### Procedure

First, 100 µl of the methanolic sample solution (in different dilutions to fit into the standard curve) was mixed with 100 µl ninhydrin solution, then heated in a water bath at 105–110°C for 10 minutes and immediately diluted with water to 1 ml. Absorbance was measured at 570 nm (UV/VIS spectrophotometer Perkin Elmer Precisely LAMBDA 25, CA, USA).

### Separation of Ephedrine-type alkaloids and quantification of E and PE by UPLC-UV

#### Chemicals

Acetonitrile Optigrade<sup>®</sup> (for HPLC), sodium lauryl sulfate (Optigrade<sup>®</sup>, for HPLC, ≥99%) and methanol (HPLC grade)

were purchased from Promochem, tetrahydrofuran (for HPLC, LiChrosolv) and o-phosphoric acid (85%, p.a.) from Merck KGaA, E-hydrochloride, and PE-hydrochloride standards from BASF, water (Chromasolv Plus, for HPLC, Sigma-Aldrich, USA).

### Instrumentation and chromatographic conditions

The UPLC-UV analysis was performed using and modifying the parameters published by Gurley et al. [12]. A component UPLC system (Acquity UPLC H-Class) consisted of a pump, vacuum degasser, reservoir of the mobile phase, a thermostated auto sampler, a thermostated column compartment, PDA eλ UV absorbance detector operated at 208 nm. A C-8 column (Acquity UPLC BEH C8, 150 × 2.1 mm; 1.7 μm) was operated with a mobile phase consisting of acetonitrile, tetrahydrofuran, and water (38:5:57, v/v/v). Sodium lauryl sulfate, an ion-pairing agent, was added to the mobile phase to achieve a final concentration of 5 mM. The mobile phase was delivered at a flow-rate of 0.3 ml/minute. Column temperature was maintained at 37°C, the pressure was around 9000 psi, the injection volume was 5 μl and the run time was 7.0 minutes. Detector output was recorded and chromatograms were analyzed by the software Acquity UPLC Console for system UPLC, Empower 2.0, Waters Corporation, USA.

### Standard preparation

E-hydrochloride standard (5.12 mg that equal to 4.192 mg E standard) was dissolved in 50 ml methanol. From that solution, 10 ml were taken and diluted with methanol up to 20 ml. PE hydrochloride was weighed (5.43 mg that equal to 4.453 mg PE standard) and prepared in the same way. The obtained standard curve for E had  $R^2 = 0.999$ , and the standard curve for PE had  $R^2 = 0.998$ .

### Validation of quantification

E and PE calibration standards were used for the validation of the quantification. Standard curves were obtained by using the area of the peak and the concentration of the corresponding standard. Analyte concentrations are expressed as mg/g dry weight. The retention times ( $t_R$ ) were 3.623 minutes for the PE standard, and 3.743 minutes for the E standard. The limit of detection was 5 ng.

### Sample preparation

The extracting solution consisted of 0.716 g sodium lauryl sulfate, 58.2 ml acetonitrile, 119 μl phosphoric acid and 91 ml water (HPLC grade). Dry and finely grounded plant material (100 mg) was suspended in 5 ml of that solution, allowed to stand for 20 minutes at room temperature and then sonicated for 25 minutes. The extracts were centrifuged, 10000 rpm for

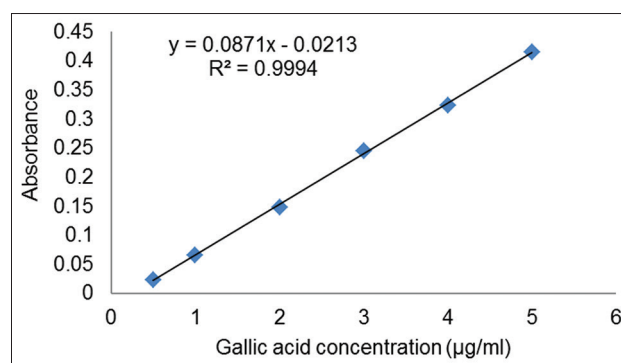
5 minutes, supernatants filtrated (membrane filter, 0.25 μm) and stored at -20°C before analysis.

## RESULTS

### TPC, TFC and TAC in *Ephedra* species

The TPCs were determined from the linear equation of a standard curve ( $R^2 = 0.999$ ) prepared with Gallic acid (Figure 1). The TPC compounds were expressed as mg GAE/g dry weight. Results of the TFC were expressed as mg QE/g dry weight and established using the standard curve ( $R^2 = 0.9961$ ) prepared with quercetin. The constructed standard curve ( $R^2 = 0.9876$ ) for the measurement of TAC plotted the absorbance of ninhydrin versus its concentration. Results of TAC are expressed as mean ± SD.

Results of TPC, TFC and TAC measurements are presented in Table 1. Among the investigated *Ephedra* species, the highest TPC and TFC were found in *E. alata* ( $53.3 \pm 0.1$  mg GAE/g dry weight and  $2.8 \pm 0.0$  mg QE/g dry weight, respectively). The lowest TPC was found in *E. foeminea* ( $6.8 \pm 0.4$  mg GAE/g dry weight), while the lowest TFC was in *E. fragilis* ( $0.5 \pm 0.2$  mg QE/g dry weight). Results showed that, depending on the species, a high TPC value was accompanied by a high TFC value, whereas a low TPC value was accompanied by a low TFC value (Figure 2). The TAC in the herb of investigated *Ephedra* species varied between  $0.1 \pm 0.0$  mg/g dry



**FIGURE 1.** A standard curve for the determination of total phenolics content.

**TABLE 1.** TPC, TFC and TAC in *Ephedra* species

<i>Ephedra</i> species	TPC (mg GAE/g dry weight)	TFC (mg QE/g dry weight)	TAC (mg/g dry weight)
<i>Ephedra alata</i>	53.3±0.1	2.8±0.0	n.a.
<i>Ephedra foliata</i>	52.6±0.1	2.5±0.0	n.a.
<i>Ephedra distachya subsp. helvetica</i>	27.0±0.4	2.1±0.3	15.8±2.0
<i>Ephedra major</i>	26.2±0.4	1.3±0.2	14.8±1.9
<i>Ephedra altissima</i>	16.4±0.1	2.0±0.0	n.a.
<i>Ephedra fragilis</i>	7.7±0.1	0.5±0.2	0.2±0.0
<i>Ephedra foeminea</i>	6.8±0.4	0.6±0.2	0.1±0.0

n.a.: Not analyzed due to insufficient plant material; TPC: Total phenolics content; TFC: Total flavonoids content; TAC: Total alkaloids content

weight and  $15.8 \pm 0.0$  mg/g dry weight. The highest content was found in *E. distachya subsp. helvetica*, while the lowest content was in *E. foeminea*. *Ephedra distachya subsp. helvetica* and *E. major* have a higher content of TAC, TPC and TFC as opposed to *E. foeminea* and *E. fragilis*. By comparing the ratio between the secondary metabolites, it can be concluded that species with a higher TAC are richer in TPC and TFC, as well. The TAC of *E. alata*, *E. altissima* and *E. foliata* was not determined in this study due to insufficiency of provided plant materials, however, they may be expected to contain a high TAC as they have an expressed metabolic activity toward the synthesis of secondary metabolites which can be seen from their high TPC and TFC values.

### Separation and quantification of authentic standards of E and PE using UPLC-UV

The contents of E and PE were calculated using the peak area of their standards (Figure 3). The results are presented in Table 2. The obtained chromatograms of *E. monosperma* as the species with highest E and PE content is presented in Figure 4, while the chromatogram of *E. foeminea* as the only species in this study that does not contain E and PE is presented in Figure 5.

Among the analyzed species, E is the dominant alkaloid in *E. major*, *E. fragilis* and *E. distachya subsp. helvetica*. *Ephedra monosperma* was the only species with a higher PE content. E and PE were not detected in *E. foeminea*. The limit of detection was 5 ng.

## DISCUSSION

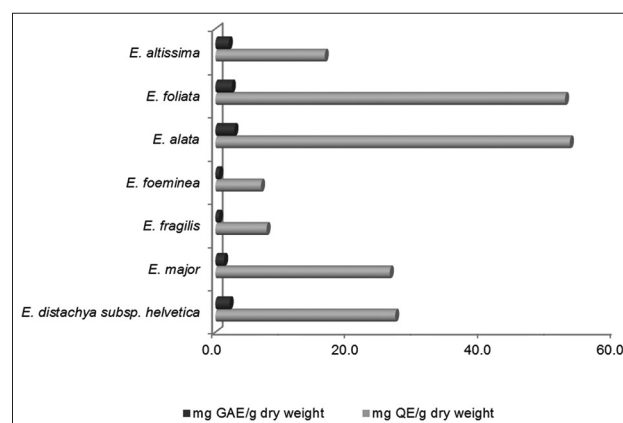
Identification of *Ephedra* species can be difficult because of their simple morphological characteristics and easy adaptation to the changes in their environment [13]. Quantitative data on the chemical composition of the alkaloids in *E. herba* are extremely variable, depending on factors such as the plant species, the amount of rainfall, soil characteristics, harvesting, storage conditions of the plant, and the analytical quantification method. For the biosynthesis of E alkaloids, plants use

**TABLE 2.** Contents of E and PE (mg/g dry weight) in *Ephedra* species

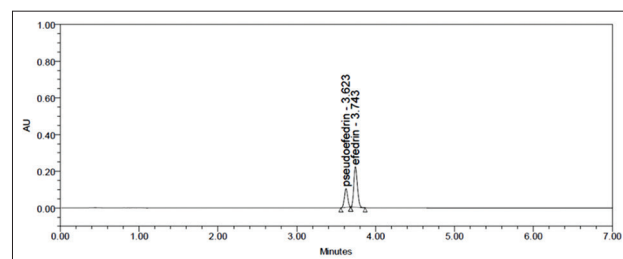
<i>Ephedra</i> species	Contents of E and PE (mg/g dry weight)		
	E	PE	E+PE
<i>Ephedra monosperma</i>	9.5	25.2	34.7
<i>Ephedra major</i>	16.3	14.3	30.6
<i>Ephedra fragilis</i>	21.0	6.1	27.1
<i>Ephedra distachya subsp. helvetica</i>	11.4	9.4	20.8
<i>Ephedra foeminea</i>	E and PE not detected. If present, their content is below the limit of detection		

E: Ephedrine; PE: Pseudoephedrine

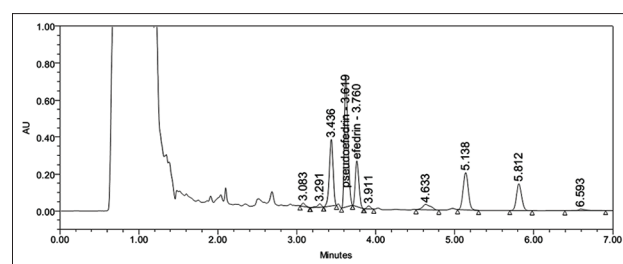
phenylalanine as a precursor; but incorporate only seven of its carbon atoms. Phenylalanine is metabolized to benzoic acid, which is then acetylated and decarboxylated to form pyruvic acid. Transamination, results in the formation of cathinone. Reduction of one carbonyl group leads to the formation of either norephedrine or norpseudoephedrine (called cathine).



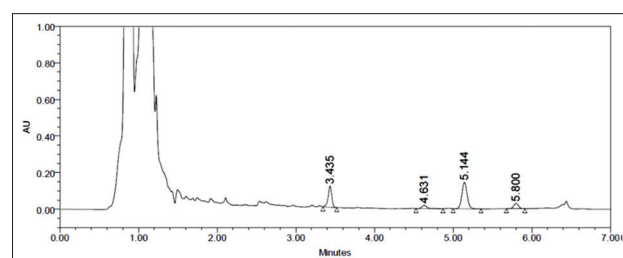
**FIGURE 2.** Comparison of total phenolics and total flavonoids content in *Ephedra* species.



**FIGURE 3.** Chromatogram of standards for pseudoephedrine (PE) (0.34 µg) and ephedrine (E) (1.1 µg). PE:  $t_R = 3.623$  minutes; E:  $t_R = 3.743$  minutes.



**FIGURE 4.** A typical chromatogram of *Ephedra monosperma* (pseudoephedrine  $t_R = 3.619$  minutes; ephedrine  $t_R = 3.760$  minutes).



**FIGURE 5.** A typical chromatogram of *Ephedra foeminea* (pseudoephedrine and ephedrine were not detected).

Finally, N-methylation would provide E or PE [2]. The content of alkaloids was first determined in East Asian *Ephedra* species. The Es are present in most Eurasian *Ephedra* species, but are more abundant in the Chinese species. American species are believed to be devoid of them [14]. The TAC is known for the following species: *Ephedra equisetina* Bunge, *Ephedra gerardiana* Wall Ex. Stapf, *Ephedra intermedia* Schrenk & C.A. Mey, *Ephedra lepidosperma* C.Y. Cheng, *Ephedra likiangensis* Florin, *Ephedra lomatolepis* Schrenk, *E. monosperma* J.G. Gmel. ex C.A. Mey, *Ephedra regeliana* Florin, *Ephedra saxatilis* (Stapf) Royle ex Florin, *Ephedra sinica* Stapf. The TAC in those species varies between 0.42 and 49 mg/g [2]. Hong et al. [15] determined and compared the TAC in *E. sinica*, *E. equisetina*, and *E. intermedia* and concluded that the content range for these species overlaps. However, the ratio E/total alkaloids and E/PE, as well as the E and ME content can be helpful for identification purposes. *Ephedra californica* S. Watson, *Ephedra distachya* L., *E. major* Host and *Ephedra viridis* Coville also belong to the *Ephedra* species containing E alkaloids [2]. Reportedly E alkaloids are present in: *E. alata* Decne., *Ephedra botschantzevii* Pachom., *E. fragilis* Desf., *Ephedra pachyclada* Boiss., and in very small amounts in *Ephedra strobilacea* Bunge and *Ephedra transitoria* Riedl [14,16].

Reports on the presence of E-type alkaloids in the *Ephedra* species used in this study are all in line with our results confirming the presence of alkaloids, including *E. foeminea*. Claims regarding the E and PE contents in that species are contrary [14]. Results obtained by the spectrophotometric method confirmed the presence of E alkaloids in *E. distachya subsp. helvetica* ( $15.8 \pm 2.0$  mg/g dry weight), *E. major* ( $14.8 \pm 1.9$  mg/g dry weight), *E. fragilis* ( $0.2 \pm 0.0$  mg/g dry weight) and *E. foeminea* ( $0.1 \pm 0.0$  mg/g dry weight). Caveney and Starratt [17] reported that *E. fragilis* may be the exception in the *Fragilis* group having a high E content, which is in line with our results ( $21.0$  mg/g dry weight) obtained by the UPLC-UV method. However, the results obtained by the spectrophotometric showed a low TAC in that species. The reason could be incomplete extraction process. The lowest TAC was found in *E. foeminea*. The summed up content of E and PE, obtained by the UPLC-UV method, varies between  $20.8$  mg/g dry weight (*E. distachya subsp. helvetica*) and  $34.7$  mg/g dry weight (*E. monosperma*). In some species, E alone amounts up to 90% and PE up to 99% of total alkaloids. Based on that, the content of E and PE obtained by the UPLC-UV method can be used to roughly estimate the TAC. In this study, among the alkaloids only E and PE were quantified due to the availability of standards. However, the UPLC-UV method proved to be adequate for successful quantification and separation of all six E-type alkaloids. The only species where PE is predominant compared to E is *E. monosperma*. There are also species

that contain only PE but no E. Ephedrine is the dominant alkaloid in *Ephedra sinica*, *Ephedra equisetina*, *E. monosperma* and *E. intermedia var. tibetica*. *Ephedra przewalskii* and *E. lepidosperma* are species with a very low content of E alkaloids [14].

The *Ephedra* genus is known for its alkaloids, hence, there are fewer studies about other phytochemicals of its species members. However, there is no doubt that phenolic compounds are constituents of *E. herba* [18]. These chemical compounds are natural pigments. Plant phenolics are mainly synthesized from phenylalanine and their most important role is in defending plants from pathogens. Flavonoids are aromatic compounds with anti-oxidative properties, present in over 70% plant species. *Ephedra* contains flavonoids (leucodelphinidin, leucopelargonine, leucoanthocyanidin, lucenine, vicenin-1, and vicenin-2), tannins, benzylmethylamine. Tannins, mainly proanthocyanidines, are constituents of many *Ephedra* species (e.g. in Eurasian species *E. intermedia*, *E. przewalskii*, *E. alata*, *E. distachya*, and *E. fragilis*; species of North America: *E. californica*, *E. fasciculata*, *E. nevadensis*, *E. torreyana*, *E. trifurca*, and *E. viridis*). Tannin deposits often induce the brown color of stems, which is helpful in species identification [19].

Literature data on TPC and TFC in individual *Ephedra* species is scarce. Harisaranraj et al. [20] have determined the TAC and TFC in *E. vulgaris* using gravimetry ( $1.24$  mg/100 g dry weight and  $1.48$  mg/100 g dry weight, respectively). In the same species, they also determined the TPC ( $1.46$  mg/100 g dry weight) by a spectrophotometric method. Recently, the focus of studies has been set toward characterization of individual phenolic constituents in different *Ephedra* species [21,22]. In this study, we determined the TPC and TFC in: *E. distachya subsp. helvetica*, *E. major*, *E. fragilis*, *E. foeminea*, *E. altissima*, *E. alata* and *E. foliata*. *Ephedra alata*, and *E. foliata* had the highest TPC as well as the highest TFC. The lowest TPC and TFC values were detected in *E. fragilis* and *E. foeminea*, which are also poor in the TAC. Comparison of TAC, TPC and TFC indicates that *E. alata* and *E. foliata* belong to the *Ephedra* species with an increased metabolic activity yielding a higher content of secondary metabolites.

## CONCLUSION

In this study, various *Ephedra* species have been investigated regarding TAC, TPC, and TFC using spectrophotometry. Chromatographic methods being simple and ensuring adequate sensitivity are more useful in phytochemical analyses. Therefore, for the first time UPLC-UV was employed to quantify E and PE, as well as to separate E-type alkaloids in the available *Ephedra* species. Contrary to most studies and reports, E alkaloids were detected in *E. foeminea*. Among

investigated species, *E. distachya subsp. helvetica* stands out as species with a high TAC, while *E. alata* has a high TPCs and TFC. The employed methods yielded a set of qualitative and quantitative parameters that can help to ascertain the identity of the plant material. Quantitative analyses have become crucial and most common approaches in the quality control of herbal preparations and plant ground materials. The described UPLC-UV method can be utilized to confirm the presence or absence of E alkaloids and employed in different analyses ranging from pharmacognosy and phytochemistry to forensic medicine. The determined content and presence/absence of specific E alkaloids determines the pharmacological and toxicological effects and reflects metabolic pathways occurring in different *Ephedra* species. By comparing the ratio between the secondary metabolites, it may be concluded that species with a high TAC are expected to be rich in TPC and TFC, as well.

## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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