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Influence of the vegetation period on sea fennel, *Crithmum maritimum* L. (Apiaceae), phenolic composition, antioxidant and anticholinesterase activities

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ABSTRACT

Since ancient times *Crithmum maritimum* L. (Apiaceae) or sea fennel, has been used in culinary practices and in folk medicine, but in the last few years it has become a rediscovered star of coastal cuisine. In this study, chemical composition (total phenolics, flavonoids, non-flavonoids, individual phenolic acids), antioxidant (FRAP, ORAC, DPPH, Briggs-Rauscher oscillating reaction) and cholinesterase (AChE, BuChE) inhibitory activities of the sea fennel samples collected in different seasons during a one-year vegetation period were investigated. The obtained results point out the great influence of the plant vegetation period on content of phenolics and consequently on plant biological activity. The sample collected before the flowering stage (in April) was, in most cases, superior in comparison to other extracts which opens a new realm of possibilities of its applications in different products and industries.

1. Introduction

Crithmum maritimum L. (Apiaceae), sea fennel, crest marine or rock samphire, is a wild, naturally salt-tolerant Mediterranean plant that can be found on cliffs and rocks or, less commonly, in shingle or sand by the sea (Maleš et al., 2003; Meot-Duros and Magné, 2009). Traditionally, in ancient times, this plant was used for culinary purposes. Due to the salty taste and sensory attributes which include some notes of celery and peel of green citrus, its leaves are usually used as a condiment and pickle, or as a salad ingredient. Also, sea fennel has been used in folk medicine due to numerous positive biological properties, while its essential oils are used in cosmetology (Atia et al., 2011; Renna and Gonnella, 2012; Senatore et al., 2000). Previous studies on sea fennel from different locations in the Mediterranean region investigated mainly the qualitative and quantitative composition of its essential oils (Baser et al., 2000; Kulišić-Bilušić et al., 2010; Musa Özcan et al., 2006; Özcan et al., 2001; Pateira et al., 1999; Ruberto et al., 2000; Senatore et al., 2000), and resulted with the hypothesis that several different chemotypes of sea fennel grow in this region. According to those studies, due to the specific chemical composition, the Croatian sea fennel could be a special chemotype (Generalić Mekinić et al., 2016; Kulišić-Bilušić et al., 2010; Siracusa et al., 2011). However, to date, only a small number of studies investigated its polar components, i.e. phenolics (Generalić Mekinić et al., 2016; Houta et al., 2011; Jallali et al., 2014; Maleš et al., 2003; Siracusa et al., 2011). Beside genetics, there are numerous other factors, abiotic and biotic, that have an impact on plant productivity and accumulation of plant metabolites, such as stage of development, vegetation period, temperature, rainfall, drought, light intensity, soil composition, wind, nutrients, interactions with pathogens and parasites, etc. (Akula and Ravishankar, 2011; Szakiel et al., 2011). On the other hand, some other factors like plant parts used for the analysis, pre-extraction treatment of plant material, solvent choice and extraction protocol also affect the plant chemical composition (Azwanida, 2015), what additionally complicates the comparison of the results from different studies.

Therefore, the aim of this study was to investigate the influence of the phenophase on the phenolic profile and related antioxidant properties of Croatian sea fennel in order to draw conclusions about which vegetation period results with highest content of phytochemicals and consequentially better biological activities, which, all in all, could bring

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to new usage possibilities of this interesting plant.

2. Material and methods

2.1. General

All used reagents, solvents and standards were of analytical grade and were obtained from Kemika (Zagreb, Croatia), T.T.T. d.o.o. (Sv. Nedjelja, Croatia), Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, Missouri, USA). Spectrophotometric measurements were performed on a SPECORD 200 Plus, Edition 2010 (Analytik Jena AG, Jena, Germany) and Synergy HTX Multi-Mode Reader (BioTek Instruments, Inc., Winooski, Vermont, USA).

2.2. Plant material and extract preparation

The plant materials, aerial parts of sea fennel, were collected during 2014 from the same location (over the area of 10 m^2) in Central Dalmatia (Split, Croatia). The samples were harvested during one-year vegetation period every two months (at first two days of each month) as follows: in vegetative phases: in February and April (before flowering), and reproductive phases: in June (beginning of flowering), August (full flowering), October (fruits) and December (seeds). The primary samples were composed of more than 10 increments and the whole plant material (500 g) was compiled and dried. The sampling protocol was the same for all samples.

Dried, pulverized sea fennel leaves (10 g) were extracted with 80% aqueous ethanol (v/v) (100 mL) at 50 °C in ultrasonic bath for 1 h. Extractions were done in three repetitions for each plant material and after the filtration, all sample extracts were combined in total extract that was used for further analysis.

2.3. Chemical composition

The total phenolic content in samples was determined according to the Folin-Ciocalteu method (Amerine and Ough, 1980). The measurement of non-flavonoids was carried out using method described by Kramling and Singleton (1969) while the content of flavonoids was calculated as the difference between total phenolics and non-flavonoids. The results are expressed as mg of gallic acid equivalents (GAE) per gram of dry plant material (mg GAE/g).

The high-performance liquid chromatography (HPLC) system used was Perkin Elmer Series 200 with UV/VIS detector (Perkin-Elmer Inc., Shelton, CT, USA), and the compounds were separated on an UltraAqueous column (C_{18}, 250 × 4.6 mm, 5 mm, Restek, Bellefonte, PA, USA). HPLC was used for identification and quantification of vanilic, caffeic, cinnamic and chlorogenic acid, in the sea fennel samples according to the procedure described in Generalić Mekinić et al. (2016).

A gradient consisting of solvent A (water/phosphoric acid, 99.8:0.2, v/v) and solvent B (methanol/acetonitrile, 50:50, v/v) was applied at flow rate of 0.8 mL/min as follows: 0.5 min 96% A and 4% B; 40 min 50% A and 50% B; 45 min 40% A and 60% B; 60 min 0% A and 100% B; 70 min 96% A and 4% B; 80 min 96% A and 4% B. The signal was monitored at 280 nm and the investigated phenolic acids were identified by comparing their retention times and absorption spectra with those acquired for corresponding standards analysed under the same conditions. Spiking of selected samples was also used to assist confirmation of the peak identity and the identified compounds were quantified using external standard calibration curves (at five concentrations). The calibration parameters for detected phenolic acids are given in Table 1. Compound concentrations are expressed in mg of compound per g of dry plant material (mg/g).

2.4. Biological activity

The reducing ability of extracts was measured as Ferric Reducing/Antioxidant Power (FRAP), using a method described by Benzie and Strain (1996) and the results are expressed as milimoles of Trolox equivalents (TE) per litre of extract (mM TE).

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging ability of the samples was measured according to the procedure reported by Katalinić et al. (2013) and the results are expressed as inhibition percentage of DPPH radical (% Inhibition).

The inhibitory activity of extracts on oscillations in Briggs-Rauscher reaction system was estimated as the length of time (in minutes) before oscillations restart (Generalić et al., 2011, 2012).

Oxygen Radical Antioxidant Capacity (ORAC) assay was performed in plate reader with 96-well plates according to the slightly modified procedure described by Prior et al. (2003). The results are expressed as mM of TE per litre of extract (mM TE).

Cholinesterase inhibitory activity measurements, both acetylcholinesterase and butyrylcholinesterase inhibition, were carried out using Ellman method as described in Generalić Mekinić et al. (2016) and the results are expressed as percentage of enzyme inhibition.

Table 1
HPLC calibration parameters for standard compounds of phenolic acids.

	Vanillic acid	Caffeic acid	Cinnamic acid	Chlorogenic acid
Retention time (min)	27.27	28.27	45.24	26.11
Regression equation	y = 63490.3x	y = 70339.4x	y = 190446.1x	y = 35838.1x
R^2	0.9999	0.9999	0.9996	0.9999
LOD (mg/L)	0.02	0.02	0.003	0.07
LOQ (mg/L)	0.06	0.06	0.009	0.21

R^2 – determination coefficient; LOD – limit of detection, LOQ – limit of quantification.

2.5. Data analysis

All analyses were performed in triplicate and the results are expressed as mean values ± standard deviation. GEN 5 software (BioTek, Winooski, Vermont, USA) was used for data collection and analysis. Statistical data analysis was performed using STATISTICA (Data Analysis Software System, v. 10, StatSoft Inc, Tulsa, OK, USA). Statistical differences between different vegetation periods and compound concentrations were determined by analysis of variance (one-way ANOVA) and followed by a least significant difference test at a 95% confidence level, while Pearson's correlation coefficient was used for determination of the relations between the variables. All results were also submitted to multivariate principal component analysis (PCA) in order to better understand the relationship between the studied variables.

3. Results and discussion

Sea fennel is an edible plant with various economic interests due to the presence of valuable bioactive phytochemicals in all plant parts. Although the apolar components from sea fennel essential oil have been largely studied, little is known about its water-soluble components (Meot Duros and Magne, 2009). Previous studies on sea fennel phenolics (Generalić Mekinić et al., 2016; Meot-Duros et al., 2010; Siracusa et al., 2011) reported that the dominant phytochemical in this plant is chlorogenic acid which has numerous beneficial health effects. The present study was conducted in order to investigate the vegetation period in which the accumulation of plant biologically active phenolics is maximal, and consequently, to investigate if samples from that period have better antioxidant and cholinesterase inhibitory activities. This information could be useful in further studies on sea fennel as well as its use in pharmaceuticals and/or for other purposes. The contents of total