

## THE EXTRACT OF FENNEL FRUIT AS A POTENTIAL NATURAL ADDITIVE IN FOOD INDUSTRY

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**Abstract:** In this study, the polyphenol profile and antioxidant activity of the hydro-ethanolic extract of the fennel fruit were examined in order to investigate the possibility of its application as a potential functional food additive. Total phenols were analyzed by the method of Folin-Ciocalteu, while total flavonoids were determined by the aluminum chloride colorimetric method. The separation and quantification of phenolic compounds were performed by LC-MS/MS analysis, using a multiple reaction monitoring (MRM) mode. The antioxidant capacity was determined by FRAP and DPPH assays.

The high values of total phenolics and flavonoids were found, as well as high antioxidant activity which amounted to  $9023.33 \pm 38.19 \mu\text{mol Fe(II)/l}$  and  $3.73 \pm 0.04 \text{ mmol TE/l}$ , tested by FRAP and DPPH assays, respectively. Among the identified phenolic compounds, *p*-hydroxybenzoic and chlorogenic acids were detected as predominant. The obtained results indicated that the hydro-ethanolic extract of the fennel fruit can be used in food industry as a potential natural antioxidant.

**Key words:** fennel fruit, hydro-ethanolic extract, antioxidant activity, total phenols, LC/MS.

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## Introduction

The application of herbs in the prevention and treatment of various diseases is as old as mankind itself. Over time, herbal compositions in the widespread traditional use have become an integral part of modern pharmacotherapy and conventional modes of treatment. Similarly, the connection between diet and health has been known since ancient times. Medicinal plants have found their place not just in the field of health preservation, but also in the food and beverage industry as their functional addition. Recent findings in the fields of biology and medicine have confirmed the hypothesis that diet plays a decisive role in the modulation and control of various body functions and in achieving and maintaining good health.

On the basis of this, the concept of functional food was developed and also a new scientific discipline, known as functional food science. Functional food means any food that, in addition to its nutritional value, contains ingredients which have positive effects on human health and reduces the risk of the disease (Kim et al., 2006). Medicinal plants, as a rich source of various bioactive compounds, exhibiting beneficial effects on human health have largely found their place in the development of functional food.

Fennel (*Foeniculum vulgare* Mill.) is a highly regarded medicinal and aromatic plant from the *Apiaceae* family. It is widespread in the Mediterranean, but it is grown in many countries of the world as well. Recent research has shown that *F. vulgare* has different pharmacological properties such as anti-allergic, analgesic, anti-inflammatory, antioxidant, antibacterial, anti-cancer, anti-stress, cytotoxicity, etc. (Kooti et al., 2015). Fennel is a highly valued spice plant. The whole plant has a very intense specific odor, and in many countries, it is cultivated and consumed as a vegetable, especially its succulent young sprouts. The fruit of fennel is used in cooking as a flavoring and odorant agent and in food industry and confectionery as well as for the production of herbal liqueurs and spirits (Timasheva and Gorbunova, 2014).

The essential oil of fennel was largely examined, both from the aspects of its chemical composition and its pharmacological activities. Various growing localities have an impact on the qualitative and quantitative composition of the fennel essential oil (Piccaglia and Marotti, 2001; Aćimović et al., 2015). Likewise, different stages of fruit maturation have a significant influence on the yield and chemical composition of the sweet fennel essential oils (Telci et al., 2009). Apart from analyzing the chemical profile of essential oils, a very strong antibacterial effect on the common foodborne pathogens was established (Dadalioglu and Evrendilek, 2004). The high antifungal and antioxidant potential of the fennel essential oil was also proven (Singh et al., 2006).

Furthermore, extracts of the fruit of fennel, prepared with different methods and different extraction agents, were examined from the aspects of their chemical composition and pharmacological activity (Kooti et al., 2015). Phenolic compounds and antioxidant activity of water and methanolic extracts (Cai et al., 2004), and extracts prepared with 80% methanol (Surveswaran et al., 2007) were examined. In the study of De Marino et al. (2007), phenolic glycosides and antioxidant activity were analyzed from the methanolic extracts of the fennel fruit. The methanolic extracts of fennel seeds were also evaluated from the points of view of their antioxidant and anti-carcinogenic effects (Mohamad et al., 2011). The antioxidant potential of methanolic extracts of different parts of the fennel plant was also tested (Barros et al., 2009) as well as total phenols and antioxidant capacity of water infusion made of numerous medicinal plants including the fennel fruit (Katalinić et al., 2006).

From a toxicological point of view, acetone, methanol and other organic solvents are not suitable as solvents for the preparation of extracts used orally. In contrast to them, hydro-ethanolic extracts may be used in the production of pharmaceutical compositions in the form of plant drops or solutions, and also in the food industry. As there is no information on *in vitro* studies of the polyphenol content and the antioxidant activity of hydro-ethanolic extracts of fennel fruits, the goal of this study was to investigate the antioxidant activity of the extract obtained by extraction with a mixture of water and ethanol (50:50), and assess the possibility of using this extract as a potential source of natural antioxidants and functional food additives.

## Materials and Methods

### Preparation of extract

The fennel fruit was purchased in dried form in the pharmacy of the Institute of Medicinal Plants Research “Dr Josif Pančić”, Belgrade (serial number: 3580611). The extract of the fennel fruit was prepared by double percolation using a modified pharmacopoeia method, in a glass percolator at room temperature, using a 50% ethanol-water solvent, where the ratio of plant material to the resulting extract was 1:1 (Pharmacopoea Jugoslavica, 1984).

### Dry matter and pH

The pH value of the tested extract was determined by a pH meter with the glass electrode (WTW inoLab), whereas the soluble dry matter (DM%) was determined using a refractometer (Gramma Libero).

#### Determination of total phenol content

The total phenol content (TPC) was determined using Folin-Ciocalteu reagent and expressed as a gallic acid (GA) equivalent (mg GAE)/l of the extract (Fu et al., 2011).

#### Determination of total flavonoid content

The content of the total flavonoids in the extract was determined by the spectrophotometric method, based on the production of complex compounds of flavonoids with aluminium chloride. The standard solution of quercetin in ethanol was used as a referent sample, and the results were expressed as mg of equivalent per liter of the extract of quercetin (Verzelloni et al., 2007).

#### Antioxidant activity

*FRAP assay:* Total antioxidant activity was investigated using the ferric reducing antioxidant power (FRAP) assay, which is based upon reduction of Fe(III) - TPTZ in acidic conditions (Fu et al., 2011). The standard curve was constructed using the FeSO<sub>4</sub> solution, and the results were expressed as  $\mu\text{mol Fe(II)/l}$  of the extract.

*DPPH radical assay:* Radical scavenging activity of the tested extract was determined by the DPPH method (Jakobek et al., 2007). The trolox solution was prepared as a reference standard and the results were presented as the TE mmol/l of extract.

#### LC analysis

The separation of phenolic compounds in the extract was carried out using a Waters Acquity UPLC system equipped with a photodiode array (PDA) detector, and interfaced to a mass spectrometer with the triple quadrupole analyser (QqQ). The separation column was a ZORBAX Eclipse XDB C18 column (150  $\times$  4.6 mm; 5  $\mu\text{m}$ ). The LC conditions were as following: mobile phase flow rate of 0.7 ml/min, column temperature of 25°C, injection volume of 1  $\mu\text{l}$ , and the solvent gradient (time [min]/ solvent B (%): 0/5, 20/16, 28/40, 32/70, 36/98, 45/98, 46/5, 55/5), where solvent A was the 0.2% solution of formic acid in deionized water (v/v) and solvent B was acetonitrile. The PDA detector range was 190–450 nm. The hydro-ethanolic extract of the fennel fruit was filtered through Premium Syringe Filters, Regenerated Cellulose 0.45  $\mu\text{m}$ , 15-mm filters, before injection.

### MS/MS-MRM analysis

The identification of phenolic compounds was performed by comparing their retention times ( $t_R$ ), UV spectra and multiple reaction monitoring (MRM) transitions with those of reference standards (Table 1). The quantification of the identified compounds was performed using the external standard method. The conditions under which the electrospray ionization (ESI) source operated were: negative ionization mode, source temperature of 150°C, desolvation temperature of 350°C, desolvation gas flow rate of 800 l/h, capillary voltage of 3.5 kV, extractor voltage of 3 V, cone voltage of 30 V (60 V for rutin), and collision energy of 9 eV (20 eV for rutin) with argon used as a collision gas. Mass Lynx 4.1 software was used for data acquisition and peak integration.

Table 1. The parameters for the identification and quantification of phenolic compounds in the hydro-ethanolic extract of the fennel fruit.

| Phenolic compound             | Molecular formula                               | Molar mass | MRM transition | $t_R$ (min) | UV <sub>max</sub> (nm) |
|-------------------------------|---|------------|----------------|-------------|------------------------|
| Protocatechuic acid           | C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>    | 154        | 153→109        | 9.14        | 218, 260               |
| <i>p</i> -hydroxybenzoic acid | C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>    | 138        | 137→93         | 13.81       | 255                    |
| Chlorogenic acid              | C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>  | 354        | 355→163        | 14.33       | 246, 325               |
| Caffeic acid                  | C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>    | 180        | 179→135        | 17.06       | 243, 323               |
| <i>p</i> -coumaric acid       | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>    | 164        | 165→91         | 23.34       | 230, 309               |
| Ferulic acid                  | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 610        | 193→134        | 25.10       | 278, 321               |
| Rutin                         | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 610        | 609→301        | 24.64       | 253, 341               |

MRM – multiple reaction monitoring,  $t_R$  – retention time, UV<sub>max</sub> – ultraviolet maximum.

### Statistical analysis

All measurements were done in triplicate and results were expressed as mean ± standard deviation.

## Results and Discussion

The hydro-ethanolic extract of the fennel fruit was obtained using the traditional percolation method. The soluble solids of this extract measured using a refractometer were 23%, whereas pH was 6.3. The amount of total flavonoids and total phenolic content of the tested extract was  $1524.86 \pm 4.81$  mg (quercetin equivalents) QUE / l and  $1534.97 \pm 6.26$  mg GAE / l of the extract, respectively (Table 2).

Oktay et al. (2003) obtained the higher value of total phenolic content in the ethanol extract of fennel seeds compared to the corresponding water extract (90.00 and 21.25 µg GAE / 1mg of the ethanol and water extracts of fennel seeds, respectively).

In the hydro-methanolic extract of fennel shoots, the significant amounts of flavonoids and high content of phenols were determined (De Marino et al., 2007).

Table 2. TPC, TFC and antioxidant activity of the extract of the fennel fruit.

| Parameter analyzed   | Value           |
|----------------------|-----------------|
| TPC (mg GAE/l)       | 1534.97 ± 6.26  |
| TFC (mg QUE/l)       | 1524.86 ± 4.81  |
| FRAP (µmol Fe(II)/l) | 9023.33 ± 38.19 |
| DPPH (mmol TE/l)     | 3.73 ± 0.04     |

TPC – total phenolic content, TFC – total flavonoid content, FRAP – ferric reducing ability of the plasma method, DPPH – 2,2-diphenyl-1-picrylhydrazil.

The antioxidant capacity of our extract was 9023.33 ± 38.19 µmol Fe (II)/l and 3.73 ± 0.04 mmol TE/l, tested by FRAP and DPPH assays, respectively (Table 2). A high antioxidant capacity has also been reported in the work of Cai et al. (2004) in hydro and methanolic extracts of the fennel fruit (TEAC: 150.6 and 105.9 µmol trolox/100g DW, respectively). In addition, the methanolic extract of fennel seeds investigated in the work of Surveswaran et al. (2007) exhibited even a stronger potential (DPPH: 2.63 mmol/100 g DW; FRAP: 1.79 µmol/g DW) compared with our results. The antioxidant activity of hydro and ethanolic extracts of fennel seeds was also evaluated by different antioxidant methods compared to standard antioxidants such as BHA, BHT, and α-tocopherol (Oktay et al., 2003). It has already been reported that the antioxidant capacities of different extracts have a strong relationship with the solvent combination used, mostly due to the different antioxidant capacities of compounds with different polarities (Boeing et al., 2014).

In the study of Cai et al. (2004), a very significant positive correlation between the content of polyphenolic compounds and antioxidant capacity was found ( $R^2$  values – 0.950), indicating that polyphenolic compounds had the greatest contribution to the activities of the plant antioxidants. In addition, a similar correlation has been shown in the work of Surveswaran et al. (2007), with R values: TPC-ABTS 0.9690; TPC-DPPH 0.9378; TPC-FRAP 0.8941.

Infusions of the 70 different medicinal plants have been tested for antioxidant capacity and total phenols and it was found that these parameters were lower for the infusion made of the fennel fruit compared to other tested plants (*Foeniculi fructus*: total phenolics– 29 mg CE/l; FRAP 142 µmol/l) (Katalinić et al., 2006). However, significantly higher values were observed in our sample in relation to those values, which may be attributed to the higher solubility of the essential oil in ethanol than in water (infusion). It has already been verified that the essential oils distilled from the fennel fruit possess a high antifungal and high antioxidant capacity (Singh et al., 2006). The essential oil was

found 100% antifungal against *Aspergillus niger*, *A. flavus*, *F. graminearum* and *F. moniliforme*.

In order to confirm that the phenolic compounds were responsible for the antioxidant activity of the hydro-ethanolic fennel fruit extract, the identification and quantification of phenolic compounds by the LC-MS/MS method in a multiple reaction monitoring (MRM) mode were performed. Extracted MRM chromatograms of quantified phenolic compounds of the tested extract are shown in Figure 1.

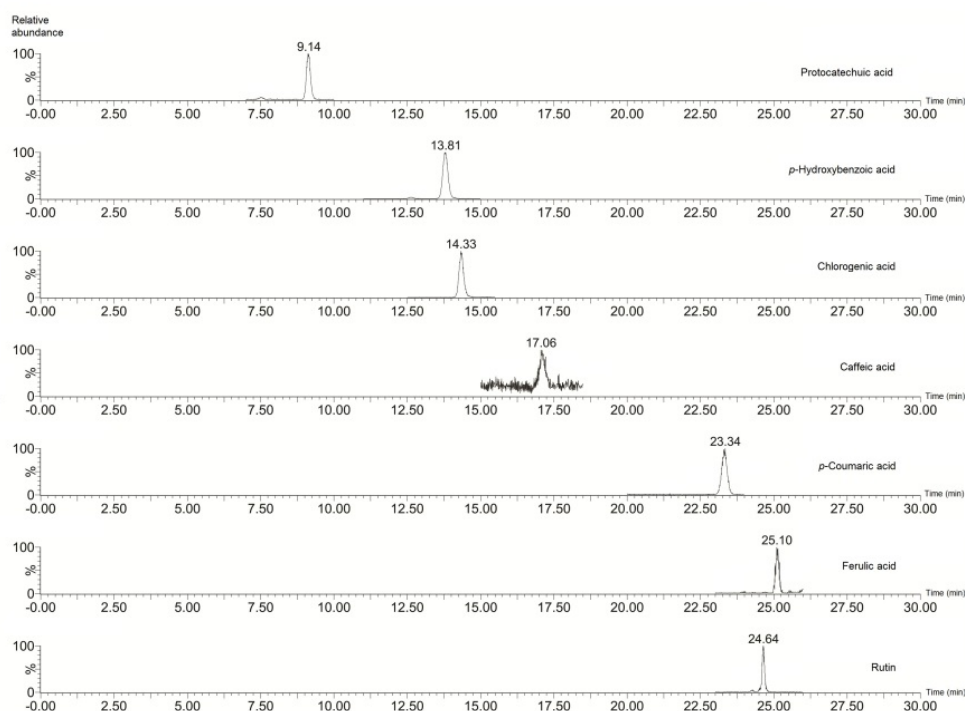


Figure 1. MRM chromatograms of protocatechuic, *p*-hydroxybenzoic, chlorogenic, caffeic, *p*-coumaric, and ferulic acids and rutin, respectively in the extract of the fennel fruit recorded in the (-) ESI mode.

As can be seen in Table 3, *p*-hydroxybenzoic, chlorogenic, *p*-coumaric, ferulic, caffeic acids, rutin and protocatechuic acid were identified, whereas gallic and ellagic acids, epicatechin, quercetin, myricetin and kaempferol as well as cinnamic and vanillic acids were not detected although these compounds were reported in the studies of other authors (Cai et al., 2004; Parejo et al., 2004).

On the other hand, a high amount (62.99 mg/l) of *p*-hydroxybenzoic acid was detected, as well as a significant amount of chlorogenic and *p*-coumaric acids. The esters of *p*-hydroxybenzoic acid are used as preservatives in pharmaceuticals, cosmetics and food industry (Aalto et al., 1953). The appreciable amounts of this acid detected in our sample indicated that this extract seemed to have a potential preservative effect. Different concentrations and isomeric mixtures of chlorogenic acid are present in coffee, wine, vegetable and fruit juices and in various herbal infusions, and it has been suggested that the consumption of these beverages can reduce the risks of different chronic diseases (Liang et al., 2016). Likewise, the antioxidant activity of ferulic, caffeic and chlorogenic acids has already been confirmed in an *in vitro* study (Milić et al., 2000).

Table 3. The content of phenolic compounds in the hydro-ethanolic extract of the fennel fruit (mg/l of extract).

| No. | Compound                      | Content±SD (mg/l) |
|-----|-------------------------------|-------------------|
| 1.  | Protocatechuic acid           | 4.04±0.18         |
| 2.  | <i>p</i> -hydroxybenzoic acid | 62.99±3.42        |
| 3.  | Chlorogenic acid              | 30.82±1.55        |
| 4.  | Caffeic acid                  | 1.31±0.06         |
| 5.  | <i>p</i> -coumaric acid       | 11.25±0.73        |
| 6.  | Ferulic acid                  | 6.12±0.82         |
| 7.  | Rutin                         | 6.81±0.34         |

The presence of chlorogenic, ferulic, caffeic acids as well as of rutin, quercetin and kaempferol was reported in the extracted residue (waste) after the production of the essential oil of the fennel from the period of flowering (Boeing et al., 2014), whereas the latter two compounds were not recorded in our sample. Differences among the detected compounds of various fennel extracts can occur due to the fact that the different fennel plant genotypes, grown in different climate conditions, were used for the extractions. Similarly, extractions were performed using different parts of the fennel plant and using different methods and solvents.

The obtained results indicate that the investigated extract represents a good source of polyphenolic compounds and can be used in food industry as a potential source of natural antioxidants.

### Conclusion

The main advantage of the application of the hydro-ethanolic extract of the fennel fruit compared to the methanolic or acetone extract is the fact that it can be used in food industry without the removal of the solvent. In this work, high values of phenols and flavonoid compounds as well as an effective degree of the



antioxidant capacity of the hydro-ethanolic extract of the fennel fruit have been obtained. The various phenolic acids were recorded, with *p*-hydroxybenzoic and chlorogenic acids as the most dominant. This extract can be used in the food industry (production of alcohol and non-alcohol beverages or confectionery), not only as a specific flavoring, but also as an effective and easily accessible source of natural antioxidants with a potential preservative effect.

### Acknowledgments

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 46001).

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Received: November 17, 2017

Accepted: April 19, 2018

EKSTRAKT PLODA MORAČA KAO POTENCIJALNI PRIRODNI ADITIV U  
PREHRAMBENOJ INDUSTRIJI

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## R e z i m e

U ovom radu određivan je sadržaj polifenola i antioksidativna aktivnost vodeno-etanolnog ekstrakta morača, sa ciljem ispitivanja mogućnosti njegove primene kao potencijalnog funkcionalnog aditiva. Ukupni fenoli su analizirani metodom po Folin-Ciocalteu, dok je ukupan sadržaj flavonoida određen kolorimetrijskom metodom primenom aluminijum hlorida. Razdvajanje i kvantifikacija fenolnih jedinjenja postignuti su upotrebom LC-MS/MS metode u režimu koji omogućava istovremeno praćenje više jonskih prelaza. Antioksidativni kapacitet je određivan primenom testova FRAP i DPPH.

U testiranom ekstraktu dobijene su visoke vrednosti za ukupne fenole i flavonoide, a dobijena je i visoka vrednost antioksidativne aktivnosti, koja je iznosila  $9023.33 \pm 38,19$  mmol Fe(II)/l i  $3,73 \pm 0,04$  mmol TE/l, računato primenom testa FRAP odnosno testa DPPH. Među fenolnim jedinjenjima, *p*-hidroksibenzoeva i hlorogena kiselina su pronađene kao dominantne. Dobijeni rezultati ukazuju na to da se ekstrakt morača može primenjivati u prehrambenoj industriji kao potencijalni prirodni antioksidans.

**Ključne reči:** morač, vodeno-etanolni ekstrakt, ukupni fenoli, antioksidativna aktivnost, LC/MS.

Primljeno: 17. novembra 2017.

Odobreno: 19. aprila 2018.

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